Defining the Roles of Serine Palmitoyltransferase-Interacting Proteins in the Regulation of Sphingolipid Homeostasis

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DEFINING THE ROLES OF SERINE PALMITOYLTRANSFERASE-INTERACTING PROTEINS IN THE REGULATION OF SPHINGOLIPID HOMEOSTASIS

By

Athen N Kimberlin

A DISSERTATION

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Sphingolipids are major structural components of the plasma membrane and endomembrane system. Research suggests that sphingolipids are involved with the formation of lipid microdomains, also known as lipid rafts, which may help to organize proteins within the membrane and may be important for membrane trafficking. Aside from their structural roles in membranes, sphingolipids and their metabolic products have been implicated in several cellular signaling responses like programmed cell death (PCD). Because of this, maintenance of sphingolipid homeostasis is critical for eukaryotic cell growth and development. Serine palmitoyltransferase (SPT) catalyzes the first step in sphingolipid biosynthesis and is the primary regulatory point for sphingolipid homeostasis.

We have characterized two sets of Arabidopsis proteins that physically interact with and impact Arabidopsis SPT activity. The first set, the ssSPTs (Arabidopsis thaliana), have been shown to be essential and redundant. Modulation of AtssSPT expression was shown to alter SPT activity, LCB accumulation and sensitivity to the mycotoxin, Fumonisin B1 (FB1), in a way that is consistent with the AtssSPTs being activators of SPT. Alternatively, modulating expression of the other set of proteins, the AtORMs, was shown to alter SPT activity, LCB accumulation and sensitivity to FB1, consistent with them acting as SPT inhibitors. Both the AtssSPTs and the AtORMs
appear to be limiting as transgenic up/down regulation of these genes leads to predictable changes to SPT activity. Interestingly, we also see changes in ceramide synthase activity with modulation of AtORM expression, suggesting a more complex regulatory role for these proteins and pointing towards coordinate regulation of SPT with downstream enzymes.

Our research also demonstrates that SPT substrate specificity can be altered through point mutations in AtssSPT and AtLCB1, leading to the production of aberrant long-chain bases (LCBs). Alteration of SPT substrate specificity may function as another regulatory control point by altering SPT products through changes in the composition of SPT subunits. We also discuss a system utilizing a point mutation in AtLCB1 that can be used as a tool to better measure SPT activity in planta. Collectively our data point towards a complex and nuanced regulatory scheme for maintaining sphingolipid homeostasis in Arabidopsis thaliana.
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CHAPTER 1

INTRODUCTION

Note: This chapter is to be published and the text has been modified from the original.

1.1 Introduction

Sphingolipids are a structurally diverse class of lipids that are major components of eukaryotic membrane systems. Originally named after the sphinx for their enigmatic nature, sphingolipids have proven to be quite interesting to the field of biochemistry. Sphingolipids have been recognized as essential components of eukaryotic cells and have been extensively studied in humans due to their association with a number of lipid storage disorders, including Tay-Sachs disease and Niemann-Pick disease (Sandhoff, 2013). Sphingolipids, however, were not identified in plants until the late 1950s (Carter et al., 1958) and for nearly four decades following this discovery, sphingolipid research in plants was limited mainly to structural and compositional analyses, including studies of sphingolipid compositional changes in response to abiotic stressors. Since the late 1990s, sphingolipids have become an increasingly interesting topic of research. Driving this heightened interest is the realization that sphingolipids are the most abundant endomembrane lipids in plant cells and that they contribute not only to membrane structure and function but also to the regulation of cellular processes.

Research on the composition and function of sphingolipids was initially limited due to challenges associated with their extraction and analysis. Consequently this has led to the quantitative significance of sphingolipids to be under appreciated. Research on plant sphingolipids, however, has been greatly advanced in the last 10-15 years as new techniques have emerged. The development and application of advanced mass spectrometry methods that enable the rapid and quantitative measurement of molecular species of specific sphingolipid classes have effectively allowed us to look at the entire
“sphingolipidome” (Markham and Jaworski, 2007). Coupling of these methods with the characterization of Arabidopsis mutants has advanced our understanding of sphingolipid metabolism and its regulation. The study of Arabidopsis mutants has provided helpful insight into the involvement of sphingolipids in plant growth, development, and response to environmental stimuli. In addition, these studies have provided evidence of unexpected connections between sphingolipids and programmed cell death along with the related hypersensitive response. Recent research has also been directed at understanding the specialized functions of sphingolipids in plasma membrane microdomains that are believed to contribute to cell surface processes such as cell wall metabolism and ion and auxin transport. These resources have allowed for advances in sphingolipid research, but there is still much to discover in plant sphingolipid metabolism.

1.2 Sphingolipid Structure

Sphingolipids typically consist of a hydrophobic ceramide backbone linked to one or more polar sugar residues to form amphipathic lipid components of membranes (Dunn et al., 2004; Chen et al., 2010)(Figure 1.1). The ceramide backbone contains a long chain amino alcohol referred to as a sphingoid long chain base (LCB) linked through an amide bond to a fatty acid. LCBs are unique to sphingolipids and are typically synthesized from serine and palmitoyl-CoA which gives a carbon chain length of 18. In plants, LCBs can contain double bonds at the Δ4 and Δ8 positions. The Δ4 double bond is found only in the trans configuration, while the Δ8 double bond can be found in either the trans or cis configurations. After its initial synthesis, an LCB has two hydroxyl groups at the C-1 and C-3 positions and is commonly referred to as a dihydroxy LCB (Carter et al., 1958;
Dunn et al., 2004). A third hydroxyl group can be enzymatically added at the C-4 position forming trihydroxy LCBs. LCBs can be phosphorylated by LCB kinases at the C-1 position to form LCB phosphates (LCB-Ps). Free LCBs and their phosphorylated forms are typically in low abundance in plant cells (Markham et al., 2006; Markham and Jaworski, 2007). Instead, the majority of LCBs can be found linked to fatty acids in ceramides. In plants, the chain lengths of fatty acids in ceramides range from 16 to 26 carbons, the majority of which contain an enzymatically added C-2 (α-position) hydroxyl group (Dunn et al., 2004; Chen et al., 2010). Analogous to the diacylglycerol backbone of glycerolipids, ceramides serve as the hydrophobic component of complex sphingolipids (Figure 1.1). The polar head group of ceramides is attached at its C-1 position and can be a phosphate residue or a variety of sugar residues (Chen et al., 2010). The latter are referred to as glycosphingolipids. The simplest glycosphingolipid in plants is glucosylceramide (GlcCer) with a single glucose residue and comprises approximately one-third of the glycosphingolipids found in Arabidopsis leaves (Markham et al., 2006; Markham and Jaworski, 2007). The most abundant glycosphingolipid in Arabidopsis contains an inositol phosphate bound to the ceramide with up to seven additional hexose and pentose residues (Cacas et al., 2013). These molecules are referred to as glycosyl inositolphosphoceramides (GIPCs) and comprise approximately two-thirds of the glycosphingolipids found in Arabidopsis leaves (Markham et al., 2006; Markham and Jaworski, 2007) (Figure 1.1). The quantitative significance of GIPCs in plants was overlooked for many years due to the difficulty in their extraction using standard lipid analytical protocols because of the high polarity of their glycosylated head groups. Between the different carbon chain-lengths and hydroxylation and unsaturation states of
LCBs and fatty acids and the array of polar head groups, hundreds of potentially different sphingolipid species can occur in plants, the individual significance of which are only beginning to be evaluated (Bure et al., 2011; Markham, 2013).
Figure 1.1 Examples of long-chain bases (LCBs) and sphingolipids found in plants. (A) Examples of LCB modifications found in plants. Shown are examples of dihydroxy and trihydroxy LCBs. The nomenclature “d18:0” indicates that the LCB has two hydroxyl groups (d) and 18 carbons with no double bonds. The nomenclature “t18:0” indicates that the LCB has three hydroxyl groups and 18 carbons with no double bonds. (B) Hydroxyceramide composed of the LCB t18:1 Δ8trans and the fatty acid 24:1 ω9cis that is hydroxylated at the C-2 position. (C) Most abundant glycosyl inositolphosphoceramide (GIPC) found in Arabidopsis leaves. (D) Glucosylceramide.

1.3 Biosynthesis of Long-Chain Bases

A plethora of complex sphingolipids exist but they all share a unique core structure that is known as the long chain base (LCB). The synthesis of LCBs occurs through a condensation reaction with serine and palmitoyl-CoA that gives rise to the typical 18 carbon intermediate 3-ketosphinganine (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008). This first step is catalyzed by the enzyme serine palmitoyltransferase (SPT), which is a member of the α-oxoamine synthase subfamily (Figure 1.2). The product of this reaction is then reduced by 3-ketosphinganine reductase (3-KSR) to form sphinganine or d18:0, the simplest LCB in plants and other eukaryotes (Chao et al., 2011). SPT is generally regarded as the main regulated step in sphingolipid biosynthesis (Hanada, 2003). Similar to other eukaryotes, the Arabidopsis thaliana SPT has been shown to exist as a heterodimer comprised of LCB1 and LCB2 subunits (Tamura et al., 2001; Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008). Although both LCB1 and LCB2 show similarity with α-oxoamine synthases, the catalytic lysine residue that forms a Schiff base with pyridoxal phosphate is found in the LCB2 subunit (Tamura et al., 2001; Hanada, 2003). A third smaller subunit, termed the small subunit of SPT (ssSPT) also interacts with the LCB1/LCB2 heterodimer and acts as an activator of the core SPT enzyme (Han et al., 2004; Kimberlin et al., 2013). The small subunit of SPT is
a tiny protein (56 amino acids) that has a single transmembrane domain and no predicted enzyme activity and does not contain any known protein domains, although it is fairly conserved in eukaryotes. It is believed that the active site of SPT occurs at the interface of LCB1 and LCB2 with LCB1 and ssSPT acting to stabilize the complex (Gable et al., 2000; Gable et al., 2002). Although SPT technically can function as a heterodimer (LCB1 and LCB2) with a minimal amount of enzyme activity, it could be regarded as a heterotrimer (LCB1, LCB2, and ssSPT) when functional, as ssSPT increases activity exponentially and is essential for cell viability in Arabidopsis (Kimberlin et al., 2013).

Studies of LCB1, LCB2, and ssSPT mutants have demonstrated that SPT activity is essential, and consequently, sphingolipids are required for the viability of plant cells (Dietrich et al., 2008; Teng et al., 2008; Kimberlin et al., 2013). In this regard, the fbr11-2 mutant of the single Arabidopsis LCB1 gene (At4g36480) displays male gametophytic lethality (Teng et al., 2008). Loss of pollen viability is also observed in double mutants of the two redundant LCB2 genes LCB2a (At5g23670) and LCB2b (At3g48780) (Dietrich et al., 2008) as well as in null mutants of ssSPTa (At1g06515), the more highly expressed of the two ssSPT genes in Arabidopsis (Kimberlin et al., 2013). Pollen deficient in sphingolipid synthesis in lcb2a/-/lcb2b/-/+ mutants lack Golgi stacks and surrounding intine layer and have vesiculated ER, all of which are consistent with the contributions of sphingolipids to the structural and functional integrity of the endomembrane system (Dietrich et al., 2008).

In the second step of LCB synthesis, the SPT product 3-ketosphinganine is reduced by the enzyme 3-ketosphinganine reductase (KSR) to form sphinganine (d18:0).
KSR is encoded by two genes in Arabidopsis thaliana, KSR-1 (At3g06060) and KSR-2 (At5g19200). Both genes are essential and contribute to the reductase activity, although KSR-1 is more highly expressed throughout the plant kingdom (Chao et al., 2011). KSR-1 and KSR-2 are functionally redundant, but KSR-1 is the primary contributor to the reductase activity (Chao et al., 2011). Loss-of-function mutants of KSR-1 are viable but display greatly reduced reductase activity (Chao et al., 2011). These mutants also display an altered leaf ionome that is associated with increased root suberization, altered root morphology, and altered root iron homeostasis (Chao et al., 2011). The sphinganine (d18:0) produced from the combined activities of SPT and KSR can be used directly by ceramide synthase or modified by hydroxylation or desaturation prior to use for ceramide synthesis.

1.4 LCB Modifications

The d18:0 LCB resulting from the sequential activities of SPT and KSR can undergo combinations of three modification reactions to generate trihydroxy and unsaturated LCBs. In Arabidopsis leaves, ~90% of the total LCBs contain three hydroxyl groups and Δ8 unsaturation. The third hydroxyl group of these LCBs occurs at the C-4 position and is introduced by a LCB C-4 hydroxylase (Sperling et al., 2001; Chen et al., 2008). This enzyme is a di-iron oxo protein with homology to desaturases and hydroxylases (Sperling et al., 2001). The two genes that encode the LCB C-4 hydroxylase in Arabidopsis are designated Sphingoid Base Hydroxylase (SBH) 1 (At1g69640) and 2 (At1g14290). Expression of these genes in mutants of the Saccharomyces cerevisiae SUR2 gene (Haak et al., 1997) that encodes a related LCB C-4
hydroxylase restores trihydroxy LCB synthesis (Sperling et al., 2001; Chen et al., 2008). It is presumed that the Arabidopsis LCB C-4 hydroxylase uses a free dihydroxy LCB as its substrate, in part, because of the prevalence of trihydroxy LCBs in the free LCB pool (Markham and Jaworski, 2007).

LCBs with Δ8 unsaturation, either in the dihydroxy or trihydroxy form, are also abundant in sphingolipids of most plant species (Dunn et al., 2004). Like the LCB C-4 hydroxylase, LCB Δ8 desaturases are di-iron oxo enzymes (Shanklin and Cahoon, 1998). The plant Δ8 LCB desaturase was originally identified in sunflower as a desaturase-like enzyme that also contains an N-terminal cytochrome b5 domain and was shown to confer production of Δ8 unsaturated LCBs when expressed in Saccharomyces cerevisiae (Sperling et al., 1995). Notably, the LCB Δ8 desaturase is not found in mammals or Saccharomyces cerevisiae, but is present in plants and filamentous or dimorphic fungi such as Pichia pastoris and Yarrowia lipolytica. Two homologs, SLD1 (At3g61580) and SLD2 (At2g46210), were identified in Arabidopsis and confirmed to be Δ8 desaturases through yeast and in planta studies (Sperling et al., 1998; Chen et al., 2012). To further add to the structural diversity found in LCBs, the Δ8 double bond can be introduced in either the cis or trans configuration (Markham et al., 2006), which likely results from presentation of LCB substrates in alternative conformations relative to the di-iron oxo atoms in the active site of these enzymes (Beckmann et al., 2002). Though evidence to date cannot preclude that at least a portion of LCB Δ8 desaturation uses free LCBs as substrates, it is presumed that these enzymes largely use LCBs bound in ceramides as substrates (Sperling et al., 1998; Beckmann et al., 2002).
Long-chain bases (LCBs) with Δ4 unsaturation are also prevalent in sphingolipids in many plant species. LCB Δ4 unsaturation occurs almost entirely in combination with LCB Δ8 unsaturation in dihydroxy LCBs. These di-unsaturated, dihydroxy LCBs (d18:2) also are found almost exclusively in ceramides of GlcCer, but absent from ceramides of GIPCs (Sperling et al., 2005; Markham et al., 2006; Markham and Jaworski, 2007). Arabidopsis contains one Δ4 desaturase gene (At4g049300) that was identified by homology to analogous genes in filamentous fungi and mammals (Ternes et al., 2002). In contrast to the LCB Δ8 desaturase, the Δ4 desaturase introduces double bonds exclusively in the trans configuration, most likely using free LCBs as substrates (Ternes et al., 2002). As a result, two d18:2 isomers occur in plants: d18:2-transΔ4, transΔ8 and d18:2-transΔ4, cisΔ8. It is notable that LCB C-4 hydroxylases and LCB Δ4 desaturase can both use d18:0 as substrates. As a result, C-4 hydroxylation precludes Δ4 desaturation, and conversely, Δ4 desaturation prevents C-4 hydroxylation.

In Arabidopsis and likely other Brassicaceae, the LCB Δ4 desaturase gene is not expressed in leaves (Michaelson et al., 2009; Luttgeharm et al., 2015). Instead, expression is limited almost entirely to flowers and, specifically, pollen, which is consistent with the occurrence of d18:2 in Arabidopsis organs (Michaelson et al., 2009; Luttgeharm et al., 2015). In most species outside of the Brassicaceae family, LCB Δ4 desaturation, as indicated by d18:2 production, occurs throughout the plant, and in species such as tomato and soybean, d18:2 is the most abundant LCB in GlcCer (Sperling et al., 2005; Markham et al., 2006).
Evidence that has emerged from sphingolipid compositional profiling of Arabidopsis mutants has shown that hydroxylation and desaturation affect metabolic outcomes in sphingolipid biosynthesis. Most notably, LCB C-4 hydroxylase mutants accumulate high levels of sphingolipids with ceramide backbones containing C16 fatty acids and dihydroxy LCBs, rather than the more typical ceramides with very long-chain fatty acids and trihydroxy LCBs (Chen et al., 2008). As discussed below, this metabolic profile arises from the substrate preferences of ceramide synthases. In addition, Arabidopsis sld1sld2 double mutants lacking LCB Δ8 unsaturation have a 50% reduction of GlcCer, perhaps due to the substrate specificity of GlcCer synthase (Chen et al., 2012). Similarly, Arabidopsis mutants for the LCB Δ4 desaturase, have a 50% reduction in GlcCer in pollen (Michaelson et al., 2009; Luttgeharm et al., 2015). This phenotype is more extreme in LCB Δ4 desaturase mutants of the yeast Pichia pastoris (Michaelson et al., 2009). Disruption of this LCB Δ4 desaturase in P. pastoris results in nearly complete loss of GlcCer, likely due to a ceramide synthase that uses exclusively LCBs with Δ4 unsaturation and dedicated to GlcCer synthesis (Michaelson et al., 2009). In addition to metabolic alterations, loss of LCB C-4 hydroxylation and Δ8 desaturation affects plant performance. LCB C-4 hydroxylase mutants have impaired growth and constitutive up-regulation of PCD (Chen et al., 2008). In addition, Arabidopsis mutants lacking LCB Δ8 unsaturation are more low temperature sensitive (Chen et al., 2012), and the relative ratio of cis-trans Δ8 unsaturation affects resistance of Arabidopsis to aluminum (Ryan et al., 2007). However, Arabidopsis LCB Δ4 desaturase mutants have no detectable impairment of pollen viability or germination or other growth phenotypes, despite the
reduction of GlcCer levels (Michaelson et al., 2009). These findings bring into question the functional importance of GlcCer for plant performance.

1.5 Sphingolipid Fatty Acid Synthesis and Structural Modifications

Carbon chain-length, unsaturation, and hydroxylation of fatty acids contribute to the structural diversity of the ceramide backbone of sphingolipids. In plants, the fatty acid component ranges from 16-26 carbon atoms (Markham and Jaworski, 2007), including small amounts of odd-chain fatty acids with 21, 23, and 25 carbon atoms (Cahoon and Lynch, 1991). In Arabidopsis leaves, C16, C24, and C26 fatty acids predominate (Markham et al., 2006; Markham and Jaworski, 2007). The C16 fatty acids of ceramides arise from palmitic acid formed by de novo fatty acid synthesis, whereas the very long-chain fatty acids or VLCFAs (i.e., fatty acids with ≥C20) of sphingolipids arise from the ER-localized reactions involving the two-carbon sequential elongation of fatty acids produced de novo in plastids (Smith et al., 2013). Each two carbon elongation cycle involves the four successive reactions catalyzed by 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase (KCR), hydroxyacyl-CoA dehydrase (HAD), and enoyl-CoA reductase (ECR). Interestingly, KCS enzymes do not occur in Saccharomyces cerevisiae (Paul et al., 2006). Instead, fatty acid elongation is initiated by ELO enzymes that are structurally unrelated, but functionally equivalent to KCS (Paul et al., 2006). Four ELO homologs occur in Arabidopsis, but no findings to date link these enzymes to the synthesis of sphingolipid VLCFAs (Smith et al., 2013). Arabidopsis mutants of the PAS2 gene (At5g10480) encoding HAD are defective in VLCFA synthesis and have been used to demonstrated the importance of sphingolipid VLCFAs
for cellular function. Partial PAS2 mutants are defective in growth and phragmoplast (cell plate) formation resulting in impaired cell division, and null PAS2 mutants display embryo lethality (Bach et al., 2008; Bach et al., 2011).

Phospholipid VLCFAs are typically saturated in the plant kingdom, but monounsaturated VLCFAs occur in sphingolipids of Brassicaceae and some Poaceae species as well as selected species from other families (Cahoon and Lynch, 1991; Dunn et al., 2004; Sperling et al., 2005; Markham et al., 2006). The double bond in sphingolipid VLCFAs of these species is at the ω-9 position (Imai et al., 2000). In Arabidopsis, this double bond is introduced by an enzyme encoded by ADS2 (At2g31360) that has homology to acyl-CoA desaturases (Smith et al., 2013). The ADS2 gene is induced by low temperatures and ADS2 null mutants display chilling sensitivity, indicating a link between sphingolipid structure and low temperature performance, as also shown for the LCB Δ8 desaturase (Chen and Thelen, 2013). Notably, ADS2-encoded enzymes do not occur in Poaceae and other monocots suggesting that a distinct pathway has evolved for biosynthesis of monounsaturated VLCFA synthesis in Poaceae.

Fatty acids in ceramides of glycosphingolipids occur almost entirely with C-2 or α-hydroxylation (Dunn et al., 2004). The C-2 hydroxyl group is introduced by a di-iron-oxo enzyme related to the Saccharomyces cerevisiae fatty acid C-2 hydroxylase encoded by the FAH1 or SCS7 gene (Haak et al., 1997; Mitchell and Martin, 1997). The Arabidopsis homologs AtFAH1 (encoded by At2g34770) and AtFAH2 (encoded by At4g20870) notably lack the N-terminal cytochrome b5 domain that is found in the Saccharomyces cerevisiae enzyme (Mitchell and Martin, 1997; König et al., 2012;
Nagano et al., 2012). Based on phenotypes in T-DNA insertion mutants and RNAi suppression lines, AtFAH1 appears to be primarily associated with hydroxylation of VLCFAs, and AtFAH2 appears to be primarily associated with hydroxylation of C16 fatty acids in planta (Nagano et al., 2012). It is presumed that AtFAH1 and AtFAH2 use fatty acids in ceramides rather than free or CoA esters of fatty acids as substrates, given that a substantial portion of fatty acids in the free ceramide pool lack C-2 hydroxylation, even though hydroxylated fatty acids predominate in glycosphingolipid ceramide backbones (Markham and Jaworski, 2007). Double mutants of the AtFAH1 and AtFAH2 genes have elevated levels of ceramides but ~25% reduction in glucosylceramide level (Konig et al., 2012). These results suggest that ceramides with C-2 hydroxylated fatty acids are important for metabolic channeling of ceramides to form glycosphingolipids, due possibly to the substrate preference of enzymes such as glucosylceramide synthase.

Suppression of PCD by ER-associated Bax inhibitor-1 protein in Arabidopsis has been shown to be dependent on functional fatty acid C-2 hydroxylases, and overexpression of the Bax inhibitor 1 gene increases fatty acid C-2 hydroxylation of ceramides through direct interaction with cytochrome b5 (Nagano et al., 2009; Nagano et al., 2012). From these findings, it has been speculated that accumulation of ceramides with fatty acids lacking the C-2 hydroxyl group initiates PCD, whereas this response is reduced when the fatty acids of these ceramides are hydroxylated (Nagano et al., 2012).

1.6 Biosynthesis of Ceramides

Ceramides are synthesized by the condensation of a long-chain base and fatty acyl-CoA through an acyltransferase-type reaction catalyzed by ceramide synthase.
Three ceramide synthases have been identified in Arabidopsis through homology with the yeast ceramide synthase encoded by LAG1 (Longevity Assurance Gene1). These enzymes are designated Lag One Homolog (LOH)-1, -2, and -3 and correspond to genes encoded by LOH1, At3g25440; LOH2, At3g19260; and LOH3, At1g13580, respectively (Markham et al., 2011; Ternes et al., 2011). Homologs of these three enzymes are found throughout the plant kingdom and appear to form two distinct evolutionary branches, LOH1/LOH3-related isoforms and LOH2-related isoforms (Markham et al., 2011; Ternes et al., 2011). Arabidopsis LOH1 and LOH3 share approximately 90% amino acid sequence identity, while LOH2 shares approximately 60% identity with LOH1 and LOH3 (Markham et al., 2011; Ternes et al., 2011). In mammals, multiple ceramide synthases are present that each have distinct specificity for fatty acyl-CoAs and/or long-chain bases (Venkataraman et al., 2002; Riebeling et al., 2003; Mizutani et al., 2005, 2006; Laviad et al., 2008). Studies of Arabidopsis LCB C-4 hydroxylase mutants initially pointed to the likelihood that two functional classes of ceramide synthases occur in plants (Chen et al., 2008). Loss of or reduced LCB C-4 hydroxylation has been shown to result in the aberrant accumulation of high levels of sphingolipids with ceramides containing C16 fatty acids bound to dihydroxy LCBs (Chen et al., 2008). Based on this observation, it was proposed that Arabidopsis has one class of ceramide synthase that links C16 fatty acyl-CoAs with dihydroxy LCBs (termed “Class I”), and a second class (“Class II”) that primarily links very long-chain fatty acyl CoAs with trihydroxy LCBs (Chen et al., 2008). This hypothesis was supported by the characterization of Arabidopsis LOH1, LOH2, and LOH3. Studies using yeast complementation showed that Arabidopsis LOH2 prefers C16 acyl-CoAs, similar to the predicted Class I ceramide synthase (Ternes et al.,
Similarly, Arabidopsis LOH2 mutants were found to be deficient in sphingolipids with ceramide backbones containing C16 fatty acids and dihydroxy fatty acids (Markham et al., 2011). Consistent with the substrate properties of Class II ceramide synthase, partial knock-out mutants of LOH1 and LOH3 contained reduced amounts of ceramides with very long-chain fatty acids and trihydroxy LCBs (Markham et al., 2011). It is notable that under ideal growth conditions, null mutants of LOH2 are viable, suggesting that the Class I ceramide synthase and hence ceramides with C16 fatty acids and dihydroxy LCBs are not essential in Arabidopsis (Markham et al., 2011). Conversely, double null mutants of LOH1 and LOH3 were not recoverable, indicating that the Class II ceramide synthase and ceramides with very long-chain fatty acids and trihydroxy LCBs are essential (Markham et al., 2011).

Ceramide synthases are known targets for competitive inhibition by sphinganine analog mycotoxins (SAMs) such as fumonisin B1 or FB1 produced by a variety of Fusarium species and AAL toxin produced by Alternaria alternata f. sp. Lycopersici (Abbas et al., 1994). These compounds, particularly FB1, have been widely used as tools for induction of programmed cell death (PCD) in plants, presumably due to the accumulation of cytotoxic LCBs from their inhibition of ceramide synthases (Stone et al., 2000). Recent evidence using FB1 treatment of Arabidopsis ceramide synthase mutants has suggested that FB1 is a more potent inhibitor of Class II ceramide synthases (i.e. LOH1 and LOH3 ceramide synthases) (Markham et al., 2011). Interestingly, in addition to accumulation of free LCBs, elevated levels of ceramides with C16 fatty acids and dihydroxy LCBs formed by Class I ceramide synthases (i.e. LOH2 ceramide synthases)
are detectable following treatment of Arabidopsis with FB1 (Markham et al., 2011). These results suggest that FB1 cytotoxicity and PCD induction may be triggered by accumulated ceramides rather than or in addition to accumulated LCBs. FB1 has also been used as a tool to study sphingolipid homeostasis in plants based on the observation that down-regulation of serine palmitoyltransferase (SPT) activity reduces FB1 cytotoxicity and up-regulation of SPT activity enhances sensitivity of plants to FB1 (Shi et al., 2007; Kimberlin et al., 2013).

1.7 Glucosylceramide Synthesis

Following its synthesis by Class I or Class II ceramide synthases, the ceramide backbone can be glycosylated at its C-1 hydroxyl group to form either of two classes of glycosphingolipids: glucosylceramides (GlcCer) or glycosylinositolphosphoceramides (GIPCs). GlcCer is the simplest glycosphingolipid and occurs broadly in eukaryotes, with the notable exception of Saccharomyces cerevisiae (Dunn et al., 2004). GlcCer consist of a glucose bound to the ceramide backbone by a 1,4-glycosidic linkage and are formed by the condensation of a ceramide substrate with UDP-glucose (Leipelt et al., 2001). This reaction is catalyzed by GlcCer synthase, an ER-localized enzyme in Arabidopsis that is encoded by At2g19980 (Melser et al., 2010). Compared to GIPCs, GlcCers are more enriched in ceramides with C16 fatty acids and dihydroxy LCBs (Sperling et al., 2005; Markham et al., 2006). In plants such as tomato and soybean, ceramides with C16 fatty acids and the LCB d18:2 predominate (Sperling et al., 2005; Markham et al., 2006). Based on this composition, it appears that a large portion of the GlcCer ceramide backbone is channeled from Class I-type ceramide synthases that have
substrate preference for C16 fatty acids and dihydroxy LCBs (Markham et al., 2011). Although it is an abundant glycosphingolipid in plants, null mutants of the LCB Δ4 desaturase in Arabidopsis have 30% reductions in GlcCer levels in flowers (Michaelson et al., 2009) and 50% reduction in GlcCer levels in pollen (Luttgeharm et al., 2015) without any apparent effect on flower and pollen physiology or function (Michaelson et al., 2009). GlcCer synthase is potently inhibited by d,l-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Melser et al., 2010). Treatment of Arabidopsis roots with PDMP results in altered Golgi morphology, including reduced numbers of Golgi stacks, and defects in endomembrane trafficking (Melser et al., 2010; Melser et al., 2011). PDMP application to Arabidopsis root cells has also been shown to result in rapid vacuolar fusion and altered vacuole morphology including the appearance of vacuolar invaginations (Kruger et al., 2013). Arabidopsis GlcCer synthase mutants devoid of GlcCer have yet to be described. Such mutants will clarify whether GlcCer is essential in plants, which is questionable because viable fungal cells can be recovered that lack GlcCer (Michaelson et al., 2009; Rittenour et al., 2011).

1.8 Inositolphosphoceramide Synthesis

As an alternative fate to GlcCer synthesis, ceramides can be used for the production of GIPCs. GIPCs, which are approximately two-fold more abundant in Arabidopsis leaves than GlcCer, are typically enriched in ceramides with VLCFAs and trihydroxy LCBs that arise from Class II ceramide synthases (Markham et al., 2006). The first step in GIPC synthesis occurs by the transfer of the inositolphosphoryl head group of phosphatidylinositol (PI) onto ceramide to form inositolphosphoceramides
(IPCs) (Wang et al., 2008; Mina et al., 2010). This activity is catalyzed by IPC synthase, a phosphatidic acid phosphatase-2 (PAP2)-related enzyme, that is encoded by three genes in Arabidopsis: IPCS1 (or ERH1), At2g37940; IPCS2, At2g37940; IPCS3, At2g29525. In contrast to the ER localization of GlcCer synthase, IPC synthases predominantly occur in Golgi bodies of Arabidopsis. Plant IPC synthases are most closely related to analogous enzymes in the protozoa Leishmania major and Trypanosoma brucei than to the Saccharomyces cerevisiae IPC synthase (encoded by the AUR1 gene) (Wang et al., 2008; Mina et al., 2010). Despite this, the three Arabidopsis IPC synthase genes are able to rescue lethality associated with the loss of IPC production in the Saccharomyces cerevisiae AUR1 mutant (Wang et al., 2008; Mina et al., 2010). Although triple mutants of the three Arabidopsis IPC synthase genes have not been reported, it is presumed that IPC biosynthesis is essential, although the three genes are likely partially redundant.

Following the synthesis of IPC, up to seven additional sugar residues can be added to the inositolphosphoryl head group to form an array of different GIPCs (Bure et al., 2011; Cacas et al., 2013). The first residue added to the inositolphosphoryl head group is a glucuronic acid moiety (Rennie et al., 2014). This reaction, which uses a UDP-glucuronic acid as substrate, was recently shown to be catalyzed by a glycosyltransferase encoded by IPUT1 (At5g18480) in Arabidopsis (Rennie et al., 2014). T-DNA null mutants of IPUT1 are not transmitted through pollen, indicating that this gene is essential in Arabidopsis (Rennie et al., 2014). The remaining glycosyltransferases associated with GIPC synthesis have yet to be identified. Interestingly, a Golgi lumen-localized GDP-mannose transporter encoded by Golgi-
Localized Nucleotide Sugar Transporter 1 (GONST1, At2g13650) in Arabidopsis was found to be deficient in GIPCs with glycosylation beyond the glucuronic acid introduced by the IPUT1-encoded glycosyltransferase (Mortimer et al., 2013). This suggests that like Saccharomyces cerevisiae, Arabidopsis GIPCs contain mannose, presumably bound predominantly to glucuronic acid moiety. Interestingly, GONST1 mutants display a dwarfed phenotype, constitutive induction of the hypersensitive response, and elevated salicylic acid levels, consistent with a connection between GIPC synthesis and plant pathogen defense (Mortimer et al., 2013).
Figure 1.2 Biosynthetic pathway for sphingolipids in plants. Abbreviations: LCB, long-chain base; Glc, glucose; PI, phosphatidylinositol; DAG, diacylglycerol; IP, inositolphosphate; GIPCase, glycosyl inositolphosphoceramidase; IPUT1, inositol phosphorylceramide glucuronosyltransferase 1.
1.9 LCB and Ceramide Phosphorylation/De-Phosphorylation

In addition to their occurrence in free form and in ceramides, LCBs are also detectable in low levels as phosphate derivatives that have been attributed to triggers of physiological responses, such as ABA-dependent guard cell closure (Ng et al., 2001; Coursol et al., 2003). Phosphorylation of LCBs at their C-1 hydroxyl group is catalyzed by LCB kinases (often referred to sphingosine kinases or SPHKs). To date three LCB kinases have been identified in Arabidopsis: SPHK1 (At5g23450), SPHK2 (At2g46090), and LCBK1 (At5g23450) (Imai and Nishiura, 2005; Worrall et al., 2008; Guo et al., 2012). Release of the phosphate group from LCB-P molecules is catalyzed by the enzyme LCB-P phosphatase, which are encoded by two genes in Arabidopsis (At3g58490 and At5g03080) (Worrall et al., 2008; Nakagawa et al., 2012). As described below, the interplay between LCB kinases and LCB-P phosphatases are believed to be important for signaling pathways in plants (Worrall et al., 2008; Nakagawa et al., 2012).

Similar to LCBs, ceramides can also be found in phosphorylated forms. Because ceramide-1-phosphates are believed to be of low abundance in plants, they have proven difficult to measure by recently developed mass spectrometry-based protocols. Mutants of the proposed ceramide kinase (encoded by At5g51290), termed accelerated death 5 or acd5 display spontaneous onset of programmed cell death or PCD in late development (Greenberg et al., 2000; Liang et al., 2003). This is accompanied by enhanced accumulation of ceramides (Greenberg et al., 2000; Liang et al., 2003). This observation led to the hypothesis, now accepted as dogma, that elevation of ceramide levels triggers PCD in plants (Greenberg et al., 2000; Liang et al., 2003) via accumulation of
mitochondrial-derived hydrogen peroxide (Bi et al., 2014). A ceramide-1-phosphate phosphatase, that would convert ceramide-1-phosphates to their free form have yet to be identified in plants.

1.10 Sphingolipid Turnover

The net content and composition of sphingolipids in membranes are determined by rates of synthesis and turnover. Little is currently known about rates of sphingolipid turnover, and the contributions of sphingolipid catabolism to membrane function and plant responses to altered environmental conditions. Also unexplored to date in plants are enzymes associated with removal of glycosphingolipid head groups, although candidate genes have been proposed (Chen et al., 2010). More is known about ceramide turnover. Enzymes known as ceramidases convert ceramides to free LCBs and fatty acids. Ceramidases are classified into three distinct forms based upon their optimal pH preferences in in vitro assays: acid, neutral, and alkaline ceramidases (Mao and Obeid, 2008). Three predicted neutral ceramidase genes and one predicted alkaline ceramidase have been identified by homology with human ceramidase genes (Chen et al., 2010; Wu et al., 2015). The Arabidopsis alkaline ceramidase homolog, AtACER (At4g22330), has been shown to function as a ceramidase with mutant and RNAi suppression lines for this gene having elevated ceramide levels and increased salt sensitivity and enhanced susceptibility to a bacterial pathogen (Wu et al., 2015). A second gene, TOD1, corresponding to At5g46220 was recently shown to encode a polypeptide with alkaline ceramidase activity, but notably, this protein lacked close homology to known alkaline ceramidases (Chen et al., 2015). Based on mutant phenotypes, this enzyme was linked to
control of turgor pressure in pollen tubes and siliques guard cells. A neutral ceramidase has also been cloned from rice and confirmed in vitro to be a member of the neutral ceramidase subclass (Pata et al., 2008).

LCBs, including those released by ceramidase activity, can be degraded following phosphorylation by LCB kinases (Figure 1.3). This process is catalyzed by LCB-P lyase (often referred to as DPL1, based on homology to the yeast enzyme), which generates C16-fatty aldehyde and phosphoethanolamine from a C18 LCB-P. Arabidopsis contains only a single DPL1 gene (At1g27980) (Tsegaye et al., 2007; Nishikawa et al., 2008; Worrall et al., 2008) that is strongly upregulated by senescence (Tsegaye et al., 2007). Null mutants of DPL1 displayed small increases in accumulation of the LCB-P t18:1-P, but surprisingly no obvious growth phenotypes (Tsegaye et al., 2007).

**Figure 1.3** Ceramide and LCB phosphorylation pathway. Dashed arrows represent enzymatic steps involved in degradation. Abbreviations: LCB, long-chain base; LCB-P, long-chain base-1-phosphate; Glc, glucose; PI, phosphatidylinositol; DAG, diacylglycerol; IP, inositolphosphate; IPCase, inositolphosphoceramidase.
1.11 Sphingolipids and Membrane Structure/Function

Sphingolipids compose an estimated ~40% of the total lipids in plasma membrane of plant tissues (Sperling et al., 2005), where they are enriched in the outer leaflet (Voorhout et al., 1991; Burger et al., 1996; Tjellstrom et al., 2010). Sphingolipids are also abundant lipid components of other endomembranes in the plant, including ER, Golgi, and tonoplast (Verhoek et al., 1983; Mongrand et al., 2004; Sperling et al., 2005; Bayer et al., 2014). GlcCer was first identified in the plasma membrane and tonoplast of plant cells in a number of studies conducted in the 1980s (Verhoek et al., 1983; Yoshida et al., 1986; Lynch and Steponkus, 1987; Cahoon and Lynch, 1991). In these membranes, GlcCer was reported to compose between 7% to 30% of plasma membrane and tonoplast, depending on the plant species and tissue type analyzed (Verhoek et al., 1983; Yoshida et al., 1986; Lynch and Steponkus, 1987; Cahoon and Lynch, 1991; Uemura and Steponkus, 1994; Uemura et al., 1995). More recently, it has been shown that GIPCs, rather than GlcCer, are the more abundant glycosphingolipid in plants (Markham et al., 2006; Markham and Jaworski, 2007). The quantitative importance of GIPCs was largely overlooked until very recently because their highly glycosylated head groups offer challenges for extraction using typical organic solvents, such as mixtures of chloroform and methanol (Markham et al., 2006). Initially, by quantification of long-chain bases of GIPCs and GlcCer and later by LC-MS/MS analysis, GIPCs were found to be nearly two-fold more abundant than GlcCer in Arabidopsis leaves, whereas amounts of GIPCs and GlcCer were nearly the same in tomato leaves (Markham et al., 2006; Markham and Jaworski, 2007). GIPCs have subsequently been identified as one of the
most abundant lipids of plant plasma membrane and are also enriched in detergent resistant membranes (DRMs) derived from isolated plasma membrane and in plasmodesmata (Cacas et al., 2012; Grison et al., 2015). Given their abundance in the plasma membrane and tonoplast, it is likely that the content and composition of sphingolipids affect the ability of plants to respond to abiotic stress, particularly osmotic stresses such as freezing, drought, and salinity. For example, GlcCer concentrations were shown to decrease by nearly half in plasma membrane during cold acclimation of rye and Arabidopsis (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Uemura et al., 1995). More recently, it was reported that GIPCs increase and GlcCer decrease in response to chilling of Arabidopsis (Nagano et al., 2014). Although this is likely an adaptive response to low temperatures, the impact of such adjustments in relative amounts of GIPCs and GlcCer on plant performance has not yet been established. In addition, the fatty acid and long-chain base composition of sphingolipids also affects plant resistance to abiotic stress. For example, Arabidopsis mutants lacking LCB Δ8 unsaturation and ceramide fatty acid unsaturation display sensitivity to low temperature growth and alterations in the relative amounts of LCB cis-trans Δ8 unsaturation affects resistance of plants to aluminum (Ryan et al., 2007; Chen et al., 2012; Chen and Thelen, 2013).

The unique structural components of sphingolipid hydrophobic ceramide backbones include VLCFAs and an abundance of hydroxyl groups distributed between LCB and fatty acid moieties. Through these structural features, sphingolipids confer rigidity to membranes. In addition, the hydroxyl groups enable the formation of
extensive hydrogen bonding networks that result in elevated phase transition temperatures and reduced ion permeability (Pascher, 1976; Lunden et al., 1977). The rigidity and high phase transition temperatures of sphingolipid micelles is moderated by interactions with other lipids, including sterols (Curatolo, 1987). Sphingolipids have been shown to cluster with sterol in membrane microdomains or lipid rafts (Cacas et al., 2012). Lipid microdomains have long been hypothesized to be present in membranes (Karnovsky et al., 1982) with sphingolipids potentially aiding in the sorting of membrane proteins, such as GPI-anchored proteins, by forming lipid domains that slow lateral protein diffusion (Simons and van Meer, 1988; van Meer and Simons, 1988; Simons and Ikonen, 1997; de Almeida et al., 2003). Pure sphingolipid membranes do form a ‘solid gel’ phase with little lateral movement that is fluidized by the presence of sterols (Estep et al., 1980; Roche et al., 2008; van Meer et al., 2008; Grosjean et al., 2015). The co-localization of sterols and sphingolipids in the membrane may be due to sphingolipids complex sugar head group’s ability to shield the non-polar sterol from the bulk solvent (Huang and Feigenson, 1999; Ali et al., 2006). Raft formation is also dependent on the various sphingolipid modifications, including fatty acid chain length and fatty acid and LCB hydroxylation (Klose et al., 2010).

Proteomic analysis of DRMS prepared from plant plasma membrane has revealed an enrichment of proteins in lipid rafts related to signaling, responses to biotic and abiotic stress, cellular trafficking, auxin transport, and cell wall synthesis and degradation (Brodersen et al., 2002; Morel et al., 2006; Lefebvre et al., 2007; Lin et al., 2008), suggesting that raft regions contribute to these cellular functions. Similar to DRMs,
plasmodesmata have also recently been shown to be enriched in sphingolipids and sterols and contain specific GPI-anchored proteins (Bayer et al., 2014; Grison et al., 2015).

Given their abundance in the endomembrane system, sphingolipids are presumed to play a major role in ER export and Golgi-mediated trafficking of proteins through the secretory system. Consistent with this, Arabidopsis pollen deficient in sphingolipids have been shown to have vesiculated ER and lack Golgi stacks (Dietrich et al., 2008). Consistent with defects in Golgi-trafficking to the plasma membrane, the sphingolipid-deficient pollen lacked a surrounding intine layer (Dietrich et al., 2008). Similarly, chemical inhibition of GlcCer synthesis has been shown to alter Golgi morphology and impair Golgi-mediated trafficking of secretory proteins to the plasma membrane (Melser et al., 2010; Melser et al., 2011). Recent studies using an Arabidopsis KSR mutant and GlcCer synthase and ceramide synthase inhibitors demonstrated the importance of sphingolipids in trafficking of ATP-binding cassette B19 (ABCB19) auxin transporter to the Golgi, trans-Golgi network, and plasma membrane (Yang et al., 2012). Similar studies targeting sterols indicated that sterols have greater significance for post-Golgi transport of ABCB19 from trans-Golgi to the plasma membrane (Yang et al., 2012). Of particular importance for trafficking of proteins through the secretory system is the presence of the very long-fatty acid (VLCFA) component of sphingolipids, which are enriched in GIPCs. Mutants defective in VLCFA synthesis or that have reduced activity of Class II ceramide synthases that incorporate VLCFAs in ceramides have impaired trafficking of secretory proteins, including PIN1 and AUX1 that are required for auxin
transport and plant growth (Zheng et al., 2005; Bach et al., 2008; Bach et al., 2011; Markham et al., 2011).

1.12 Sphingolipids as Physiological Mediators

In addition to the contributions of the glycosphingolipids GIPCs and GlcCer to membrane structure and function, the less abundant sphingolipid biosynthetic intermediates LCBs, LCBPs, ceramides, and ceramide-1-phosphates have been linked to the mediation of numerous, diverse physiological processes in plant cells. An important contributor to the formation of these physiological mediators are kinase and phosphatase reactions that convert LCBs and ceramides between their phosphorylated and free forms, as described above. The phosphorylation status of LCBs and ceramides are key to the particular physiological process that they regulate.

One of the first links between sphingolipids and control of cellular processes was the observation that the LCB-P sphingosine-1-phosphate or S1P participates in the ABA-mediated signaling pathway that controls stomatal aperture by elevating cytosolic Ca$^{2+}$ levels which, in turn, activates ion channels in guard cell membranes, with the resulting K$^+$ efflux causing loss of guard cell turgor pressure and stomatal closure (Kim et al., 2013). After drought treatment of the plant Commelina communis, S1P levels were found to increase in leaves, and when applied exogenously, S1P resulted in a Ca$^{2+}$ spike followed by stomatal closure (Ng et al., 2001). Phytosphingosine-1-phosphate elicits the same response, although sphinganine-1-phosphate does not, indicating some level of LCB specificity in the mediation of guard cell closure (Coursol et al., 2003). Treatment of Arabidopsis plants with ABA was found to activate LCB kinase, and this activity was
sensitive to the mammalian LCB kinase inhibitor, N, N-dimethylsphingosine (Coursol et al., 2003). As in mammals, the target of S1P in plants is presumed to be a G-protein coupled receptor, and Arabidopsis mutants lacking the G-protein α-subunit (GPA1) did not respond upon exogenous S1P application (Coursol et al., 2005). More recently, a connection between phospholipids and sphingolipids in the signaling pathway for ABA-dependent guard cell closure has been proposed. In this regard, ABA and phosphatidic acid (PA) produced by phospholipase Dα1 (PLDα1) have been shown to activate sphingosine kinase (SPK) to promote production of LCB-P (Guo et. al., 2012; Worral et. al., 2008). PA enhancement of SPK activity was found to occur by direct interaction of PA with this enzyme (Guo et. al., 2012; Guo and Wang, 2012). Given that LCB-P induction of guard cell closure requires a functional PLDα1, it was proposed that LCB-P functions upstream of PLDα1 in this signaling pathway (Guo et. al. 2012; Guo and Wang, 2012).

Sphingolipids, primarily in the form of ceramides and LCBs, have also been strongly implicated in mediation of programmed cell death (PCD) in plants. As described above, an initial indication of the role of ceramides as PCD triggers was obtained from the Arabidopsis acd5 mutant that is defective in a proposed ceramide kinase (Greenberg et. al., 2000; Liang et. al., 2003). This mutant accumulates enhanced levels of free ceramides and displays early onset of PCD relative to wild-type controls, resulting in part to the enhanced release of mitochondrial reactive oxygen species (Bi et. al., 2014). PCD induction in the acd11 mutant has also been linked to ceramide accumulation associated with defects in ceramide-1-phosphate transport in this mutant.
(Simanshu et. al., 2014). Similar findings have been obtained by treatment of Arabidopsis cell cultures with C2 ceramide at a concentration of 50 µM (Townley et. al., 2005). This treatment induces a transient increase in cytosolic Ca\(^{2+}\) and hydrogen peroxide production, followed by cell death, which was reversed by inhibition of Ca\(^{2+}\) release (Townley et. al., 2005). These findings implicate Ca\(^{2+}\) as an essential component of ceramide induction of PCD. Notably, C2 ceramides containing 2- or α-hydroxylated fatty acids were not effective in PCD induction in Arabidopsis cell cultures (Townley et. al., 2005). Consistent with this observation, the ability of Bax inhibitor-1 (BI-1) to suppress cell death in Arabidopsis is dependent of 2-hydroxylation of ceramide VLCFAs (Nagano et. al., 2012).

Similar to results with ceramides, application of the free LCBs d18:1, d18:0, and t18:0 to Arabidopsis leaves also induces PCD, albeit at concentrations lower than that observed with ceramides (Shi et. al., 2007). This induction of PCD was also dependent on ROS generation, but was suppressed by application of LCB-P along with free LCBs (Alden et. al. 2011; Shi et. al., 2007). These findings suggest that the ratio of free LCB to LCB-P, mediated by LCB kinases and LCB-P phosphatases, is an important “rheostat” for regulation of PCD (Alden et. al. 2011; Shi et. al., 2007). This is analogous to the dependence of PCD induction on relative levels of ceramides and ceramide-1-phosphates (Greenberg et. al., 2000; Liang et. al., 2003). The transduction pathway for elicitation of PCD by free LCBs has been shown to be dependent in Arabidopsis on mitogen-activated protein kinase 6 (MPK6) (Saucedo-Garcia et. al., 2011) as well as 14-3-3 protein
phosphorylation by calcium-dependent kinase 3 (CPK3) that is activated by LCB-triggered release of cytosolic Ca\(^{2+}\) (Lachaud et al., 2013).

Fungal-derived sphingosine-analog mycotoxins (SAMs) including fumonisin B1 produced by Fusarium species and AAL toxin produced by Alternaria alternata lycopersici are also potent triggers of PCD in plants. These molecules competitively inhibit ceramide synthases leading to the accumulation of free LCBs that, in turn, elicit PCD (Abbas et al., 1994). Consistent with this, reduction of LCB synthesis by chemical inhibition of serine palmitoyltransferase (SPT) activity enhances resistance of plants to SAMs (Spassieva et al., 2002). Increased resistance to FB1-triggered PCD induction has also been observed in an Arabidopsis LCB1 mutant and in ssSPT RNAi suppression lines that have reduced SPT activity (Kimberlin et al., 2013; Shi et al., 2007). Recent evidence has also emerged that FB1 not only increases levels of free LCBs in plant cells but also elevates levels of ceramides containing C16 fatty acids (Markham et al., 2011; Ternes et al., 2011). This finding suggests that FB1 most effectively inhibits Class II ceramide synthases (i.e., LOH1, LOH3) that produce ceramides with VLCFAs and are less effective inhibitors of Class I ceramide synthase (i.e., LOH2) that produces ceramides with C16 fatty acids (Markham et al., 2011; Ternes et al., 2011). These findings suggest that the potency of SAMs for PCD-induction is due to their ability to enhance accumulation of LCBs and ceramides.

The hypersensitive response (HR) is an important process for resistance to bacterial and fungal pathogens that is characterized by localized induction of PCD that reduces or prevents the spread of pathogens in plants. Given the importance of LCBs and
ceramides to PCD induction, a considerable body of research has emerged linking sphingolipids to bacterial and fungal pathogen resistance as described in a recent review (Berkey et. al., 2012). Notably, ceramide accumulation in acd5 and acd11 mutants has been shown to be associated with salicylic acid (SA)-dependent upregulation of HR-type PCD and pathogen-resistance genes, including genes for PR1, ERD11, and chitinase (Brodersen et. al., 2002; Greenberg et. al., 2000). More recently, Arabidopsis mutants defective in 2-hydroxylation of ceramide fatty acids were found to have elevated LCB and ceramide levels, as well as, increased levels of free and glycosylated SA and constitutive induction of PR1 and PR2 genes (Konig et. al., 2012). These mutants also displayed enhanced resistance to the biotrophic fungal pathogen Golovinomyces cichoracearum (Konig et. al., 2012). In addition, infection of Arabidopsis with the bacterial pathogen Pseudomonas syringae was accompanied by transient increases in the LCB phytosphingosine (t18:0) and induction of ROS and cell death (Bach et. al., 2011; Peer et. al., 2010). Furthermore, resistance to the bacterial pathogen Pseudomonas cichorii was compromised in tobacco upon chemical inhibition of SPT and an accompanying reduction in LCB synthesis (Takahashi et. al., 2009). This resistance appears to be mediated by MPK6, as FB1-elicited Arabidopsis mpk6 mutants displayed reduced resistance to the bacterial pathogen Pseudomonas syringae pv. tomato avrRpm1 due to compromised induction of PCD in this mutant (Saucedo-Garcia et. al., 2011).

Sphingolipids as abundant components of plasma membrane and tonoplast contribute to the ability of plants to resist chilling and freezing stresses. As evidence of this, Arabidopsis mutants lacking LCB Δ8 unsaturation have increased sensitivity to
prolonged exposure to low, non-freezing temperatures (Chen et. al., 2012). In addition to their roles as membrane components, recent studies have implicated sphingolipids in cold stress signaling pathways (Cantrel et. al., 2011; Guillas et. al., 2011). Exposure of Arabidopsis plants to 4°C resulted in accumulation of PA and nitrous oxide (NO). In addition, within five minutes of this cold treatment amounts of the LCB-P phytosphingosine phosphate and ceramide-1-phosphate increased by ~50% (Cantrel et. al., 2011). This increase was negatively regulated by NO, as chemical inhibition of NO production enhanced the accumulation of these molecules but chemically-induced enhancement of NO levels reduced accumulation of the phosphorylated LCB and ceramides (Cantrel et. al., 2011). From these findings, it was suggested that NO may regulate the relative levels of phosphorylated and dephosphorylated LCBs and ceramides as part of a rapid signaling response pathway to low, non-freezing temperatures (Cantrel et. al., 2011; Guillas et. al., 2011). The mechanistic details of this potential signaling pathway remain uncharacterized.

1.13 SPT Regulation

SPT is generally regarded as the main regulated step in sphingolipid biosynthesis with regulation potentially occurring at the transcriptional and post-transcriptional levels. Studies of knockout mutants in Arabidopsis thaliana have definitively shown that sphingolipid synthesis is essential for the viability of plant cells (Dietrich et. al., 2008; Kimberlin et. al., 2013; Teng et. al., 2008). The Arabidopsis thaliana LCB1 is encoded by one gene (At4g36480), whose RNAi suppression leads to reduced growth and whose single mutant, fbr11-2, displays male gametophytic lethality (Chen et. al., 2006; Teng et.
al., 2008). The Arabidopsis thaliana LCB2 is encoded by two genes designated $LCB2a$ (At5g23670) and $LCB2b$ (At3g48780) that are constitutively expressed and encode redundant polypeptides (Dietrich et. al., 2008). Mutants containing a homozygous knock-out of one gene and a heterozygous knock-out for the second gene contain 50% aborted pollen, consistent with male gametophytic lethality resulting from loss of sphingolipid synthesis (Dietrich et. al., 2008). The Arabidopsis thaliana ssSPT is encoded by two genes designated $ssSPTa$ (At1g06515) and $ssSPTb$ (At2g30942) that are constitutively expressed and encode redundant polypeptides (Kimberlin et. al., 2013). While both ssSPT genes are constitutively expressed, ssSPTa is predominantly expressed throughout the plant and is the primary gene expressed in pollen. Mutants containing homozygous knock-out of ssSPTb are viable and appear normal. Mutants containing a heterozygous knock-out of ssSPTa contain 50% aborted pollen, consistent with male gametophytic lethality associated with loss of sphingolipid synthesis (Kimberlin et. al., 2013). Increased expression of ssSPTb allows for mutants containing homozygous knock-out of ssSPTa to be viable. The ssSPT genes appear to be limiting in Arabidopsis thaliana as modulation of their expression leads to predictable changes in SPT activity.

In yeast, the ORM proteins have been shown to act as homeostatic negative regulators of SPT in response to sphingolipid levels (Breslow et. al., 2010; Han et. al., 2010; Roelants et. al., 2011). The mechanism has recently been established in Saccharomyces cerevisiae and involves TORC2-dependent phosphorylation of ORM to gradually relieve ORM suppression of SPT (Muir et. al., 2014; Roelants et. al., 2011). It is unclear if this mechanism is conserved in other eukaryotes as the phosphorylatable
residues in the N-terminal region of yeast ORM are not conserved (Roelants et. al., 2011). Arabidopsis has two potential homologs, ORM1 (At1g01230) and ORM2 (At5g42000), that have been identified by sequence homology and have been shown to inhibit SPT activity and, interestingly, impact ceramide synthase activity. In yeast, double knockout of both ORMs leads to sickly, but viable, cells with severe sensitivities to temperature and other stressors. Arabidopsis ORM has been shown to complement the yeast knockout phenotype indicating that Arabidopsis ORM can at least inhibit yeast SPT. RNAi suppression mutants of ORM in rice show temperature sensitivity and pollen abnormalities which are consistent with altered sphingolipid synthesis (Chueasiri et. al., 2014). It is possible that this is a conserved function, but to date no other plant ORMs have been examined leaving the regulation of SPT activity an active area of research.

The regulation of SPT is thought to primarily occur through the ssSPT and ORM proteins. The ssSPTs appear to be rate limiting and so modulation of their expression alters SPT activity. This has been shown synthetically in Arabidopsis thaliana with over-expression and RNAi suppression of ssSPTs leading to increased and reduced SPT activity respectively (Kimberlin et. al., 2013). The ssSPTs may also serve a more subtle role by regulating the type of LCBs produced by SPT. It is unclear if this occurs in plants, but human ssSPTs have been shown to confer different acyl-CoA specificity when bound to SPT leading to the production of either C18 or C20 LCBs (Han et. al., 2009). This specificity was also shown to be caused by a single amino acid residue that when changed from methionine to valine or valine to methionine could alter SPT acyl-CoA preference (Han et. al., 2009). In Arabidopsis thaliana ssSPT this residue is a methionine
in both isoforms, but when mutated to valine confers the production of unusual C20 LCBs in the plant. This type of regulation, caused by the ssSPT amino acid difference, is unlikely to occur in *Arabidopsis thaliana* given that nearly all LCB produced is C18, but it may occur in other plants and act as a regulatory control point for the types of LCBs produced. The ssSPTs in *Arabidopsis thaliana* may also serve to regulate SPT through differential expression of each isoform, as in vitro enzymatic assays have shown different SPT activity levels with each isoform (Kimberlin et. al., 2013). There is currently no evidence for post-translational modifications playing a role in ssSPT regulatory function, and it does not appear that ssSPT has any known enzymatic activity.

Regulation of SPT by the ORM proteins appears to be more complex and the mechanism is unclear in plants and other eukaryotes outside of yeast. In yeast, the primary regulatory mechanism of ORM occurs through TORC2 dependent YPK1 phosphorylation of ORM that relieves inhibition of SPT that can be reversed by SAC1 phosphatase activity that restores inhibition of SPT (Muir et. al., 2014; Roelants et. al., 2011). This mechanism is adjustable and dependent on sphingolipid levels and has been shown to be coordinated with ceramide synthase activity (Muir et. al., 2014). Unfortunately the mechanism established in yeast does not appear to occur in plants and it is unclear even if ORMs in plants are phosphorylated. An N-terminal extension of approximately 80 amino acids in yeast ORM was found to contain several serines that are responsible for this phosphorylation mechanism (Breslow et. al., 2010; Han et. al., 2010). All other eukaryotic ORMs outside of yeast are lacking this N-terminal extension. While ORM homologs can be found throughout the plant kingdom the lack of the yeast N-
terminal extension makes it hard to extrapolate ORM regulation of SPT from yeast, ultimately more research is needed to fully understand how the plant ORMs impact sphingolipid metabolism.
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Chapter 2:

Arabidopsis 56-amino acid serine palmitoyltransferase-interacting proteins stimulate sphingolipid synthesis, are essential, and affect mycotoxin sensitivity.

Note: The results in this chapter have been previously published, some text has been modified from the original.

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2.1 Abstract

Maintenance of sphingolipid homeostasis is critical for cell growth and programmed cell death (PCD). Serine palmitoyltransferase (SPT), composed of long chain base subunit 1 (LCB1) and long chain base subunit 2 (LCB2), catalyzes the primary regulatory point for sphingolipid synthesis. Small subunits of SPT (ssSPT) that strongly stimulate SPT activity have been identified in mammals, but the role of ssSPT in eukaryotic cells is unclear. Candidate Arabidopsis thaliana ssSPTs, ssSPTa and ssSPTb, were identified and characterized. Expression of these 56-amino acid polypeptides in a yeast SPT null mutant stimulated SPT activity from the Arabidopsis LCB1/LCB2 heterodimer by >100-fold through physical interaction with LCB1/LCB2. ssSPTa transcripts were more enriched in all organs, and >400-fold more abundant in pollen than ssSPTb transcripts. Accordingly, homozygous ssSPTa T-DNA mutants were not recoverable, and 50% non-viable pollen was detected in heterozygous ssspta mutants. Pollen viability was recovered by expression of wild-type ssSPTa or ssSPTb under control of the ssSPTa promoter, indicating ssSPTa and ssSPTb functional redundancy. SPT activity and sensitivity to the PCD-inducing mycotoxin fumonisin B1 (FB1) were increased by ssSPTa overexpression. Conversely, SPT activity and FB1 sensitivity were reduced in ssSPTa RNAi lines. These results demonstrate that ssSPTs are essential for male gametophytes, are important for FB1 sensitivity, and limit sphingolipid synthesis in planta.
2.2 Introduction

Sphingolipids are essential components of eukaryotic cells with diverse roles in membrane structure and function and mediation of basic cellular processes such as programmed cell death (PCD) (Brodersen et al., 2002; Liang et al., 2003; Alden et al., 2011; Markham, 2013). In plants, sphingolipids are major lipid components of the endomembrane system, plasma membrane, and tonoplast and contribute to membrane physical properties that are important for environmental stress tolerance (Verhoek et al., 1983; Lynch and Steponkus, 1987; Sperling et al., 2005; Chao et al., 2011; Chen et al., 2012). Endomembrane-associated sphingolipids also participate in Golgi-mediated protein trafficking that affects processes such as polar auxin transport (Borner et al., 2005; Aubert et al., 2011; Markham et al., 2011; Yang et al., 2012). In addition, sphingolipids contribute to the structural integrity of raft-like domains in the plasma membrane that are important for cell surface activities including cell wall synthesis and degradation, signaling, and trafficking (Mongrand et al., 2004; Borner et al., 2005; Melser et al., 2011). Beyond their functions in membranes, sphingolipids, acting through their ceramide and long-chain base precursors and metabolites, are increasingly regarded as signaling molecules for regulation of a number of physiological processes (Liang et al., 2003; Coursol et al., 2005; Donahue et al., 2010). Ceramide and long-chain base accumulation has been shown to trigger programmed cell death, which may be important for the hypersensitive response for pathogen defense (Liang et al., 2003; Saucedo-Garcia et al., 2011; Konig et al., 2012). PCD induction by long-chain base accumulation appears to be the mode of action for sphinganine analog mycotoxins (SAM) including fumonisins.
B1 (FB1) and *Alternaria alternata lycopersici* (AAL) toxin (Abnet et al., 2001; Brandwagt et al., 2002). In addition, phosphorylated forms of long-chain bases have been implicated in ABA-dependent guard cell closure and low temperature signaling in plants (Coursol et al., 2003; Coursol et al., 2005; Chen et al., 2012; Guillas et al., 2012).

Maintenance of sphingolipid homeostasis is critical for all eukaryotic cells. Sphingolipid homeostasis, for example, is a central component of the regulation of apoptotic pathways in human cells (Rotolo et al., 2005; Chipuk et al., 2012). In plants, cell growth via expansion is dependent on sphingolipid synthesis, and elimination of sphingolipid biosynthesis results in loss of gametophytic and sporophytic cell viability (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008). Conversely, accumulation of ceramides and long-chain bases triggers PCD (Liang et al., 2003; Saucedo-Garcia et al., 2011). Sphingolipid homeostasis is generally believed to be mediated by regulation of serine palmitoyltransferase (SPT), the first enzyme in long-chain base biosynthesis that catalyzes the condensation of serine with typically palmitoyl (16:0)- or stearoyl (18:0)-CoA (Hanada, 2003). Similar to other eukaryotes, the *Arabidopsis* SPT is a heterodimer consisting of long chain base subunit 1 (LCB1) and long chain base subunit 2 (LCB2) (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008). Together, LCB1 and LCB2 form a heterodimer with pyridoxal phosphate-dependent activity. LCB1 is encoded by a single gene (Chen et al., 2006), in *Arabidopsis* designated *LCB1*, while LCB2 is encoded by two functionally redundant genes designated *LCB2a* and *LCB2b* (Dietrich et al., 2008). How SPT activity is finely regulated in plants to support growth and modulate
PCD initiation is unclear. Public microarray data, for example, suggest little transcriptional regulation of *LCB1, LCB2a*, or *LCB2b* in response to most stresses.

A major advance in understanding the regulation of SPT activity was the discovery of yeast (*Saccharomyces cerevisiae*) Tsc3p (Gable et al., 2000). This 80 amino acid polypeptide has no catalytic activity but stimulates SPT activity and is essential for high temperature growth (Gable et al., 2000). Structurally unrelated but functional equivalents of Tsc3p, small subunits of SPT (ssSPT), have recently been identified in humans and other mammals (Han et al., 2009). In humans, two ssSPTs have been identified: ssSPTa, a 68 amino acid polypeptide, and ssSPTb, a 76 amino acid polypeptide (Han et al., 2009). Co-expression of either human ssSPTa or human ssSPTb with the human LCB1 and LCB2a or LCB2b in an *lcb1Δlcb2A* yeast mutant results in the stimulation of in vitro SPT activity by 100- to 500-fold (Han et al., 2009). In addition, it was observed that ssSPTb co-expression yielded C18 and C20 LCBs from activity with palmitoyl-CoA and stearoyl-CoA, respectively, but ssSPTa expression in this system resulted in the production of only C18 LCBs. A single amino acid difference between ssSPTa and ssSPTb was found to account for the altered substrate specificity of SPT (Harmon et al., 2013). In addition, ssSPTa and ssSPTb were found to physically interact with LCB1 and with LCB2a and LCB2b in the SPT heterodimer (Han et al., 2009).

However, the mechanism by which ssSPTs enhance SPT activity and contribute to SPT acyl-CoA substrate specificity has yet to be elucidated. It is also unclear if ssSPT polypeptides are essential in more complex eukaryotes or whether alteration in their expression can affect SPT activity in vivo and alter physiological properties of cells. A
more complete understanding of ssSPT properties and functions is important for understanding the fine-tuned maintenance of sphingolipid homeostasis in all eukaryotes.

Research reported here was conducted to provide insights into the regulatory system for sphingolipid synthesis in plants and to examine whether ssSPTs have critical functions in higher eukaryotic cells. *Arabidopsis* is particularly amenable as a eukaryotic model system for exploring ssSPT function because of the availability of insertion mutants and the relative ease of genetic manipulation and propagation. Here we describe the identification of two 56 amino acid *Arabidopsis thaliana* ssSPTs, ssSPTa and ssSPTb, based on limited homology with human ssSPTs. Both polypeptides strongly stimulate Arabidopsis SPT activity in yeast SPT mutants through direct interaction with the Arabidopsis LCB1/LCB2 heterodimer. In addition, mutant and altered expression lines for the *Arabidopsis* ssSPT genes display strong phenotypes ranging from loss of pollen viability to altered sensitivity to FB1, which are attributable to suppression or enhancement of SPT activity.
2.3 Methods

2.3.1 Yeast Cell Growth and Expression Plasmids.  Yeast (Saccharomyces cerevisiae) strain TDY9113 (Mat a tsc3Δ:NAT lcb1Δ:KAN 1 ura3 leu2 lys2 trp1Δ) lacking endogenous SPT was used for expression and characterization of the Arabidopsis ssSPT subunits.  The mutant was cultured in medium containing 15 μM phytosphingosine (PHS) and 0.2% tergitol.  The At ssSPTa and At ssSPTb cDNA open reading frames were inserted after the 3x HA tag in pADH1 (Kohlwein et al., 2001).  pAL2-TRP was constructed for divergent constitutive expression of At LCB1-FLAG and Myc-At LCB2a or Myc-At LCB2b by replacing the Gal1 and Gal 10 promoters of pESC-TRP (Stratagene) with the yeast LCB2 and ADH promoters, respectively.  The At ssSPTb M19V mutation was introduced by QuikChange mutagenesis (Stratagene).

2.3.2 SPT Assay.  Yeast microsomes were prepared and SPT was assayed as described except that 75 μM myristoyl-, palmitoyl-, or stearoyl-CoA was used for the yeast microsomal SPT assays and 50 μM palmitoyl-CoA was used for the Arabidopsis microsomal SPT assays.  SPT activity was measured using [3H] serine and palmitoyl-CoA.

2.3.3 Protein-Protein Interactions.  Immunoprecipitation was conducted as described (Breslow et al., 2010) with minor modifications.  Microsomal membrane proteins were prepared from yeast cells expressing Flag-tagged At LCB1, Myc-tagged At LCB2a with HA-tagged At ssSPTa.  Untagged At LCB1 was used to verify that At ssSPTa itself does not bind to the anti-Flag resin.  Microsomal membrane proteins were resuspended at 1 mg/ml in IP buffer (50 mM Heps-KOH, pH 6.8, 150 mM KOAc, 2 mM MgOAc, 1 mM...
CaCl2, 15% glycerol) supplemented with 1 mM PMSF, 2 mg/ml Pepstatin A, 1 mg/ml leupeptin, and 1 mg/ml protinin and solubilized using 1% digitonin at 4°C for 2.5 hours. 1 ml of solubilized microsomes were incubated with 25 µl of anti-FLAG beads (Sigma) at 4°C for 4 h and the beads were washed four times with IP buffer containing 0.1% digitonin. The bound proteins were eluted in IP buffer containing 0.25% digitonin and 200 µg/ml FLAG peptide, resolved on a 4-12% Bis-Tris NuPAGE gel (Invitrogen) and detected by immunoblotting with antibodies, anti-HA (Covance 1:5000 dilution), anti-Myc (Sigma 1:3000 dilution), and anti-FLAG (GeneScript 1:5000 dilution).

2.3.4 Membrane Association and Glycosylation Cassette Mobility Shift Assays. The assays were conducted as described (Harmon et al., 2013) with minor modifications. Microsomes prepared from yeast cells expressing HA-tagged At ssSPTa/b, At Lcb1, and At Lcb2a were incubated on ice in buffer containing 1 M NaCl, 0.2 M Na2CO3, 5 M urea, 0.4% Nonidet P-40 or 2% Triton X-100 for 60 min. The samples were subjected to centrifugation at 100,000 x g for 30 min, and equal proportions of the supernatants and pellets were resolved by SDS-PAGE. For topology mapping, a glycosylation cassette (GC) containing three consensus AsnX (Ser/Thr) glycosylation sites was appended to the C-terminus of At ssSPTb. HA-At ssSPTb, and HA-At ssSPTb-GC (along with At LCB1 and At LCB2) were expressed in yeast, and glycosylation was assessed using EndoH as previously described (Harmon et al., 2013).

2.3.5 Plant Material and Growth Conditions. Wild-type and mutant Arabidopsis thaliana (Col-0) were grown on soil or surface sterilized and sown on Linsmaier and Skoog (LS) agar plates. After stratification at 4°C for 4 days, plants were maintained at
22°C and 50% humidity with a 16-h-light (100 µmol/m-2/s-1)/8-h-dark cycle.

Arabidopsis transformation and selection. Binary vectors were introduced into Agrobacterium tumefaciens GV3101 by electroporation. Transgenic plants were created by floral dip of Arabidopsis thaliana (Col-0) or heterozygous SALK_104888 T-DNA mutant plants (Clough and Bent, 1998). Seeds were screened with a green LED light and a Red 2 camera filter to identify positive (DsRed) transformed seeds.

2.3.6 RNA Isolation and qPCR. For analyses of organ-specific expression of ssSPTa and ssSPTb, 6- to 8-week old Col-0 plants were used as sources of plant material. Pollen was harvested as described previously (Johnson-Brousseau and McCormick, 2004). RNA extraction was performed using the RNeasy Plant Kit (Qiagen) according to the manufacturer’s protocol. RNA (1 µg) was treated with DNaseI (Invitrogen) according to manufacturer’s protocol. Treated RNA was then reverse transcribed to cDNA with the iScript cDNA synthesis kit (BioRad) according to manufacturer’s protocol. qPCR was performed on the resulting cDNA using the BioRad MyiQ iCycler qPCR instrument. Values shown are the average of three independent measurements ± SD. SYBR green was used as the fluorophore in a qPCR supermix (Qiagen). QuantiTect (Qiagen) primer sets (primers P1, P2, and P3; see Supplemental Table 1 online) were used for relative quantification. PP2AA3 (At1g13320) was used as an internal reference gene.

2.3.7 Subcellular Localization of ssSPTa and ssPTb. YFP fusion proteins with ssSPTa and ssSPTb were prepared by amplification of the ssSPTa and ssSPTb open reading frames using gene-specific primers (primers P4 through P7). PCR products were first cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting plasmids were
combined with the destination vector pEarlyGate 104 (Earley et al., 2006) to generate the
YFP N-terminal fusion constructs. Agrobacterium tumefaciens-mediated infiltration of
Nicotiana benthamiana leaves was performed with ssSPTa-YFP and ssSPTb-YFP
separately and in conjunction with the ER marker CD3-959 (HDEL-mCherry) (Nelson et
al., 2007). Sequential imaging was performed using a Nikon A1 confocal imaging
system mounted on a Nikon Eclipse 90i microscope. Excitation/emission wavelengths
for YFP and mCherry were 488 nm/500-550 nm and 561.6 nm/570-620 nm, respectively.

2.3.8 Arabidopsis Mutant Genotyping. T-DNA insertion mutants were obtained from
the Arabidopsis Biological Resource Center and the GABI-Kat collections. Genomic
DNA was extracted from plants using the REDextract-N-Amp Tissue PCR kit (Sigma).
Genotyping was performed by PCR using gene-specific and T-DNA-specific primer sets
(primers P8 through P15).

2.3.9 Genetic Complementation of ssspta-1. For genomic complementation of
SALK_104888 (ssspta-1) heterozygous T-DNA mutant plants, a ~2.0-kb fragment of
ssSPTa was amplified from Arabidopsis (Col-0) genomic DNA (primers P16 and P17).
The amplified product was then digested with AscI and cloned into the binary vector
pB110. Heterozygous T-DNA mutants (SALK_104888) were then transformed with the
pB110 construct by the floral dip method (Clough and Bent, 1998). Primary
transformants were selected (DsRed) and genotyped. Selected heterozygous plants were
also genotyped in the next generation (primers P18 and P19). The ADS1 gene was used
as a genotyping control (primers P20 and P21). Pollen was analyzed from the primary
and homozygous transformants. The ssSPTa promoter: ssSPTb cDNA fusion construct
was generated by amplification of ~1.0-kb region upstream of the ssSPTa start codon (primers P22 and P23; Supplemental Table 1 online). The product was digested with BamHI and EcoRI and cloned into the corresponding sites of pBinGlyRed2. The ssSPTb cDNA fragment was amplified from Col-0 cDNA, digested with EcoRI and XhoI, and cloned into pBinGlyRed2 (primers P24 and P25). Transformants were selected and genotyped, and pollen was assessed for viability. The gametophyte-specific DMC1 promoter (Klimyuk and Jones, 1997) was amplified from pPTN850 (provided by Dr. Tom Clemente, U. of Nebraska-Lincoln) and cloned into the pBinGlyRed3-35S:ssSPTa cDNA construct using the BamHI and EcoRI sites, replacing the CaMV35S promoter with DMC1 promoter (primers P26 and P27).

2.3.10 Microscopy. Pollen imaging was performed using an Olympus AX70 optical microscope. Anthers and siliques of mature plants were isolated using a Nikon SMZ745T dissection microscope. Anthers were smeared on a glass slide and incubated with Alexander stain (Alexander, 1969) at 4˚C for 45 min before viewing. Pollen viability was assessed by shape and color.

2.3.11 Fumonisin B1 Screening of Arabidopsis ssSPTa Overexpression and RNAi lines. ssSPTa overexpressing plants were generated by transforming Col-0 with the CaMV35S promoter:ssSPTa cDNA constructs. ssSPTa was cloned into the binary vector pBinGlyRed3-35S using the EcoRI/XbaI restriction sites (primers P28 and P29). ssSPTa RNAi lines were generated by overexpressing a hairpin with the ssSPTa 3’UTR. The ssSPTa 3’ UTR fragments were amplified (primers P30 and P31) and cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting vector was digested with ApaLI.
This vector was then combined with the destination vector, pFGCGW-red, to generate the final \textit{ssSPTa} RNAi construct. \textit{Arabidopsis} (Col-0) plants were transformed with these constructs, and the resulting transformants were selected and screened by qPCR for \textit{ssSPTa} transcript. Sensitivity screening relative to a wild type control was done at 0 µM, 0.3 µM, and 0.5 µM FB1 (Sigma) concentrations in LS media. Sensitivity was determined by plant growth rate and germination at the varying FB1 concentrations.

\textbf{2.3.12 Plant Microsomal Membrane Isolation.} Microsomal membrane isolation from soil grown 3-week old \textit{Arabidopsis} rosettes was performed as described previously (Lynch and Fairfield, 1993) and protein concentration was measured using the BCA method (Smith et al., 1985).

\textbf{2.3.13 Generation of At\textit{ssSPTb M19V} construct.} The At \textit{ssSPTb M19V}mutant cDNA was amplified from the corresponding yeast vector described above (primers P32 and P33) and cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting vector was linearized with \textit{ApaLI} and combined with the destination vector, pCD3-724-Red, resulting in the final vector pCD3-724-Red-\textit{ssSPTb M19V}. The vector was transformed into \textit{A. thaliana} Col-0 as described above.

\textbf{2.3.14 Sphingolipid Analysis.} Sphingolipids were extracted from 2-15 mg of lyophilized seedling tissue as described (Markham and Jaworski, 2007). Sphingolipid profiling by LC/ESI-MS/MS was performed as described with modifications, using a Shimadzu Prominence UPLC system and a QTRAP4000 mass spectrometer (AB SCIEX). Sphingolipids were separated on a Zorbx Eclipse Plus narrow bore RRHT C18 column, 2.1 x 100 mm, 1.8 µm particle size (Agilent) column at 40°C and a flow rate of
0.2 ml/min. Binary gradients were generated as described (Markham and Jaworski, 2007) using tetrahydrofuran (THF)/methanol/5mM ammonium formate (3:2:5) + 0.1% formic acid (Solvent A) and THF/methanol/5mM ammonium formate (7:2:1) + 0.1% formic acid (Solvent B). Sphingolipid species were detected using a 4000QTRAP mass spectrometer (AB SCIEX) with instrument settings as follows: ion spray voltage 5000V, source temperature 350°C (for GIPCs) and 400C, GS1 30, GS2 60, curtain gas 13 psi, interface heater on at 100°C. Voltage potentials (DP, CE, EP, CXP) were adjusted slightly from values given by Markham and Jaworski (2007) to account for differences in column dimensions, flow rate, and source temperature. Optimized voltage potentials were determined using Arabidopsis leaf sphingolipids as a calibrant. Multiple reaction monitoring (MRM) Q1/Q3 transitions for t18:0, t18:1, d18:0 and d18:1 sphingolipid species were as described by Markham and Jaworski (2007). t20:0 free long-chain bases were monitored using a 346.3/328.3 m/z MRM. MRM Q1/Q3 transitions for t20:0-containing sphingolipids are shown in Supplemental Table 2. Instrument settings for t18:0 species were used for t20:0 species. Data analysis was performed using Analyst 1.5 and Multiquant 2.1 software (AB SCIEX), as described by Markham and Jaworski (2007).

2.3.15 Phylogenetic Analysis. Amino acid sequences were aligned using ClustalW (Thompson et al., 1994) using Gonnet protein weight matrix (gap open penalty = 10, gap extension penalty = 0.1, gap separation distance = 4). Sequence alignments are provided in Supplemental Figure 12. Phylogenetic analysis of the aligned full-length sequences was conducted with MEGA 5 using the neighbor-joining method (Tamura et al., 2011).
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 40 positions in the final dataset.

2.3.16 Accession numbers. Sequence data from this thesis can be found in the GenBank/EMBL libraries under the following accession numbers: ssSPTa (At1g06515), ssSPTb (At2g30942), LCB1 (At4g36480), LCB2a (At5g23670), LCB2b (At3g48780).
2.4 Results

2.4.1 Two functional homologs of mammalian ssSPTs occur in Arabidopsis.

Two genes, designated \textit{ssSPTa} and \textit{ssSPTb}, encoding 56 amino acid polypeptides were identified in homology searches using the human ssSPTs as query. The amino acid sequences of the \textit{Arabidopsis} polypeptides share 88% identity and have predicted homologs throughout the plant kingdom (Fig. 2.1). \textit{ssSPTa} and \textit{ssSPTb} also share 25% to 30% amino acid sequence identity with human \textit{ssSPTa} and 18 to 25% identity with human \textit{ssSPTb} but <10% identity with the \textit{Saccharomyces cerevisiae} Tsc3p. \textit{ssSPTa} and \textit{ssSPTb} have one likely transmembrane domain encompassing 19 to 23 amino acids in the central portion of the polypeptides, based on predictions from the SOSUI (Hirokawa et al., 1998), TopPred (Claros and von Heijne, 1994) and TMpred programs (Ikeda et al., 2003) (Fig 2.1). This is also consistent with mammalian ssSPTs, which have been shown to contain a single transmembrane domain (Han et al., 2009; Harmon et al., 2013).

![Amino acid sequence alignment of ssSPTs from Arabidopsis thaliana (At) and Homo sapiens (Hs).](image)

\textbf{Figure 2.1} Amino acid sequence alignment of ssSPTs from \textit{Arabidopsis thaliana} (At) and \textit{Homo sapiens} (Hs). Translated protein sequences were aligned using ClustalW. The divergent amino acid shown to influence SPT substrate specificity (M vs. V) is indicated with an asterisk and the predicted transmembrane domain (TMD) is indicated with a bar. Shading represents the degree of sequence conservation.

To establish their functions, \textit{Arabidopsis} HA-tagged \textit{ssSPTa} and \textit{ssSPTb} were expressed along with the \textit{Arabidopsis} core SPT subunits LCB1 with a FLAG tag and LCB2a or LCB2b with Myc tags in a yeast mutant lacking endogenous SPT. Expression
of LCB1-FLAG with the Myc-LCB2 subunits failed to complement the long-chain base auxotrophy of the yeast mutant, but co-expression of either HA-ssSPTa or HA-ssSPTb resulted in robust growth, even at 37°C where the requirement for SPT activity is relatively high (Fig. 2.2). Immunoprecipitation of the LCB1-FLAG subunit resulted in co-purification of the HA-ssSPTs as well as the Myc-LCB2 subunits (Fig. 2.2, left panel). This reflects specific binding of HA-ssSPTa with the LCB1/LCB2 heterodimer, because no HA-ssSPTa bound to the beads when solubilized microsomes prepared from cells expressing untagged LCB1, MYC-LCB2 and HA-ssSPTa were used for the immunoprecipitation (Fig. 2.2, right panel). The ssSPTs behave like integral membrane proteins when coexpressed with LCB1 and LCB2, as the proteins are solubilized with detergents and urea, but not by salt or bicarbonate (Fig. 2.2). By analogy to the human ssSPTs (Harmon et al., 2013), we predicted that the Arabidopsis ssSPTs would have a single transmembrane domain and that their C-termini would reside in the ER lumen. To directly address the location of the C-terminus of ssSPTb, a glycosylation cassette (GC) containing three consensus AsnX(Ser/Thr) glycosylation sites was appended to the C terminus of HA-ssSPTb and tagged protein was assessed for glycosylation based on sensitivity to EndoH treatment. The increased electrophoretic mobility of HA-ssSPTb following EndoH treatment (Fig. 2.2) provides strong evidence that the C-terminus is in the ER lumen. Consistent with the complementation data, microsomes prepared from yeast expressing the At LCB1/LCB2 heterodimers had very low SPT activity (Fig. 2.2), whereas co-expression of either At ssSPTa or At ssSPTb increased the microsomal SPT activities >100-fold. With all four combinations of LCB2, ssSPT, and LCB1 subunits, a high preference for palmitoyl (16:0)-CoA was observed. Activities measured with
myristoyl (14:0) - or stearoyl (18:0)-CoA were <10% of the activity with palmitoyl-CoA (see Supplemental Fig. 3 in Appendix A). The higher SPT activities measured with ssSPTa are likely due to higher expression levels achieved with ssSPTa versus ssSPTb (Fig. 2.2), as indicated by western blot analysis of microsomes from the recombinant yeast (see Supplemental Fig. 3 in Appendix A), rather than to an intrinsic functional difference between these polypeptides.
Figure 2.2 Co-expression of *Arabidopsis* ssSPTa and ssSPTb with the *Arabidopsis* SPT subunits LCB1/LCB2a or LCB1/LCB2b complements loss of cell viability in yeast lacking endogenous SPT by activating SPT through physical interaction with the core SPT heterodimer. As shown in (A), yeast lacking endogenous SPT activity and expressing either the *Arabidopsis* LCB1/LCB2a or LCB1/LCB2b are viable only when provided with the long-chain base phytosphingosine (t18:0). The long-chain base auxotrophy of these cells is rescued at 26°C and 37°C when the *Arabidopsis* SPT heterodimers are co-expressed with either ssSPTa or ssSPTb. (B) Solubilized
microsomes from cells expressing At-LCB1-FLAG, Myc-At-LCB2, and HA-At-ssSPTa (left panel), or untagged At-LCB1, Myc-At-LCB2, and HA-At-ssSPTa (right panel), were incubated with anti-FLAG beads, the beads were washed and eluted with FLAG peptide. Aliquots of the solubilized microsomes (input) and eluent (Flag IP) were resolved by SDS-PAGE and the Arabidopsis SPT subunits were detected by immunoblotting with anti-Flag, anti-Myc, anti-HA, and anti-Elo3p (negative control) antibodies. (C) Microsomes from yeast expressing HA-At-ssSPTa or HA-At-ssSPTb, along with At-LCB1 and At-LCB2a, were extracted on ice with an equal volume of buffer or buffer containing 1 M NaCl, 0.2 M Na2CO3, 5 M urea, 0.4% Nonidet P-40 or 2% Triton X-100 for 60 min. The samples were subjected to centrifugation at 100,000 x g for 30 min and equal proportions of the supernatants and pellets were resolved by SDS-PAGE. HA-At-ssSPTa and HA-At-ssSPTb were detected by immunoblotting with anti-HA antibody. (D) HA-At-ssSPTb and HA-At-ssSPTb-GC were expressed in yeast along with At-LCB1 and At-LCB2a. 20 μg of microsomal protein (with or without EndoH treatment) were resolved by SDS-PAGE and HA-At-ssSPTb was detected by immunoblotting. (E) SPT activity was measured in yeast microsomes expressing At-LCB1 + At-LCB2a or At-LCB1 + At-LCB2b with or without At-ssSPTa or At-ssSPTb to assess their ability to enhance SPT activity. SPT activity was measured using [3H] serine and palmitoyl-CoA. Values shown are the average of three independent assays ± SD.

2.4.2 ssSPT polypeptides are ER-localized but ssSPTa and ssSPTb are differentially expressed in Arabidopsis. Confocal microscopy studies were conducted on transiently expressed ssSPTa and ssSPTb with N-terminal YFP tags in Nicotiana benthamiana to establish the intracellular localization of these polypeptides. Both polypeptides were found to co-localize with the ER marker mCherry-HDEL (Fig. 3A-F). This is consistent with ER localization of LCB1, LCB2a, and LCB2b, as previously reported (Tamura et al., 2001; Chen et al., 2006).

To assess the in planta contributions of each gene to SPT activation, transcript levels of ssSPTa and ssSPTb were measured in different organs of Arabidopsis (Fig. 3G). It is notable that neither gene is represented in any of the public microarray databases. Our analyses revealed that ssSPTa is more highly expressed than ssSPTb in all organs examined (Fig. 3G). Of most significance, ssSPTa transcript levels were nearly 500-fold
more abundant in pollen than those for ssSPTb. ssSPTa transcripts were also 60-fold and 35-fold more abundant in flowers and leaves, respectively, than ssSPTb transcripts (Fig. 3G).

**Figure 2.3** ssSPTa and ssSPTb are ER-localized and the corresponding genes are differentially expressed in Arabidopsis thaliana. (A) to (F) Subcellular localization of ssSPTs was determined by transient expression in Nicotiana benthamiana. ssSPT-YFP and ER marker-mCherry fusion constructs were created in binary transformation vectors, co-expressed in tobacco by transient Agrobacterium tumefaciens-mediated transformation, and visualized by confocal microscopy. Yellow from the YFP-ssSPTa and YFP-ssSPTb fusions ([A] and [B], respectively) co-localize with red from the ER marker-mCherry fusion ([C] and [D]) as shown in the merged micrographs for YFP-ssSPTa /ER marker-mCherry (E) and YFP-ssSPTb /ER marker-mCherry (F). Bars = 5 µm. (G) Relative expression of ssSPTa and ssSPTb. Tissues were collected from wild-type Col-0 grown under our standard conditions. Quantitative RT-PCR (qPCR) was used to determine ssSPTa and ssSPTb transcript levels. Protein phosphatase 2A subunit A3 (PP2AA3) was used as a reference gene. Values shown are expression levels of ssSPTa (relative to PP2AA3) ± SD for three independent measurements.
2.4.3 T-DNA disruption of ssSPTa results in loss of pollen viability. To further examine the relative in planta contributions of ssSPTa and ssSPTb, T-DNA insertion mutants for each gene were characterized. ssSPTa is on chromosome 1 and has two introns within the coding sequence, whereas ssSPTb is on chromosome 2 and has two introns within the coding sequence (Fig. 2.4A). T-DNA insertion mutants were identified for each gene: SALK_104888 for ssSPTa (designated ssspta-1) and SALK_028181 and GAB_169D12 for ssSPTb (designated sssptb-1 and sssptb-2, respectively). In the case of sssptb-1 and sssptb-2, lines homozygous for the T-DNA insertion and lacking detectable ssSPTb transcript were identified (see Supplemental Fig. 4 in Appendix A). Homozygous sssptb-1 and sssptb-2 plants were indistinguishable in their growth and pollen viability from wild type plants (see Supplemental Fig. 5 in Appendix A). By contrast, no homozygous ssspta-1 mutants were identified by genotyping 250 plants over three generations, suggesting that ssSPTa is an essential gene (see Supplemental Fig. 6). Examination of pollen with viability stain revealed 50% non-viable pollen in ssspta-1 plants genotyped as heterozygous for the T-DNA insertion (Fig. 2.4B). Chi-square goodness-of-fit tests confirmed that the defect observed in pollen collected from ssspta-1 heterozygous plants segregated at the expected 1:1 ratio for a male gametophytic lethal mutation within a 90%-95% confidence interval ($\chi^2=2.627; n=521$). However, the relative numbers of aborted ovules in these plants were low and not significantly different from those in wild-type plants (see Supplemental Fig. 7). Levels of pollen viability were restored to wild-type levels by introduction of a wild-type copy of ssSPTa (including its native promoter) into ssspta-1 plants genotyped as homozygous for the ssspta-1 allele (Fig. 2.4B; see Supplemental Fig. 8). Overall, these
results demonstrate that ssSPTa, but not ssSPTb, is essential for pollen viability. The much higher expression of ssSPTa relative to ssSPTb in pollen (Fig. 2.4B) likely accounts for the inability of ssSPTb to compensate for loss of ssSPTa to maintain normal male gametophytic development. To test this hypothesis, the ssSPTb cDNA was placed under control of the ssSPTa promoter and introduced into the heterozygous ssspta-1 mutant. Based on genotyping data, plants were recovered that expressed this transgene and contained the homozygous ssspta-1 allele and lacked defective pollen (Fig. 2.4B; see Supplemental Fig. 9 in Appendix A). These findings indicate that the ssSPTa and ssSPTb polypeptides are functionally redundant. We also tested whether ssSPTa is also essential for sporophytic (vegetative) growth by expression of an ssSPTa cDNA under control of the pollen- and ovule (meiotic cell)-specific DMC1 (Klimyuk and Jones, 1997) promoter in the ssspta-1 mutant to advance the ssspta-1 -/- allele beyond pollen development. No transgenic plants genotyped as ssspta-1 -/- and lacking the ssSPTa transcript in leaves were recovered. Instead, four independent ssspta-1 -/- lines were obtained that were severely dwarfed and had low levels of ssSPTa expression in leaves, presumably due to leaky expression from the DMC1 promoter (Fig. 2.5). These plants had pollen with high levels of viability, and the dwarf phenotype persisted into the subsequent generation (Fig. 2.5). This result suggests that ssSPTa is also essential for sporophytic growth.
Figure 2.4 T-DNA disruption of AtssSPTa results in loss of pollen viability. Introns and exons are shown as white and black boxes, respectively, and the UTRs are shown as solid lines. AtssSPTa (At1g06515) contains an intron in the 5’UTR. T-DNA insertion mutant alleles for AtssSPTa (At1g06515) and AtssSPTb (At2g30942) were confirmed by PCR-based genotyping (Fig. S5), as shown in (A). (B) Mutation of AtssSPTa leads to male gametophytic lethality. Pollen obtained from mature flowers of the respective genotypes were treated with Alexander stain and visualized by light microscopy. Defective pollen stains green and is folded and shriveled, whereas viable pollen is purple and round. Representative pollen collected from heterozygous plants segregating for the T-DNA insertion in the sspta-1 mutant are shown. Pollen from segregating plants genotyped as wild-type (sspta-1 +/) are uniformly healthy, whereas those from the heterozygous plants (sspta-1 +/-) yield defective pollen at ~50% frequency (black arrows). Transgenic complementation of the pollen defect was accomplished in sspta-1 -/- plants with constructs expressing either a genomic copy of AtssSPTa with native promoter.


(\textit{ssspta-1} -/- + AtssSPTa) or the AtssSPTb cDNA under control of the AtssSPTa promoter (\textit{ssspta-1} -/- + pAtssSPTa::AtssSPTb).

**Figure 2.5** Complementation of \textit{ssspta-1} +/- with ssSPTa cDNA under the control of the gametophyte specific promoter, DMC1, yields \textit{ssspta-1} -/- plants that have viable pollen and are dwarfed. (A) Segregating plants from a complemented \textit{ssspta-1} line. The two larger plants are \textit{ssspta-1} +/- while the small plant (red arrow) is \textit{ssspta-1} -/- . (B) The progeny from the indicated \textit{ssspta-1} -/- plant in (A) are all \textit{ssspta-1} -/- and display the dwarf phenotype. (C) Pollen from \textit{ssspta-1} -/- complemented plants appear normal, indicating successful complementation.

**2.4.6 ssSPT expression levels affect SPT activity \textit{in planta} and sensitivity to the fungal mycotoxin \textit{fumonisin B1}**. ssSPTa over-expression and suppression lines were generated in wild-type Arabidopsis to determine the impact on SPT activity in planta. Over-expression lines were prepared by placing the ssSPTa cDNA under control of the CaMV35S promoter, and RNAi lines were generated using hairpin-generating constructs that targeted the ssSPTa gene under control of the CaMV35S promoter. Homozygous lines from the introduction of these transgenes in wild-type Arabidopsis were screened by qPCR to identify those with strongly enhanced or suppressed expression of ssSPTa (see Supplemental Fig. 10). Enzyme assays conducted with microsomes prepared from rosettes revealed an approximately two-fold increase in SPT activity in a selected over-expression line and up to a two-fold decrease in SPT activity in two independent suppression lines (Fig. 2.6A and 2.6B). These results indicated that SPT
activity in planta can be modulated by altered expression of ssSPTa, and based on the findings from the over-expression line, levels of ssSPTa expression are limiting for maximal SPT activity in planta.

Based on results from LCB1 and LCB2 Arabidopsis mutants (Shi et al., 2007; Saucedo-Garcia et al., 2011), altered SPT activity should affect resistance to the ceramide synthase mycotoxin inhibitor Fumonisin B1 (FB1) (Stone et al., 2000). Enhanced SPT activity is hypothesized to increase FB1 sensitivity by heightened production of cytotoxic long-chain bases (LCBs), whereas reduced SPT activity is hypothesized to decrease FB1 sensitivity by lessened production of LCBs (Shi et al., 2007; Saucedo-Garcia et al., 2011). Consistent with these hypotheses, ssSPTa suppression lines were observed to have increased resistance to FB1: suppression lines maintained viability at 0.5 µM FB1, which was toxic to wild-type plants (Fig. 2.6C). Conversely, ssSPTa over-expression lines displayed strong sensitivity to 0.3 µM FB1, but wild-type plants retained viability at this FB1 concentration (Fig. 2.6C). Consistent with this, free LCB and LCB-phosphate accumulation in the ssSPTa RNAi line was strongly reduced relative to that in wild type plants in response to FB1, whereas free LCB and LCB-phosphate accumulation was approximately 3-fold higher in the ssSPTa over-expression line relative to that in wild type plants in response to FB1 (Fig. 2.6D). These results indicate that altered ssSPTa expression is an effective means of modulating sensitivity of plants to FB1 and likely other SAM-mycotoxins by increasing or decreasing SPT activity.
Figure 2.6 In planta SPT activity and sensitivity to fumonisin B1 (FB1) is modulated by altered ssSPTa expression. ([A],[B]) SPT activity assayed from wild type, transgenic plants overexpressing ssSPTa, and transgenic plants with reduced expression of ssSPTa (silenced by RNAi) indicated proportional regulation of SPT activity by ssSPTa. Microsomes were prepared from the respective plant lines and SPT activity was measured with [3H] serine and palmitoyl-CoA (C16). Overexpression of ssSPTa increased SPT activity (A), whereas suppression of ssSPTa resulted in reduced SPT
activity (B). Values shown are the average of assays of three independent samples (± SD). Student t-test was performed, * = P < 0.02, ** = P < 0.002. (C) Altered expression of ssSPTa affected sensitivity to Fumonisins B1 (FB1), a competitive inhibitor of ceramide synthase. Seeds were sown on LS-agar plates supplemented with FB1 at 0.3 μM and 0.5 μM as indicated, which distinguish between the FB1-resistant (fbr) and FB1-sensitive (fbs) phenotypes, respectively (Stone et al., 2000). Wild-type (Col-0) is extremely sensitive to FB1 at 0.5 μM FB1, and less affected at 0.3 μM FB1. Upregulation of ssSPTa by transgenic overexpression causes an fbs phenotype (ssSPTa OE), whereas down-regulation of ssSPTa by RNA-interference (RNAi) causes an fbr phenotype (ssSPTa RNAi). Images were taken 14 days after sowing seeds and are representative of four independent experiments. (D) Altered ssSPTa expression impacts accumulation of cytotoxic free long-chain base and long-chain base phosphate [LCB(P)] levels in response to FB1 treatment. Wild-type plants (wt) show increased LCB(P) levels when treated with FB1. Compared to WT, ssSPTa over-expression (OE) plants display strong sensitivity to FB1 and show elevated LCB(P) levels. Alternatively, ssSPTa RNAi suppression plants display resistance to FB1 and show reduced LCB(P) levels, indicating that modulation of LCB(P) levels affects FB1 sensitivity. ESI-MS/MS analyses were performed with a sample size of 6 plants per treatment with three independent biological replicates ± SD. Plants were grown on LS plates +/- FB1. Plants were grown for 2 weeks before tissue collection.

2.4.7 Altered SPT substrate specificity conferred by M19V mutation of ssSPTs is conserved among eukaryotes. Human ssSPTa and ssSPTb confer distinct substrate specificities to human SPT: ssSPTa expression with human LCB1/LCB2 results in substrate preference for palmitoyl-CoA to generate C18 LCBs, whereas ssSPTb expression with LCB1/LCB2 results in preference for stearoyl-CoA to generate C20 LCBs (Han et al., 2009; Harmon et al., 2013). The basis for this is due to a single amino acid difference in human ssSPTa (Met 25) and ssSPTb (Val 25) (Harmon et al., 2013). Like human ssSPTa, At ssSPTa and At ssSPTb have Met at the equivalent position (Met 19), and expression of either At ssSPTa or At ssSPTb with At LCB1/At LCB2a or At Lcb2b yields activity primarily with palmitoyl-CoA (Fig. 2.1, 2.2C). To test the importance of amino acid 19 of At ssSPTs for SPT substrate specificity, Met 19 in At ssSPTb was mutated to Val and expressed in a yeast SPT-null mutant along with At
LCB1/At LCB2a and in wild-type Arabidopsis (Col-0). Consistent with findings with
type Arabidopsis (Col-0). Consistent with findings with
human SPT (Han et al., 2009; Harmon et al., 2013), yeast co-expressing wild-type
Arabidopsis ssSPTb and At LCB/At LCB2a accumulated exclusively C18-LCBs
(produced from condensation of serine with palmitoyl-CoA), while those expressing the
ssSPTb M19V mutant accumulated high levels of C20-LCBs, reflecting condensation of
serine with stearoyl-CoA (Fig. 2.7A). Similarly, expression of ssSPTb M19V in
Arabidopsis resulted in the aberrant production of C20 LCBs that accumulated in all
sphingolipid classes (Fig. 2.7B; see Supplemental Figure 11). These findings indicate
that a single conserved amino acid in human and Arabidopsis ssSPTs is critical for SPT
substrate specificity even though these polypeptides share ≤30% amino acid sequence
identity. In addition, Arabidopsis lines expressing the ssSPTb M19V mutant displayed
enhanced sensitivity to FB1 and enhanced overall accumulation of free and
phosphorylated LCBs in response to FB1 relative to wild-type plants (see Supplemental
Fig. 12). This effect is similar to that described above for ssSPTa over-expression.
Figure 2.7 The ssSPTb M19V mutant has altered SPT acyl-CoA preference. (A) Yeast expressing wild-type ssSPTb along with LCB1 and LCB2a accumulated exclusively C18-LCBs (solid line), while those expressing the ssSPTb M19V mutant accumulated high levels of C20-LCBs reflecting condensation of serine with stearoyl-CoA (broken line). (B) Plants overexpressing the ssSPTb M19V mutant also accumulated C20-LCBs which were nearly undetectable in wild-type Arabidopsis. Plants overexpressing the ssSPTb M19V mutant accumulated C20-LCBs which were nearly undetectable in wild-type Arabidopsis. Shown is multiple reaction monitoring (MRM) for free t20:0 in ESI-MS/MS of leaves of wild-type plants and plants expressing ssSPTb M19V. Anhydrous (An) forms are also shown.
2.5 Discussion

Here we show the occurrence of 56 amino acid polypeptides in Arabidopsis that share low, but significant, sequence identity with human ssSPTs. Like the human ssSPTs, the Arabidopsis ssSPT homologs physically interact with and greatly stimulate the activity of LCB1/LCB2 heterodimers. Our findings also demonstrate the importance of ssSPTs to cellular function in eukaryotes, which is apart from the role of the yeast Tsc3p for high temperature viability, has not been previously clarified in eukaryotes (Gable et al., 2000). In this regard, we show that ssSPTa is essential for male gametophyte (pollen) development and likely for sporophytic viability. In addition, our findings indicate that SPT activity can be enhanced in planta by over-expression of ssSPTa, suggesting that ssSPT levels limit SPT activity in cells of complex eukaryotes and thus that their increased expression may, in part, underlie regulation of SPT activity.

Similar to what has been reported in humans (Han et al., 2009), Arabidopsis also has two functional ssSPT genes (Fig. 2.1), both of which are able to strongly increase SPT activity when co-expressed with the Arabidopsis LCB1 and LCB2a or 2b in a yeast SPT null mutant (Fig 2.2E). In addition, like the human ssSPT polypeptides, we show that Arabidopsis ssSPT polypeptides are integral membrane proteins with their C-termini oriented toward the ER lumen. The Arabidopsis ssSPT polypeptides ssSPTa and ssSPTb, however, share considerably higher identity with each other (88%) than do the human ssSPTa and ssSPTb polypeptides, which are 41% identical, and also unlike the human ssSPTs, Arabidopsis ssSPTa and ssSPTb contain the same number of amino acids. In addition, the Arabidopsis ssSPTs both have a conserved Met at amino acid 19, whereas
the analogous position of the human ssSPTs is either a Met or Val (Han et al., 2009). It has been shown that this amino acid difference is associated with defining the substrate properties of SPT. The presence of Val at this position confers an increased ability of SPT to use the C18 substrate stearoyl-CoA (18:0-CoA), rather than the more typical C16 palmitoyl-CoA (16:0-CoA), to generate C20 LCBs in vitro and in vivo (Han et al., 2009). We show that this amino acid substitution also expands the ability of the Arabidopsis SPT to produce C20 LCBs, which is not detectable in wild-type Arabidopsis (Fig 2.7B).

The most striking difference between ssSPTa and ssSPTb is their expression levels in Arabidopsis plant tissues (Fig. 2.3G). In all organs examined, ssSPTa is more highly expressed than ssSPTb. The largest difference (~400-fold) was detected in pollen, which is consistent with the pollen lethality displayed in the ssspta-1 mutant. The higher expression levels of ssSPTa suggest that this gene is of more importance for both reproductive and vegetative growth and viability under normal growth conditions. Expression of the ssSPTb cDNA under control of the ssSPTa promoter is able to rescue the pollen lethality in ssspta-1, indicating that the ssSPTa and ssSPTb polypeptides are functionally redundant in Arabidopsis. Unclear is whether ssSPTb has a more specialized function for the response of cells to stress and altered sphingolipid homeostasis. Complicating resolution of this question is the absence of public microarray data for Arabidopsis ssSPTa and ssSPTb, likely due in part to the small size of their transcripts. More extensive expression profiling of Arabidopsis under a wide array of stresses as well as profiling of sphingolipid homeostatic mutants is needed to clarify differential roles of ssSPTa and ssSPTb in the in vivo regulation of SPT.
The pollen lethality observed in ssspta-1+/- is similar to that previously described in double mutants of the LCB2a and LCB2b genes (Dietrich et al., 2008; Teng et al., 2008). In the case of lcb2a-1/-lcb2b-1+/- mutants, approximately 50% of microspores did not progress to the bicellular stage of pollen development (Dietrich et al., 2008). The defective pollen lacked a well-developed endomembrane system, as indicated by vesiculated ER and the absence of Golgi stacks, and also lacked a surrounding intine layer, suggestive of disrupted Golgi trafficking (Dietrich et al., 2008). Our current findings with the ssspta-1 mutant and previous findings with LCB2a/LCB2b mutants demonstrate the absolute requirement of sufficient SPT activity for sphingolipid synthesis to support male gametophyte development. In addition, like previous results with double mutants of LCB2a and LCB2b (Dietrich et al., 2008), ssSPTa appears to also be essential for sporophytic growth due to our failure to obtain ssspta-1 homozygous mutants devoid of ssSPTa transcript upon expression of an ssSPTa cDNA under control of the meiotic cell-specific DMC1 promoter in the ssspta-1+/- background.

Emerging evidence, particularly from studies in yeast, points to an intricate regulation of SPT activity, which is responsive to intracellular sphingolipid levels. The current model comprises the SPT heterodimer and Tsc3p at the core of a complex that also includes the Orm1 or Orm2 polypeptide and SacI phosphatase, which is referred to as the “SPOTS complex” (Fig. 2.8) (Walther, 2010). In this model, Orm1/2 polypeptides and SacI phosphatase physically interact with the SPT heterodimer to regulate its activity (Breslow et al., 2010; Han et al., 2010). Orm1 and 2 polypeptides most recently have been shown to down-regulate SPT activity in response to high intracellular sphingolipid
levels (Breslow et al., 2010; Roelants et al., 2011). This suppression of SPT activity is alleviated by phosphorylation of the Orm1/2 N-terminus by the protein kinase Ypk1 in response to low intracellular sphingolipid levels (Roelants et al., 2011). Tsc3p in yeast and ssSPTs in complex eukaryotes function in converse of Orm1/2 to stimulate SPT activity. Orm 1/2 homologs have been identified in Arabidopsis (Hjelmqvist et al., 2002), but their functions have yet to be characterized. In our studies, we show that overexpression and suppression of ssSPTa can effectively mediate SPT activity and alter mycotoxin sensitivity. Given our demonstration that the Arabidopsis ssSPT and the previous report that human ssSPT physically interact with the SPT heterodimer, it is possible that these polypeptides, which lack any known catalytic activity, facilitate or stabilize interaction of LCB1 and LCB2 to enhance SPT activity. As such, alteration in ssSPT protein levels in response to perturbations in intracellular sphingolipid levels may contribute, along with other components of the SPOTS complex, to regulation of SPT. Consistent with this, we show that SPT activity in planta can be modulated by altering ssSPTa expression. It remains to be determined whether SPT is regulated in part by mediation of ssSPT protein levels in response to perturbations in sphingolipid homeostasis.

Finally, we show that up- or down-regulation of Arabidopsis ssSPT gene expression provides a simple, single gene approach to altering cellular functions. In the example shown, transgenic alteration of ssSPT gene expression yielded plants that are more or less tolerant to the PCD-inducing mycotoxin FB1. There is little evidence to suggest that up regulation of LCB1 and LCB2, individually or in combination, can
increase plant SPT activity in vivo, although modest increases in SPT activity have been reported in mammalian cells with combined over-expression of LCB1 and LCB2 (Han et al., 2004). It is envisioned that ssSPT genes will be useful biotechnological or breeding targets for physiological processes and stresses that are mediated by sphingolipids, either in their structural roles in membranes and Golgi trafficking or in their signaling roles in plants.
2.6 Works Cited


Chapter 3:

Modulation of ORM Expression Alters Fumonisin B₁-Induced Perturbations of Sphingolipid Homeostasis and Differentially Affects Ceramide Synthase Activities

Note: This chapter is to unpublished.
3.1 Abstract

Sphingolipid synthesis is tightly regulated in eukaryotes. This regulation in plants ensures sufficient sphingolipids to support growth, while limiting accumulation of sphingolipid metabolites that induce programmed cell death (PCD). Serine palmitoyltransferase (SPT) catalyzes the first step in sphingolipid biosynthesis and is considered the primary sphingolipid homeostatic regulatory point. In this report, Arabidopsis putative SPT regulatory proteins, orosomucoid-like proteins AtORM1 and AtORM2 were found to physically interact with the Arabidopsis SPT and to suppress SPT activity when co-expressed with Arabidopsis SPT subunits LCB1 and LCB2 and the small subunit of SPT in a yeast SPT-deficient mutant. Consistent with a role in SPT suppression, AtORM1 and AtORM2 overexpression lines displayed increased resistance to the PCD-inducing mycotoxin fumonisin B1 (FB1), with an accompanying reduced accumulation of long-chain bases (LCBs) and C16-fatty acid-containing ceramide accumulation relative to wild type plants. Conversely, RNAi suppression lines of AtORM1 and AtORM2 displayed increased sensitivity to FB1 and an accompanying strong increase in LCBs and C16 fatty acid-containing ceramides relative to wild-type plants. Overexpression lines were also found to have reduced activity of the Class I ceramide synthase that uses C16-fatty acid acyl-CoA and dihydroxy LCB substrates, but increased activity of Class II ceramide synthases that use very long-chain fatty acyl-CoA and trihydroxy LCB substrates. RNAi suppression lines, in contrast, displayed increased Class I ceramide synthase activity, but reduced Class II ceramide synthase activity. These findings indicate that ORM-mediation of SPT activity differentially regulates
functionally distinct ceramide synthase activities as part of a broader sphingolipid homeostatic regulatory network.
3.2 Introduction

Sphingolipids play critical roles in plant growth and development as essential components of endomembranes, including the plasma membrane where they comprise more than 40% of the total lipid (Sperling et al., 2005; Cacas et al., 2015). Sphingolipids are also highly enriched in detergent insoluble membrane fractions of the plasma membrane that form microdomains for proteins with important cell surface activities, including cell wall biosynthesis and hormone transport (Cacas et al., 2012; Perraki et al., 2012; Bayer et al., 2014; Cacas et al., 2015). In addition, sphingolipids, particularly those with very long-chain fatty acids (VLCFAs), are integrally-associated with Golgi-mediated protein trafficking that underlies processes related to the growth of plant cells (Bach et al., 2008; Bach et al., 2011; Markham et al., 2011; Melser et al., 2011). Furthermore, sphingolipids function through their bioactive long-chain base (LCB) and ceramide metabolites to initiate programmed-cell death (PCD), important for mediating plant pathogen resistance through the hypersensitive response (HR) (Greenberg et al., 2000; Liang et al., 2003; Shi et al., 2007; Bi et al., 2014; Simanshu et al., 2014).

Sphingolipid biosynthesis is highly regulated in all eukaryotes. In plants, maintenance of sphingolipid homeostasis is vital to ensure sufficient sphingolipids for growth (Chen et al., 2006; Kimberlin et al., 2013) while restricting the accumulation of PCD-inducing ceramides and long-chain bases (LCBs), until required for processes such as pathogen-triggered HR. Serine palmitoyltransferase (SPT), which catalyzes the first step in LCB synthesis, is generally believed to be the primary control point for sphingolipid homeostasis (Hanada, 2003). SPT synthesizes LCBs, unique components of
sphingolipids, by catalyzing a pyridoxal phosphate-dependent condensation of serine and palmitoyl (16:0)-CoA in plants (Markham et al., 2013). Similar to other eukaryotes, the Arabidopsis SPT is a heterodimer consisting of LCB1 and LCB2 subunits (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008). Research to date has shown that SPT is regulated primarily by post-translational mechanisms involving physical interactions with non-catalytic, membrane-associated proteins that confer positive and negative regulation of SPT activity (Han et al., 2009; Breslow et al., 2010; Han et al., 2010). These proteins include a 56-amino acid small subunit of SPT (ssSPT) in Arabidopsis, which was recently shown to stimulate SPT activity and to be essential for generating sufficient amounts of sphingolipids for pollen and sporophytic cell viability (Kimberlin et al., 2013).

Evidence from yeast and mammalian research points to a more critical role for proteins termed ORMs in sphingolipid homeostatic regulation (Breslow et al., 2010; Han et al., 2010). The Saccharomyces cerevisiae Orm1p and Orm2p negatively regulate SPT through reversible phosphorylation of these polypeptides in response to intracellular sphingolipid levels (Breslow et al., 2010; Han et al., 2010; Roelants et al., 2011; Gururaj et al., 2013; Muir et al., 2014). Phosphorylation/de-phosphorylation of ORMs in S. cerevisiae presumably affects the higher order assembly of SPT to mediate flux through this enzyme for LCB synthesis (Breslow, 2013). In this sphingolipid homeostatic regulatory mechanism, the S. cerevisiae ORM1p and ORM2p are phosphorylated at their N-termini by Ypk1, a TORC2-dependent protein kinase (Han et al., 2010; Roelants et al., 2011). The absence of this phosphorylation domain in mammalian and plant ORM
homologs brings into question the nature of SPT reversible regulation by ORMs in other eukaryotic systems (Hjelmqvist et al., 2002).

Sphingolipid synthesis is also mediated by N-acylation of LCBs by ceramide synthases to form ceramides, the hydrophobic backbone of the major plant glycosphingolipids glucosylceramides (GlcCer) and glycosyl inositolphosphoceramides (GIPCs). Two functionally distinct classes of ceramide synthases occur in Arabidopsis, designated Class I and II (Chen et al., 2008). Class I ceramide synthase activity resulting from the Longevity Assurance Gene One Homolog2 (LOH2)-encoded ceramide synthase acylates, almost exclusively, LCBs containing two hydroxyl groups (“dihydroxy” LCBs) with 16:0-CoA, which are primarily used for GlcCer synthesis (Markham et al., 2011; Ternes et al., 2011; Luttgeharm et al., 2015). Class II ceramide synthase activity resulting from the LOH1- and LOH3-encoded ceramide synthases are most active in the acylation of LCBs containing three hydroxyl-groups (“trihydroxy” LCBs) with very long-chain fatty acyl (VLCFA)-CoAs, including primarily C24 and C26 acyl-CoAs (Markham et al., 2011; Ternes et al., 2011; Luttgeharm et al., 2015). Class II (LOH1 and LOH3) ceramide synthase activity is essential for producing VLCFA-containing glycosphingolipids to support growth of plant cells, whereas Class I (LOH2) ceramide synthase activity is non-essential under normal growth conditions (Markham et al., 2011; Luttgeharm et al., 2015). It was recently speculated that LOH2 ceramide synthase functions, in part, as a “safety valve” to acylate excess LCBs for glycosylation to a less cytotoxic form (Luttgeharm et al., 2015; Msanne et al., 2015). Recent studies have shown that the Lag1/Lac1 components of the S. cerevisiae ceramide synthase, Orm1p and
Orm2p, are phosphorylated by Ypk1, and this phosphorylation stimulates ceramide synthase activity in response to heat and reduced intracellular sphingolipid levels (Muir et al., 2014). This finding points to possible coordinate regulation of ORM-mediated SPT and ceramide synthase activities to regulate sphingolipid homeostasis, which is likely more complicated in plants and mammals due to the occurrence of functionally distinct ceramide synthases in these systems (Stiban et al., 2010; Markham et al., 2011; Ternes et al., 2011; Luttgeharm et al., 2015).

RNAi suppression of ORM genes in rice has been shown to affect pollen viability (Chueasiri et al., 2014), but no mechanistic characterization of ORM proteins in plants has yet to be reported. Here we describe, two Arabidopsis ORMs, AtORM1 and AtORM2, that suppress SPT activity through direct interaction with the LCB1/LCB2 heterodimer. We also show that strong upregulation of AtORM expression impairs growth. In addition, up- or down-regulation of ORMs is shown to differentially affect sensitivity of Arabidopsis to the PCD-inducing mycotoxin fumonisin B1, a ceramide synthase inhibitor, and to also differentially affect activities of Class I and II ceramide synthases as a possible additional mechanism for regulating sphingolipid homeostasis.
3.3 Methods

3.3.1 Yeast Growth and Expression Plasmids. Yeast (Saccharomyces cerevisiae) strain TDY9113 (Mat a tsc3Δ:NAT lcb1Δ:KAN ura3 leu2 lys2 trp1Δ) lacking endogenous SPT was used for expression and characterization of the Arabidopsis thaliana SPT subunits and ORM proteins. The mutant was cultured in medium containing 15 μM phytosphingosine and 0.2% (w/v) tergitol. The AtORM1 (At1g01230) and AtORM2 (At5g42000) open reading frames were amplified by PCR and inserted into pPR3-N (Dualsystems Biotech) with HA tagged at N-terminus. The pAL2-URA was constructed for divergent constitutive expression of AtLCB1-FLAG and Myc-At LCB2a by replacing the GAL1 and GAL10 promoters of pESC-URA (Stratagene) with the yeast LCB2 and ADH promoters, respectively. The AtssSPTa cDNA open reading frame was inserted after the 3xHA tag in pADH1 (Kohlwein et al., 2001; Kimberlin et al., 2013). For deleting the first transmembrane domain (TMD1) of AtLCB1, an AvrII restriction site was inserted at codon 33 and 53 respectively by QuikChange II site directed mutagenesis (Agilent technologies). The deletion of TMD1 was achieved by cutting with AvrII and religation. The deletion was confirmed by sequencing.

3.3.2 Immunoprecipitation. Immunoprecipitation was conducted as described (Kimberlin et al., 2013) with minor modifications. Microsomal membrane proteins were prepared from yeast cells expressing FLAG-tagged At LCB1 and Myc-tagged AtLCB2a with HA-tagged AtssSPTa and HA-tagged AtORM1 or AtORM2. Microsomal membrane proteins were solubilized in 1.5% digitonin at 4°C for 2.5 h and incubated with Flag-beads (Sigma-Aldrich) overnight. The bound proteins were eluted in IP buffer
(50 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM calcium chloride, and 15% glycerol) containing 0.25% digitonin and 200 μg/mL of FLAG peptide, resolved on a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen), and detected by immunoblotting with antibodies, anti-HA (Covance; 1:5000 dilution), anti-Myc (Sigma-Aldrich; 1:3000 dilution), and anti-FLAG (GenScript; 1:5000 dilution).

3.3.3 SPT Assay. Plant microsomes were prepared and SPT activity was assayed as described (Kimberlin et al., 2013) except that 50 μM palmitoyl-CoA and 20 μM BSA were used for the Arabidopsis microsomal SPT assays. SPT activity was measured using [3H] Ser and palmitoyl-CoA.

3.3.4 Yeast Complementation. Synthetic complete (SC) media was used to grow yeast (BY4741). SC media was supplemented with 15 μM phytosphingosine (PHS) and 0.1% tergitol. Yeast knockout mutants, Δorm1 and Δorm2, were obtained from the S. cerevisiae knockout library kindly provided by Professor Jaekwon Lee (University of Nebraska-Lincoln). AtORM1/2 cDNA was cloned into the centromeric plasmid pSH15 using the XhoI and PstI restriction sites using primers P1-P4 (Supplemental Table 1 in Appendix B). The plasmid, pSH15, containing native S. cerevisiae ORM2 was received as a gift from Amy Chang (University of Michigan). Cells were grown at 30°C and then normalized to OD600=0.1 before being serially diluted and plated.

3.3.5 Plant Material and Growth Conditions. All Arabidopsis thaliana Col-0 lines used in this study were stratified at 4°C for 4 days before growth and were maintained at 22°C with a 16 h light (100 μmol/m-2/s-1)/8 h dark cycle. Plants sown on Linsmaier and Skoog (LS) agar plates were surface sterilized before stratification. Plants
that were grown hydroponically were grown on modified Hoagland’s solution as described previously, in custom made hydroponics tanks (Conn et al., 2013).

3.3.6 Arabidopsis Transformation and Selection. Binary vectors were transformed into Agrobacterium tumefaciens GV3101 by electroporation. The floral dip method was used to create transgenic plants in Arabidopsis thaliana (Col-0) (Clough and Bent, 1998). A green LED light and a Red 2 camera filter were used to screen seeds and identify transformed seed that contained DsRed.

3.3.7 RNA Isolation and qPCR. For expression analyses of ORM1 and ORM2, RNA extraction was done using the RNeasy Plant Kit (Qiagen) according to the manufacturer’s protocol. RNA (1 µg) was treated with DNaseI (Invitrogen) according to the manufacturer’s protocol. Treated RNA was then reverse transcribed to cDNA with the iScript cDNA synthesis kit (BioRad) according to the manufacturer’s protocol. For tissue-specific expression analysis, 6- to 8-week old Col-0 plants were used as sources of plant material. qPCR was performed on cDNA using the BioRad MyiQ iCycler qPCR instrument. Values shown are the average of three independent measurements ± SD. SYBR green was used as the fluorophore in a qPCR supermix (Qiagen). QuantiTect (Qiagen) primer sets P5-P7 (Supplemental Table 1) were used for relative quantification. PP2AA3 (At1g13320) was used as an internal reference gene. RT-PCR analysis of homozygous T-DNA mutant lines was performed on cDNA using primers P8-P11 (Supplemental Table 1).

3.3.8 Analysis of Promoter GUS Expressing Plants. To generate the AtORM1 promoter::GUS and AtORM2 promoter::GUS constructs, a ~1kb region upstream of the
start codon was PCR amplified from genomic DNA (primers P12-P15; Supplemental Table 1), and cloned into a pBinGlyRed2 vector containing the GUS gene using the BamHI and EcoRI restriction sites. This vector was then transformed into Agrobacterium tumefaciens C58, and cells harboring the binary vector were used to transform wild-type Arabidopsis as described previously (Clough and Bent, 1998). GUS staining solution was comprised of 20 mM sodium phosphate (monobasic), 30 mM sodium phosphate (dibasic), 2 mM potassium ferricyanide and 2 mM potassium ferrocyanide, along with 1 μl/ml Triton X-100 and 1mg/ml 5-bromo-4-chloro-3-indolyl-γ-D-glucuronide (X-GLUC). Tissues were pre-incubated in chilled 90% acetone for 10 minutes then vacuum infiltrated with chilled GUS staining solution for 10 minutes. The tissues were then incubated overnight at 37°C and then cleared with 100% ethanol followed by 70% ethanol. Images of GUS analyzed tissue were taken with an Olympus AX70 optical microscope.

3.3.9 Subcellular Localization of ORM1 and ORM2. YFP fusion proteins with ORM1 and ORM2 were prepared by amplification of the ORM1 and ORM2 open reading frames using gene-specific primers (P16-P19; Supplemental Table 1). PCR products were cloned into the 35S-pFAST-eYFP vector using the SacI and KpnI restriction sites generating C-terminal YFP fusion constructs. Agrobacterium tumefaciens-mediated infiltration of Nicotiana benthamiana leaves was performed with ORM1-YFP and ORM2-YFP constructs separately and in conjunction with the ER marker CD3-959 (HDEL-mCherry). Sequential imaging was performed using a Nikon A1 confocal imaging system mounted on a Nikon Eclipse 90i microscope.
Excitation/emission wavelengths for YFP and mCherry were 488 nm/500-550 nm and 561.6 nm/570-620 nm, respectively.

**3.3.10 Arabidopsis Mutant Genotyping.** T-DNA insertion mutants were acquired from the Arabidopsis Biological Resource Center and the GABI-Kat collections. The REDextract-N-Amp Tissue PCR kit (Sigma) was used to extract genomic DNA from leaf tissue. Genotyping was performed by PCR using gene-specific and T-DNA-specific primer sets P20-P28 (Supplementary Table 1).

**3.3.11 Fumonisin B1 Screening of Arabidopsis ORM1 and ORM2**

**Overexpression and RNAi lines.** ORM overexpressing plants were generated by transforming Col-0 with the CaMV35S promoter:ORM cDNA constructs. AtORM1 and AtORM2 cDNAs were cloned into the binary vector pBinGlyRed3-35S using the EcoRI/XbaI restriction sites (P29-P32). ORM RNAi lines were generated by overexpressing a hairpin composed of an ORM1 or ORM2 gene fragment. The ORM gene fragments were cloned into the pINTRON vector using the XbaI, XhoI, SpeI, and HindIII restriction sites forming a hairpin using primers P33-P36 (Supplemental Table 1). The pINTRON fragment containing the hairpin was amplified using primers P37 and P38 (Supplemental Table 1) and cloned into pBinGlyRed3-35S using the EcoRI/XbaI restriction sites. Arabidopsis (Col-0) plants were transformed with these constructs, and the resulting transformants were selected and screened by qPCR for ORM1 and ORM2 expression. Sensitivity screening relative to a wild type control was done at 0 µM, 0.3 µM, and 0.5 µM FB1 (Sigma) concentrations in Linnsmaier-Skoog (LS media).
Sensitivity was determined by plant growth rate and germination at the varying FB1 concentrations. Plants were grown for two weeks on FB1 before analysis.

### 3.3.12 Plant Microsomal Membrane Isolation.
Microsomal membrane isolation from hydroponically grown 4-week old Arabidopsis roots was performed as described previously and protein concentration was measured using the BCA method (Smith et al., 1985; Lynch and Fairfield, 1993).

### 3.3.13 Ceramide Synthase Assays.
Ceramide synthase assays were performed on microsomal protein derived from hydroponically grown root. The reactions were performed as described previously using 10 µg of microsomal protein (Luttgeharm et al., 2015). Reaction substrates for class I ceramide synthase assays were d18:0 LCB and C16 fatty acid, while substrates for class II ceramide synthase assays were t18:0 LCB and C24 fatty acid. After incubation and extraction, sphingolipids produced in the assay were analyzed by mass spectrometry.

### 3.3.14 Sphingolipid Analysis.
Sphingolipids were extracted from 2-15 mg of lyophilized seedling tissue as described previously (Kimberlin et al., 2013).
3.4 Results

3.4.1 Two Functional Homologs of Mammalian ORMDLs Physically Interact with and Inhibit Arabidopsis SPT. Two genes, designated AtORM1 (At1g01230) and AtORM2 (At5g42000), encoding 157 and 154 amino acid polypeptides respectively, were identified in homology searches using human ORMDLs as query (Hjelmqvist et al., 2002). The amino acid sequences of the Arabidopsis polypeptides share 81% identity and have predicted homologs throughout the plant kingdom. Arabidopsis ORM1 (AtORM1) and ORM2 (AtORM2) share 38 to 43% identity with human ORMDL1, ORMDL2, and ORMDL3. AtORM1 and AtORM2 also share 35-39% identity with S. cerevisiae ORM1 and ORM2, but interestingly lack the N-terminal phosphorylation domain found in the yeast ORMs (Figure 3.1A). Notably, this N-terminal domain is absent from ORMDL proteins from human and other mammals. Similar to the mammalian ORMDL proteins, AtORM1 and AtORM2 are predicted to have two to three transmembrane domains based on in silico analyses using TopPred II, SOSUI, and TMPred programs (von Heijne, 1992; Hirokawa et al., 1998)(Figure 3.1).

To test whether the AtORM polypeptides physically interact with AtSPT, FLAG-tagged AtLCB1 was expressed along with Myc-AtLCB2a, HA-AtssSPTa, and HA-tagged AtORM1 or -AtORM2 in a yeast mutant that lacks endogenous SPT from knockout of the yeast LCB1 and TSC3 genes. Pull-down assays using anti-FLAG antibodies with microsomes of cells expressing these polypeptides resulted in the detection of not only AtLCB1, but also AtLCB2a, and AtssSPTa, AtORM1 or AtORM2, but no detection of ELO3, a polypeptide not known to associate with SPT (Figure 3.1).
These results are consistent with the physical interaction of AtORM1 and AtORM2 with the core SPT complex.

To examine their ability to function as suppressors of SPT activity, Arabidopsis ORM1 and ORM2 were expressed in the S. cerevisiae Δorm2 mutant. The Δorm2 mutant in yeast has a strong sensitivity to excess LCB, and the inclusion of 15 µM phytosphingosine in media is toxic to this mutant (Han et al., 2010). Expression of AtORM1 or AtORM2 in the Δorm2 mutant rescued the sensitivity of the mutant to exogenous LCB, consistent with the ability of the Arabidopsis proteins to suppress SPT activity (Figure 3.1). In addition, Arabidopsis ORM1 and ORM2 were co-expressed along with AtLCB1C144W, AtLCB2a, and AtssSPTa in a yeast mutant strain that lacks endogenous SPT from knockout of the yeast LCB1 and TSC3 genes (Figure 3.2). The AtLCB1C144W contains a single amino acid substitution linked to human sensory neuropathy in the human LCB1 (Gable et. al., 2010). Co-expression of AtLCB1C144W and AtLCB2 results in the ability of SPT to use serine as well as alanine as substrates to form normal LCB (d18:0) and deoxy-LCB (m18:0), respectively (Figure 3.2). Deoxy-LCBs cannot be degraded due to the missing hydroxyl group and serve as an in situ read-out of SPT activity. When expressed in this background, Arabidopsis ORM1 and ORM2 markedly decrease the amount of deoxy-LCB produced, indicating that they act as SPT inhibitors (Figure 3.2). The inhibitory effect of the AtORMs can also be seen on native AtSPT expressed in a yeast mutant lacking endogenous SPT (Figure 3.2). Interestingly this inhibitory effect can be almost completely abolished with the removal of transmembrane domain 1 of AtLCB1 (ΔTMD1)(Figure 3.2). This evidence suggests that
TMD1 of AtLCB1 is important for ORM inhibitory function or ORM binding to the SPT complex, although the exact function of TMD1 to the SPT complex is currently unclear.

Figure 3.1 Arabidopsis ORMs complement S. cerevisiae knockout mutant and physically interact with AtSPT. (A) Amino acid sequence alignment for ORM polypeptides from Saccharomyces cerevisiae (Sc), Arabidopsis thaliana (At), and Homo sapiens (Hs). The
alignments shows the N-terminal extension, found only in yeast, responsible for reversible phosphorylation (at residues marked with asterisks) that impacts SPT activity. (Breslow et al., 2010). The dotted lines mark the positions of 4 potential transmembrane domains identified by hydropathy analyses. (B) Topology mapping of ScORM2. Glycosylation cassettes (GC) were inserted after the indicated amino acid and the GC-tagged proteins were expressed in yeast. Increased mobility following treatment of microsomes with Endoglycosidase H (EndoH) revealed that the GCs at residue 100 and 169 are glycosylated and therefore reside in the lumen of the ER. (C) Model of ORM protein topology. The figure shows the experimentally determined membrane topology of ScORM2. (D) Co-immunoprecipitation of FLAG-tagged AtLCB1 in yeast expressing AtLCB1-FLAG, AtLCB2a-Myc, AtssSPTa-HA, and either AtORM1-HA or AtORM2-HA. Solubilized yeast microsomes were incubated with anti-FLAG beads and protein was eluted with FLAG peptide. Solubilized microsomes (Input) and the eluent (IP-FLAG) were analyzed by SDS-PAGE and the AtORM1/2 peptides were detected by immunoblotting. Elo3p was used as a negative control. (E) AtORM1 and AtORM2 complement the phytosphingosine (15μM) sensitivity of the S. cerevisiae Δorm2 mutant.
AtORM1 and AtORM2 inhibit activity of AtSPT in S. cerevisiae. (A) Schematic representation of the core SPT complex consisting of AtLCB1, AtLCB2a/b, AtssSPT, and AtORM. The complex resides in the ER membrane and catalyzes a condensation reaction between serine and palmitoyl-CoA to produce LCB. The C144W (HSN1) mutation in AtLCB1 allows SPT to utilize alanine as well as serine. LCB produced with alanine lacks the hydroxyl group that is needed for LCB degradation and is referred to as deoxy-sphinganine (DoxSA). (B) AtSPT activity was measured in a yeast mutant that lacks endogenous SPT. Microsomes expressing AtLCB1C144W, AtLCB2a, AtssSPTa with or without AtORM1 or AtORM2 were used to demonstrate an inhibitory effect on SPT activity. The activity is measured by accumulation of deoxy-sphinganine (DOXSA), produced by the AtLCB1C144W-containing mutant SPT enzyme. The DOXSA product is not naturally produced and is not degraded. Values shown are the average of three independent assays ± SD. **P<0.01. (C) Immunoblot of yeast microsomes expressing AtLCB1C144W-FLAG, AtLCB2a-Myc, AtORM1/2-HA, and
AtSPT activity measured in microsomes of yeast lacking endogenous SPT but expressing AtLCB1, AtLCB2a, AtssSPTa and either AtORM1 or AtORM2. The activity is measured through the accumulation of total LCB produced. (E) AtSPT activity measured as in (D), except transmembrane domain 1 of AtLCB1 was removed.

3.4.2 AtORM1 and AtORM2 are Constitutively Expressed and AtORM1 and AtORM2 Polypeptides are ER-Associated. To assess the in planta contributions of each gene to SPT inhibition, transcript levels of AtORM1 and AtORM2 were measured in different organs of Arabidopsis. Our analyses revealed that AtORM1 is more highly expressed in all tissues tested except for pollen, although the differences in expression are modest (Figure 3.3A). AtORM1 transcript was approximately 3-4 fold more abundant in all tissues tested except pollen, where AtORM2 transcript was approximately 4-fold more abundant (Figure 3.3A). Our results are consistent with those for these genes in the AtGenExpress public microarray database (Supplemental Figure 1 in Appendix B).

Using AtORM1 and AtORM2 promoter::GUS fusion constructs, the location of in planta expression was examined. Promoters for both genes conferred expression in vegetative tissues, with GUS staining most pronounced in vascular tissues. Differences in promoter activity for AtORM1 and AtORM2 were observed in floral tissues and roots. Based on the intensity of GUS staining, AtORM1 promoter conferred higher expression in the anther and developing embryos than the AtORM1 promoter (Figure 3.3H-J) while AtORM2 promoter conferred greater expression than the AtORM1 promoter in filaments, petals, sepals, pistil and siliques (Figure 3.3M-O). Both AtORM1 and AtORM2 promoters yielded GUS expression in mature pollen grains and in roots. However, the
AtORM1 promoter, but not the AtORM2 promoter, conferred detectable GUS expression in lateral root buds (Figure 3.3K, L, P, Q).

The subcellular localization of ORM1 and ORM2 polypeptides was visualized using N-terminal and C-terminal yellow fluorescent protein (YFP) tags with transient expression in *Nicotiana benthamiana*. Tagged-ORM1 and -ORM2 co-localized with the ER marker mCherry-HDEL (Figure 3.3D, G), consistent with the known ER localization of AtLCB1, AtLCB2a/2b, and AtssSPTa/b (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008; Kimberlin et al., 2013), but were also detected in other subcellular locations, including the cytosol (Figure 3.3B-G).
3.4.3 Overexpression of At ORM1 and 2 Results in Dwarfed Growth. To examine the in planta functions of AtORM1 and AtORM2, three available T-DNA mutant lines for these genes were initially characterized: SALK_046054 (predicted T-DNA insertion in the first intron of AtORM1), GK-143A01 (predicted T-DNA insertion in the first exon of AtORM1), and SAIL_1286_D09 (predicted T-DNA insertion in the 5’UTR of AtORM2). Homozygous lines were identified for each of these mutants, but full length transcripts were still detected, indicating that these lines are not null mutants for the AtORM1 and AtORM2 genes (Supplemental Figure 2 in Appendix B). As an alternative approach for characterization of the in planta functions of AtORM1 and AtORM2, overexpression and RNAi suppression lines for these genes were created in Arabidopsis Col-0. Overexpression lines were prepared by placing the AtORM1 or AtORM2 cDNA under control of the cauliflower mosaic virus 35S (CaMV35S) promoter. Homozygous lines were screened by qPCR to identify those with increased
expression. A portion of the lines with confirmed overexpression of AtORM1 or AtORM2 were visually indistinguishable from Col-0 plants, including those with ~5-fold overexpression of AtORM1 and ~35-fold overexpression of AtORM2 in leaves (Figure 3.4B, E). However, a second class of overexpression lines for both genes was observed with strong dwarfing. These lines included those with >80-fold overexpression of AtORM1 and ~800-fold overexpression of AtORM2 in leaves relative to expression levels of these genes in Col-0 leaves (Figure 3.4C, F). These results suggest that a threshold of AtORM1 and AtORM2 levels can be reached to suppress SPT activity sufficiently to reduce growth, as previously observed for AtLCB1 RNAi suppression (Chen et al., 2006) and reduced AtssSPTa expression (Kimberlin et al., 2013). While Col-0 and OE ORM lines showed lower SPT activity, they were not significantly different (Supplemental Figure 3 in Appendix B). Sphingolipidomic profiling revealed little difference between Col-0 and ORM overexpression lines and virtually no difference between ORM overexpression lines with or without a growth phenotype (Supplemental Figures 4-7 in Appendix B). ORM RNAi suppression lines showed significant suppression of both ORM1 and ORM2 transcript (Supplemental Figure 8 in Appendix B). Consistent with a role of ORMs as SPT repressors, SPT activity assayed from root microsomes of RNAi suppression lines showed increased SPT activity (Supplemental Figure 3). No reductions in growth and no significant differences was seen in the sphingolipidome of ORM RNAi lines relative to the Col-0 controls (Supplemental Figures 5-7 in Appendix B).
Figure 3.4 Phenotype Associated with ORM overexpression. (A) Sufficient upregulation of ORM by transgenic overexpression causes a phenotype that is characterized by slow growth, smaller plant size, early leaf senescence, and early plant death. Representative pictures are shown in (A)-(F). Comparatively grown wild type (Col-0) is shown in (A) and (D). AtORM1 overexpression line 1 is shown in panel (B) while AtORM2 overexpression line 1 is shown in panel (E). AtORM1 overexpression line 2 is shown in panel (C) while AtORM2 overexpression line 2 is shown in panel (F). This phenotype appears to correlate with ORM expression level, as the strongest overexpressors display a phenotype, while relatively weaker overexpressors do not show a noticeable phenotype. (G)-(H) Expression levels of ORM1 (G) and ORM2 (H) in overexpressing lines. Tissue was collected from wild-type Col-0 and lines overexpressing ORM1 and ORM2 grown under standard conditions. qPCR was used to determine relative ORM1 and ORM2 transcript levels by comparison with Col-0. Protein phosphatase 2A subunit A3 (PP2AA3) was used as a reference gene. Values shown are ± SD for three independent measurements and indicate relative fold increase of ORM1 or ORM2 compared to wild type levels.
3.4.4 Modulation of ORM Expression Affects Sensitivity to FB1. The PCD-inducing mycotoxin FB1, a ceramide synthase inhibitor, has been routinely used as means of perturbing sphingolipid homeostasis for study of SPT activity. From these studies, reduced SPT activity, such as that achieved by suppression of AtssSPTa, results in enhanced resistance to FB1 due presumably to reduced accumulation of cytotoxic LCBs (Kimberlin et al., 2013). Conversely, increased SPT activity, such as that obtained by overexpression of AtssSPTa, results in enhanced sensitivity to FB1 due presumably to accumulation of cytotoxic LCBs (Kimberlin et al., 2013). Consistent with suppression of SPT activity, AtORM1 or AtORM2 overexpression lines displayed increased resistance to FB1, along with a decrease in accumulation of free LCBs and LCB-phosphates (LCBPs) (Figure 3.5A, 3.5B). These lines were viable at 0.5 µM FB1, a concentration that was toxic to wild-type Arabidopsis (Figure 3.5A). This phenotype was observed in a range of overexpression lines, including those with and without the dwarfing phenotype described above (Supplemental Figure 9 in Appendix B). Conversely, AtORM RNAi suppression lines were not viable on media containing 0.3 µM FB1, but no toxicity was observed in wild type plants at this concentration (Figure 3.5A). RNAi suppression lines of ORM were observed to have a large increase in free LCBs and LCBPs when grown on FB1, which was particularly accentuated relative to AtORM overexpression lines and wild type Arabidopsis on media containing 0.5 µM FB1 (Figure 3.5B). Mirroring alterations in LCB concentrations in transgenic and wild type plants in response to FB1, concentrations of ceramides containing C16 fatty acids were lower in lines with overexpression of ORM and higher in RNAi suppression lines (Figure 3.5C). These
results indicate that altered ORM expression is an effective way of modulating plant sensitivity to FB1 and indicates that AtORMs act as inhibitors of SPT.

Figure 3.5 Modulation of ORM expression changes sensitivity to FB1 and alters LCB(P) and ceramide accumulation. (A) Altered expression of ORM affected sensitivity to FB1,
a competitive inhibitor of ceramide synthase. Seeds were sown on LS agar plates supplemented with FB1 at 0.3 and 0.5 μM as indicated. The wild type (Col-0) is extremely sensitive to FB1 at 0.5 μM and less affected at 0.3 μM. Upregulation of ORM by transgenic overexpression (OE) causes an FB1 resistant phenotype, whereas downregulation of ORM by RNAi causes an FB1 sensitive phenotype (ORM RNAi). Images were taken 14 days after sowing seeds and are representative of three independent experiments. (B) Altered ORM expression affects accumulation of cytotoxic free LCB and LCB-phosphate (LCB-P) levels in response to FB1 treatment. Wild-type plants show increased total LCB levels when treated with FB1. Compared with the wild type, ORM overexpression (OE) plants display FB1 resistance and reduced total LCB level. Alternatively, ORM RNAi suppression plants display FB1 sensitivity and increased total LCB level. Electrospray ionization-tandem mass spectrometry analyses were performed with three independent biological replicates ± SD. Plants were grown on LS plates ± FB1 for 2 weeks before tissue collection. DW, dry weight. *P<0.05. **P<0.01. (C) Total C16 ceramide levels are affected by modulation of ORM expression. As in (B), except C16 ceramide is quantified. ORM1 overexpression plants show decreased accumulation of C16 ceramide when compared with wild type comparatively grown on LS ± FB1, while ORM RNAi plants show increased accumulation of C16 ceramide. DW, dry weight. *P<0.05.

3.4.5 Modulation of ORM Expression Impacts Ceramide Synthase Activity.

Recent studies in S. cerevisiae have suggested a role for ceramide synthase in maintenance of sphingolipid homeostasis through a phosphorylation/de-phosphorylation mechanism similar to that used for reversible modulation of ORMs (Muir et al., 2014). In contrast to S. cerevisiae, Arabidopsis has two functionally distinct ceramide synthase classes: Class I and Class II. This difference likely results in more complexity of potential ceramide synthase regulation of sphingolipid homeostasis in Arabidopsis. To gain insights into coordinate regulation of ORMs and ceramide synthases, Class I ceramide synthase was assayed in microsomes from roots of AtORM overexpression and RNAi lines using 16:0-CoA and d18:0, the preferred substrates of this enzyme class. Class II ceramide synthase activity was assayed using 24:0-CoA and t18:0, preferred substrates of this enzyme class. Lower Class I ceramide synthase activity was detected in microsomes of AtORM overexpression lines and increased activity was detected in microsomes from
AtORM RNAi suppression lines (Figure 3.6A). Conversely, lower Class II ceramide synthase activity was increased in microsomes from AtORM RNAi suppression lines, but reduced in AtORM overexpression lines (Figure 3.6B). These results suggest preferential metabolic flux through Class I ceramide synthase for the synthesis of C16 fatty acid-containing ceramides with increased SPT activity resulting from disrupted ORM-mediation of SPT in AtORM RNAi lines, and preferential flux through Class II ceramide synthases for production of VLCFA-containing ceramides when SPT activity is limited by suppression from increased ORMs in AtORM overexpression lines (Figure 3.7). These findings are consistent with the increased concentrations of C16 fatty acid-containing ceramides detected in AtORM RNAi lines in response to FB1 treatment (Figure 3.6B).

**Figure 3.6** Ceramide Synthase Activity is Altered by Modulation of ORM Expression. (A) Altered ORM expression impacts ceramide synthase activity. Ceramide synthase activity was assayed on microsomal protein prepared from hydroponically grown root tissue. Increase in ORM expression resulted in a decrease in class I ceramide synthase activity, while RNAi suppression of ORM resulted in an increase in activity. *P<0.05, **P<0.01. (B) Increase in ORM expression resulted in an increase in class II ceramide
synthase activity, while RNAi suppression of ORM resulted in a decrease in activity. **P<0.01.

**Figure 3.7** Model of How AtORM1/2 Impacts SPT and Ceramide Synthase Activity. The figure shown represents the core synthesis pathway of ceramides in Arabidopsis. Serine palmitoyltransferase (SPT) catalyzes a condensation reaction between serine and palmitoyl-CoA leading to the production of 3-ketosphinganine which is reduced to dihydroxy long chain base (d18:0) through 3-ketosphinganine reductase activity. Dihydroxy LCBs can be used by class I ceramide synthase along with palmitoyl-CoA to produce “short-chain” C16 ceramide. Alternatively dihydroxy LCBs may be hydroxylated by C-4 hydroxylase to form trihydroxy LCBs (t18:0) which can be used by class II ceramide synthases to produce “long-chain” ceramide (C18-C26). Both class I and class II have activity under normal conditions in which SPT is active, but modulation of ORM expression leads to alterations in ceramide synthase activity. ORM RNAi suppression, indicated by “GO”, results in increased SPT activity and increased generation of LCBs with a concomitant increase in class I activity and decreases in class II activity. Conversely, overexpression of ORM, indicated by “STOP”, results in decreased SPT activity and reduced LCB generation with a concomitant decrease in class I activity and increases in class II activity.
3.5 Discussion

Research reported here demonstrates the existence of two ORM proteins in Arabidopsis that share significant identity with human and yeast homologs. Our findings provide evidence for a conserved function of ORM, as an inhibitor of SPT, across different eukaryotes. Although AtORMs complemented the yeast mutant PHS sensitivity, it is unclear if AtORMs can actually de-repress in yeast or if they are simply binding to and inhibiting yeast SPT (Figure 3.1). If a de-repression mechanism were to occur it would have to occur through a novel mechanism distinct from the one found in yeast ORMs, as the N-terminal region responsible for this mechanisms is missing. It is also possible that other proteins are involved with the SPT complex and they in turn may be responsible for de-repressing ORM inhibition. Further studies are needed to identify all of the interacting proteins of SPT in planta. We also show that the Arabidopsis ORMs physically interact with the Arabidopsis SPT heterodimer and effectively inhibit activity (Figures 3.1 & 3.2). Furthermore, we show that the inhibitory action of the ORMs is dependent upon transmembrane domain 1 of LCB1, which suggests that this first transmembrane domain of AtLCB1 is important for SPT regulation (Figure 3.2). It is unclear at this moment if TMD1 of AtLCB1 merely serves as an interaction point for the AtORMs or has some higher function. Recent evidence in yeast points to a regulatory mechanism of ORM that “senses” intracellular sphingolipid levels and adjusts SPT activity accordingly (Roelants et al., 2011; Muir et al., 2014). ORMs in yeast have been shown to inhibit SPT activity, although their inhibition of SPT can be gradually relieved through phosphorylation of the ORM N-terminus by ypk1, which can also be reversed again through de-phosphorylation.
by sac1. The general notion is that complete phosphorylation of ORM occurs when sphingolipid levels are low, thus allowing SPT to function at a higher capacity, while complete de-phosphorylation occurs when sphingolipid levels are very high, fully inhibiting SPT. This regulatory system is versatile and would allow for adjustments and fine tuning of SPT activity to match current cellular demands for sphingolipids. Although the AtORMs lack the N-terminal extension region found in yeast, we have shown them to be functional inhibitors of SPT. There is not enough evidence to refute the notion that ORM in Arabidopsis still operate based on a reversible phosphorylation mechanism, like the one found in yeast. Although evidence certainly suggests that if they do it most likely involves other proteins. It is also possible that ORM inhibition of SPT occurs through a different mode of regulation that may include transcriptional regulation or other post-translation mechanisms (Chueasiri et al., 2014). It is possible that AtORM1 and AtORM2 are not completely redundant allowing for fine tuning of SPT activity through transcriptional modulation of the AtORMs. Our studies do seem to indicate that AtORM1 may potentially be a more potent inhibitor of SPT activity as AtORM1 overexpressing lines show a more robust FB1 resistance and show a more severe phenotype with increased overexpression (Figures 3.4, 3.5). Although mechanistically still unclear, our findings unequivocally demonstrate that the ORM proteins in Arabidopsis inhibit SPT activity through physical interaction with SPT.

Numerous recent studies have demonstrated the essential nature of sphingolipids and specifically the absolute requirement for functional SPT activity for cell viability (Chen et al., 2006; Dietrich et al., 2008; Kimberlin et al., 2013). It is because of this that
loss of SPT functionality leads to severe and often times lethal phenotypes. We have shown here that, once again, a severe phenotype is associated with a loss of SPT activity (Figure 3.4). Interestingly this phenotype correlates quite well with high expression of AtORMs, suggesting a threshold of SPT activity needed for plant viability. Consistent with this notion, ORM RNAi suppression lines show no obvious phenotype presumably due to adequate SPT activity (Supplemental Figure 3 in Appendix B). We do not see a significant decrease in SPT activity in root microsomes with ORM overexpression and this is likely due to the limitations of the assay as SPT activity is naturally very low making it difficult to detect further decreases. While we have identified homozygous T-DNA mutant lines for AtORM1/2, we have not found a T-DNA mutant devoid of transcript (Supplemental Figure 1 in Appendix B). Due to the lack of complete knockout mutants for the Arabidopsis ORMs, it is unclear if these genes are essential and it is possible they have other important functions. Indeed, the subcellular localization of the AtORMs indicates that may be localized to other areas in the cell besides the ER.

Interestingly, our research suggests that the ORM proteins in Arabidopsis may have other functions, as indicated by their modulation of ceramide synthase activity (Figure 3.6). It is unknown if this occurs through direct activity or interaction of ORMs with ceramide synthase or whether this change in activity is mediated through other indirect protein interactions. Either way this points to complex and intricately regulated sphingolipid synthesis. These findings are also consistent with the yeast regulatory model in which phosphorylation and subsequent activation of ceramide synthase is coordinated with phosphorylation of ORM and de-repression of SPT activity. This ultimately has the effect of increasing LCB flux through the pathway. This is consistent with current
knowledge as spikes in free LCB concentrations have been shown to trigger PCD, so in order to avoid cell death, free LCBs produced by SPT need to be rapidly incorporated into ceramides and other downstream sphingolipids. It is unclear if ceramide synthase in Arabidopsis is phosphorylated or if this mechanism is conserved in plants. Our findings point to something similar though, albeit a bit more complex. With overexpression of ORM we see a shift in activity, with an increase in class II ceramide synthase activity and a decrease in class I ceramide synthase activity. We also see the reverse in ORM RNAi suppression lines with an increase in class I ceramide synthase activity and a decrease in class II ceramide synthase activity (Figure 3.7). Short-chain C16 ceramides produced by the class I enzyme are not essential and their function is unknown, although evidence suggests that their accumulation is detrimental (Luttgeharm et al., 2015). Longer-chain ceramides produced by the class II enzymes are essential and appear to be vital to cell viability (Ternes et al., 2011). The ceramide synthase activity shift seen with the ORM mutants may be a compensatory regulatory mechanism similar to the one seen in yeast. Increases in SPT activity, through ORM RNAi suppression, may activate class I ceramide synthase as a safety mechanism to avoid accumulation of toxic free LCBs (Figure 3.7). Whereas, limited SPT activity, through ORM overexpression, may upregulate class II ceramide synthase activity as these ceramides are essential for cell viability. Further research is needed to fully elucidate these regulatory mechanisms.

In addition, we have demonstrated that modulation of ORM expression can lead to predictable changes in FB1 sensitivity paired with changes in SPT activity and subsequent LCB accumulation (Figure 3.5). Consistent with our hypotheses, we show the
inhibitory effect AtORMs have on AtSPT and that AtORMs modulate accumulation of free LCBs and LCBPs. This suggests that ORM protein levels in Arabidopsis are not limiting and that it is possible that transcriptional regulation of ORMs may exist as a mechanism to regulate SPT activity. Further studies are needed to determine if transcriptional regulation of the AtORMs is important for regulating SPT activity. Interestingly, the core SPT complex does have activity without ORM indicating that ORM is not needed to stabilize the complex. The nature of this inhibitory action though, is still unclear as it is unknown if AtORMs have any enzymatic activity. It is possible that they have no direct activity by themselves but merely act to physically block SPT’s access to substrates. Post-translational modifications may alter ORM protein conformation, allowing for better substrate access for SPT. Without the N-terminal extension found in yeast, AtORMs likely inhibit AtSPT through a different mechanism. One predicted phosphorylation site does occur in AtORM1 and AtORM2, so phosphorylation cannot be ruled out and further research is needed to determine if these proteins are phosphorylated in planta. While regulation of SPT is critical, other enzymes in this pathway, including ceramide synthase, are most likely coordinately regulated in order to maintain sphingolipid homeostasis and promote cell viability. The AtORMs remain an interesting target for future studies as they may be a vital regulatory component for integrating sphingolipid biosynthesis and homeostasis.
3.6 Works Cited


Han S, Lone MA, Schneider R, Chang A (2010). Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. Proc Natl Acad Sci U S A 107: 5851-5856


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Chapter 4:

Designing a Better System to Analyze SPT Activity

Note: This chapter may be part of a publication in the future.
4.1 Abstract:

The activity of serine palmitoyltransferase (SPT) is essential for eukaryotic organisms and represents a key regulatory point for maintaining sphingolipid homeostasis. SPT catalyzes a condensation reaction between serine and palmitoyl-CoA, creating long-chain base (LCB), the unique component of sphingolipids. Measurement of SPT activity, therefore, remains an important aspect of studying and understanding the complex regulation of sphingolipid biosynthesis. Unfortunately getting good SPT activity measurements has proven to be a challenge in plant tissues. SPT activity from Arabidopsis tissues is detectable, although quite low, especially when compared to Arabidopsis SPT activity from yeast expressing the enzyme. Activity measured through the synthesis of LCBs also does not take into account the degradation component of the pathway which could potentially lead to misinterpretation. Here we discuss the design of a better system to measure SPT activity in planta using a disease associated mutation found in human LCB1. The C144W mutation found in human LCB1 is associated with Hereditary Sensory Neuropathies (HSN) and allows SPT to use alanine as well as serine. This allows for the production of deoxysphinganine (DoxSA), which is not normally present in plants and, as a result of the loss of hydroxyl group, cannot be degraded. DoxSA accumulation then provides a potentially better readout for SPT activity in plants.
4.2 Introduction:

Sphingolipids are a diverse class of essential lipid that are important for plant growth, development, and immune responses and are highly abundant in endomembranes and the plasma membrane, where they make up more than 40% of the total lipid (Sperling et al., 2005). Sphingolipids are also enriched in lipid microdomains which are important for protein organization at the plasma membrane (Cacas et al., 2012; Perraki et al., 2012; Cacas et al., 2016). Outside of their structural roles in membranes, sphingolipids and their metabolites can act as signaling molecules that modulate programmed cell death which can impact development and pathogen resistance (Greenberg et al., 2000; Liang et al., 2003; Shi et al., 2007; Bi et al., 2014). It is therefore imperative to maintain sphingolipid homeostasis for cell viability, with dysregulation leading to cell death, abnormal growth, and gametophytic lethality (Greenberg et al., 2000; Liang et al., 2003; Chen et al., 2006; Chen et al., 2008; Dietrich et al., 2008; Kimberlin et al., 2013).

The main regulatory point for maintaining sphingolipid homeostasis is proposed to be serine palmitoyltransferase (SPT), the first committed step in sphingolipid biosynthesis (Hanada, 2003). SPT catalyzes a condensation reaction between serine and palmitoyl-CoA producing a long-chain base (LCB), which is the unique component of sphingolipids (Markham, 2013). The core SPT enzyme is a heterodimer consisting of LCB1 and LCB2 subunits, although other proteins interact with this heterodimer and can greatly impact its activity, including ssSPT and ORM proteins (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008; Han et al., 2009; Breslow et al., 2010; Kimberlin
et al., 2013). Although previous works has focused on the regulatory aspects of these proteins that affect SPT, a core problem in studying this enzyme in plants is its low activity in in vitro assays (Kohlwein et al., 2001; Breslow et al., 2010; Han et al., 2010; Kimberlin et al., 2013). Both SPT activity and free LCB concentrations are normally quite low, making analysis of this enzyme’s activity quite difficult. Artificial elevation of LCBs and LCBPs can be achieved by using the mycotoxin, fumonisin B1, to inhibit ceramide synthase (Stone et al., 2000; Markham et al., 2011; Kimberlin et al., 2013). This does not provide an accurate picture of SPT activity under normal circumstances and fails to account for the degradative pathway that may be influencing both SPT regulation and LCB accumulation. Given how important SPT modulation is to maintaining cell viability we sought to design a system to more accurately and easily look at SPT activity. We wanted to utilize a known mutant variant of LCB1 that is associated with hereditary sensory neuropathy type 1A (HSN1) in humans, which alters SPT substrate specificity (Bejaoui et al., 2002; Gable et al., 2002; Jun et al., 2015). HSN1 is a dominantly inherited degenerative disorder of the peripheral nerves and, in humans, is associated with several point mutations that occur in LCB1 (Bejaoui et al., 2002; Stimpson et al., 2015). The detrimental effects associated with HSN1 are thought to be caused by accumulation of the unusual LCB, deoxysphinganine (DoxSA) (Ernst et al., 2015; Othman et al., 2015). The HSN1 mutation broadens the substrate specificity of the SPT heterodimer to include alanine as well as serine (Eichler et al., 2009). While normal LCB is still produced by the HSN1 SPT heterodimer, it also produces some DoxSA by utilizing alanine instead of serine. Interestingly, DoxSA cannot be degraded as it is
missing the critical hydroxyl group required for LCB degradation and hence accumulates over time.

Incorporating the HSN1 mutation into the Arabidopsis SPT would allow the production of the non-degradable LCB, DoxSA. Importantly, DoxSA is also not normally present in Arabidopsis making it a viable readout of SPT activity, as any detected would be produced by the mutant enzyme. Modulation of SPT activity in these HSN1 mutant lines then, would in theory allow us to see proportional changes in DoxSA accumulation that reflect changes in SPT activity. Evidence in yeast points to a system like this operating, although it has not been tested in planta (Chapter 3). Here we show that there is a conserved HSN1 mutation that occurs in the Arabidopsis LCB1. We also show that overexpression of HSN1 in Col-0 leads to the production of DoxSA. In addition we describe a strategy to replace native LCB1 with HSN1 in Arabidopsis utilizing the SALK_077745 LCB1 T-DNA mutant.
4.3 Methods

4.3.1 Plant Material and Growth Conditions. All Arabidopsis thaliana Col-0 lines used in this study were stratified at 4°C for 4 days before growth and were maintained at 22°C with a 16 h light (100 µmol/m-2/s-1)/8 h dark cycle. Plants that were grown hydroponically were grown on modified Hoagland’s solution as described previously, in custom made hydroponics tanks (Conn et al., 2013).

4.3.2 Yeast Growth and Expression Plasmids. Yeast (Saccharomyces cerevisiae) strain TDY9113 (Mat a tsc3Δ:NAT lcb1Δ:KAN ura3 leu2 lys2 trp1Δ) lacking endogenous SPT was used for expression and characterization of the Arabidopsis thaliana LCB1C144W-containing SPT. The mutant was cultured in medium containing 15 µM phytosphingosine and 0.2% (w/v) tergitol. The AtssSPTa (At1g06151) and AtssSPTb (At2g30942) open reading frames were amplified by PCR and inserted into pPR3-N (Dualsystems Biotech) with HA tagged at N-terminus. The pAL2-URA was constructed for divergent constitutive expression of AtLCB1-FLAG and Myc-At LCB2a by replacing the GAL1 and GAL10 promoters of pESC-URA (Stratagene) with the yeast LCB2 and ADH promoters, respectively.

4.3.3 HSN1 (C144W) Construct Creation and Transformation. The HSN mutation was created in native AtLCB1 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutated AtLCB1 (HSN) was then cloned into the pBinGlyRed3 binary vector using the XhoI and Xbal sites. Binary vectors were transformed into Agrobacterium tumefaciens GV3101 by electroporation. The floral dip method was used to create transgenic plants in Arabidopsis thaliana (Col-0) (Clough and Bent, 1998). A
green LED light and a Red 2 camera filter were used to screen seeds and identify transformed seed that contained DsRed.

4.3.4 Arabidopsis Mutant Genotyping. T-DNA insertion mutants were acquired from the Arabidopsis Biological Resource Center. The REDextract-N-Amp Tissue PCR kit (Sigma) was used to extract genomic DNA from leaf tissue. Genotyping was performed by PCR using gene-specific and T-DNA-specific primer sets.

4.3.5 Microsome Preparation and SPT Assay. Plant microsomes were prepared and SPT activity was assayed as described (Kimberlin et al., 2013) except that 50 µM palmitoyl-CoA and 20 µM BSA were used for the Arabidopsis microsomal SPT assays. SPT activity was measured using [3H] Ser and palmitoyl-CoA.

4.3.6 Sphingolipid Analysis. Sphingolipids were extracted from 2-15 mg of lyophilized leaf tissue as described previously (Kimberlin et al., 2013).
4.4 Results

4.4.1 SPT Activity is Low in Arabidopsis Microsomes. SPT activity from both leaf and root tissue has been described previously. SPT activity in microsomes from root is marginally higher than leaf with roughly twice the amount of activity (Figure 1). Although activity of Arabidopsis SPT expressed in yeast is higher still, being roughly tenfold higher (Figure 4.1). This suggests that the Arabidopsis SPT enzyme itself is capable of higher activity and that the lower activity seen in plant tissues is most likely attributable to an unknown inhibitory compound found in microsomal fractions from plant tissues.
Figure 4.1 AtSPT activity measured in different tissues. Leaf tissue was harvested from rosettes of 3-week old soil grown Arabidopsis. Root tissue was harvested from 3-week old hydroponically grown Arabidopsis. AtSPT was expressed in a yeast mutant lacking endogenous SPT. All measurements were performed on normalized microsomal protein.

4.4.2 An HSN1 mutation in LCB1 is conserved in Arabidopsis. One of the mutations in human LCB1 responsible for HSN1 is C133W. Human and Arabidopsis LCB1 share significant homology, being 44% identical. Although there is no cysteine at position 133 in Arabidopsis, there is a cysteine at position 144 that coincides with a conserved domain found in the human LCB1 (Figure 4.2). Mutating this residue then should confer the effect and allow for the production of DoxSA.

Figure 4.2 Partial amino acid sequence alignment of *Arabidopsis thaliana* LCB1 (*AtLCB1*) and *Homo sapiens* LCB1 (*HsLCB1*). The red arrow indicates one residue responsible for the HSN1 phenotype which corresponds to C133 in humans and C144 in Arabidopsis.

4.4.3 Overexpression of HSN1 (C144W) mutated AtLCB1 in Col-0 allows for production of deoxysphinganine. Overexpressing Arabidopsis LCB1^{C144W} in Col-0 produced normal plants with no obvious phenotype. These lines did accumulate some DoxSA, albeit a small amount, indicating that the LCB1^{C144W} mutation is analogous to
the LCB1\textsuperscript{C133W} mutation found in humans (Figure 4.3). The low amount of DoxSA produced could be attributable to the native enzyme still being present competing for substrate. Expressing AtLCB1\textsuperscript{C144W} in a yeast strain lacking endogenous yeast has been used previously and shows accumulation of DoxSA as well (Chapter 3).

![Ceramide profile of Arabidopsis (Col-0) overexpressing AtLCB1\textsuperscript{C144W} showing the production of DoxSA (m18:1).](image)

**Figure 4.3** Ceramide profile of Arabidopsis (Col-0) overexpressing AtLCB1\textsuperscript{C144W} showing the production of DoxSA (m18:1).

### 4.4.4 Complementation of SALK\textsubscript{077745} mutant requires the native promoter

We sought to increase the amount of DoxSA produced and designed a complementation construct with the native promoter of LCB1 and transformed it into heterozygous LCB1 mutants (SALK\textsubscript{077745}). Complete knockout of LCB1 is lethal, so we are relying on the LCB1p::LCB1\textsuperscript{C144W} construct to complement this mutant (Chen et al., 2006). If complementation is successful then we will have lines that contain only LCB1\textsuperscript{C144W}, lacking native LCB1. Complementation is unsuccessful when using the 35S promoter, most likely due to problems with the promoter being expressed in pollen.
Complementation has been achieved with WT LCB1 under control of its native promoter indicating that the promoter region chosen is adequate for complementation (Figure 4.4).

**Figure 4.4** Complementation of SALK_077745. SALK_077745 (LCB1) is homozygous lethal and is maintained as a heterozygous mutant. A construct containing the wild-type AtLCB1 under control of the upstream native promoter region (~1kb) is sufficient to complement the SALK_077745 T-DNA mutant. Amplification with gene specific primers indicates presence of wild type AtLCB1, while amplification with T-DNA primers indicates presence of T-DNA. Amplification with both primer sets indicates heterozygous line. Amplification with gene specific only indicates wild type. Amplification with T-DNA only indicates homozygous mutant (complemented).
4.5 Discussion

We have shown that a disease causing mutation found in human LCB1 is conserved in Arabidopsis LCB1 and is responsible for producing the aberrant LCB, DoxSA. We discuss the design of a potential tool in studying SPT in planta. This would be a valuable asset in studying sphingolipid metabolism as SPT activity is considered a key regulatory point for sphingolipid biosynthesis. Ideally complementation of the SALK_077745 mutant would be successful, although the mutant LCB1 may not successfully complement. In this case it would still provide us with some valuable insight, pointing to the importance of native SPT activity. It would also suggest that a disease causing mutation found in humans is also problematic in plants, emphasizing the importance of this highly conserved region of LCB1. It is also possible that the decrease in overall SPT activity found in the mutant heterodimer (Figure 1), may not allow for successful complementation. The use of the native promoter of AtLCB1 should allow for functional expression of AtLCB1C144W although other promoters could be tried.

The use of DoxSA as a readout has been shown to work in yeast and it appears to be a promising mechanism in plants. The benefit of using DoxSA is twofold. For one, DoxSA is not normally produced in Arabidopsis and so when looking at DoxSA production, one can safely assume it is reflective of SPT activity and not due to background. Also, DoxSA lacks the hydroxyl group responsible for phosphorylation of LCBs and thus prevents degradation of DoxSA. This is quite important as LCBs are most likely in constant flux and so measurements may not be entirely reflective of SPT activity itself, they may be more indicative of a change in LCB degradation. It is hard for
us to know which contributes more to LCB levels in certain circumstances; modulation of
SPT or modulation of LCB degradation. DoxSA alleviates this problem as it cannot be
degraded and so accumulation is indicative of SPT activity, not a decrease in degradation.
It will also be interesting to see if DoxSA is incorporated into more complex
sphingolipids like glucosylceramide or the glycosyl inositolphosphoceramides (GIPCs) in
plants. This could possibly allow for further characterization of the entire pathway as
you could potentially quantify flux of LCB under differing conditions.
4.6 Works Cited


mutation in serine palmitoyltransferase resides at a putative phosphorylation site that is involved in regulating substrate specificity. Neuromolecular Med 17: 47-57


Kimberlin AN, Majumder S, Han G, Chen M, Cahoon RE, Stone JM, Dunn TM, Cahoon EB (2013) Arabidopsis 56-amino acid serine palmitoyltransferase-interacting proteins stimulate sphingolipid synthesis, are essential, and affect mycotoxin sensitivity. The Plant cell 25: 4627-4639


5.1 Final Discussion

The results discussed in this thesis point towards a complex regulatory scheme for maintaining sphingolipid homeostasis. We have shown that two distinct protein pairs act oppositely of each other in their impact on SPT activity. We have shown that SPT activity is essential for viability and have provided evidence suggesting a connection between SPT activity and downstream sphingolipid metabolism. We have described a potential regulatory strategy, through modulation of these proteins, in altering SPT activity and have laid the groundwork for establishing a more detailed picture of the SPT protein complex.

In chapter two we identified two small proteins, AtssSPTa/b, that physically interact with SPT and impact SPT activity. We show that AtssSPTa/b are redundant and essential, consistent with their function as activators of SPT. Our research also suggests that they are limiting as modulation of their expression leads to consistent changes in SPT activity. This would suggest transcriptional regulation as a means to control SPT activity however, due to their small size the AtssSPTs are not represented in public expression databases making it difficult to determine if SPT is regulated in this way. It remains to be determined if the transcriptional regulation of AtssSPT is a mechanism that occurs to adjust AtSPT activity in response to certain stressors.

The phenotype seen in the AtssSPTa heterozygous mutant is consistent with loss of SPT activity. The pollen lethality observed is comparable to that found in the LCB2a/b mutant in which the pollen did not progress to the bicellular stage of pollen (Dietrich et al., 2008). While not directly observed in this case, it is most likely that loss
of SPT activity, through loss of the activator AtssSPT, results in the same defective pollen, with an underdeveloped endomembrane system and loss of intine layer (Dietrich et al., 2008). This is suggestive of a disruption in Golgi trafficking and is most likely reflective of the biophysical roles sphingolipids play in maintaining membrane structure and fluidity. While the specific function of sphingolipids in membrane organization has not been define in mechanistic detail, it appears that alteration or loss of sphingolipids can have significant impacts on membrane trafficking and protein organization in membranes (Melser et al., 2010; Markham et al., 2011; Melser et al., 2011; Yang et al., 2012).

While AtssSPTb did rescue the pollen defect found in the AtssSPTa mutant with sufficient expression, it is still possible that AtssSPTa and AtssSPTb are not entirely redundant. Although the AtssSPTs are nearly identical, there are residue differences that could be responsible for differences in SPT activation. Evidence for this can be seen in the human ssSPTs, in which ssSPTb shifts SPT substrate preference towards steryl-CoA (Han et al., 2009). This shift was found to be the result of a point mutation (M19V) that is conserved in Arabidopsis. While we show this mutation in AtssSPTb confers production of C20 LCB, other residues may shift activity as well. Interestingly though the substrate specificity shift is not the only regulatory difference noted with the human ssSPTs, as the overall SPT activity is altered depending on the heterotrimer expressed (Han et al., 2009). With LCB1, LCB2a, and ssSPTa providing the most SPT activity and LCB1, LCB2b, and ssSPTb providing the least SPT activity (Han et al., 2009). It is unclear if this effect is an artifact or if it actually occurs in vivo. It is possible that
different SPT complex combinations lead to different SPT activities and while both AtssSPTa and AtssSPTb activate SPT, they may activate it to different degrees, which would add another layer of complexity to SPT regulation.

In chapter three we identify another pair of proteins that physically interact with AtSPT and act as inhibitors. Similarly to the AtssSPTs, modulation of AtORM expression has a consistent impact on SPT activity. This would suggest that the AtORMs, like the AtssSPTs, are limiting and that transcriptional regulation may be a viable strategy to alter SPT activity. Public microarrays do show some transcriptional change of the AtORMs under different conditions, although further studies need to be done. Modulation of AtORM expression leads to predictable changes in FB1 sensitivity and LCB accumulation that are consistent with the AtORMs acting as SPT inhibitors. Interestingly, with sufficient overexpression of AtORMs a dwarf phenotype arises which is consistent with reduction in SPT activity. It is most likely the case that a threshold of SPT activity must be met to maintain normal cell growth, and with sufficient attenuation of SPT activity you reduce growth and eventually cell viability (Chen et al., 2006; Chen et al., 2008).

In chapter three we also provide evidence for a potential connection between SPT activity and downstream sphingolipid metabolism. The hypothesis is that SPT is intricately regulated in coordination with downstream enzymes such as ceramide synthase. This is thought occur because LCB and ceramide metabolites can be detrimental to cell growth, triggering PCD, and seem to be tied with immune responses (Greenberg et al., 2000; Coursol et al., 2003; Liang et al., 2003; Nagano et al., 2014).
Studies in yeast point towards a possible coordination between SPT activity and ceramide synthase activity, pairing activity to lessen LCB accumulation (Muir et al., 2014). We find that modulation of AtORM expression leads to changes in ceramide synthase activity, although it is unclear as to why. The situation is more complex in Arabidopsis as there are two distinct classes of ceramide synthase that are responsible for producing different ceramides. The change in ceramide synthase activity found with modulation of AtORM expression is indicative of a connection and points towards a more complex regulatory scheme involving the AtORMs. It is also unclear if the AtORMs impact and physically interact with other proteins. Some preliminary evidence suggests that the ORM proteins can move from different subcellular compartments, and may interact with other enzymes (Wang et al., 2015). The AtORMs may directly impact ceramide synthase activity though physical interaction or indirectly impact their activities through other proteins. Further study is needed to fully characterize the regulatory mechanisms of the AtORMs.

One main question that remains unanswered is how the complete SPT complex is physically organized and functions in a biophysical context. While we show that AtssSPTs and AtORMs physically interact with the core heterodimer, the exact binding and organization of the proteins is unclear. AtssSPTs and AtORMs may physically impact each other and could potentially physically interact. Other proteins most likely interact with the complex and may alter the function or binding of the SPT heterodimer and/or the AtssSPTs and AtORMs. Post-translational modifications, which are known to occur and serve a purpose in yeast, may also play a role in plants. This has yet to be
determined though and it is possible that other proteins, besides the AtORMs, may be phosphorylated. It is also possible that one or more of the ceramide synthases in Arabidopsis are phosphorylated. The exact mechanism for coordinate regulation of SPT and ceramide synthase is also currently unknown. It’s possible that a downstream sphingolipid metabolite acts to control upstream production either directly, or indirectly through another set of proteins. Identifying other proteins binding partners of the SPT complex and establishing the phosphorylation status of the SPT subunits is the next logical step to unraveling the entangled web that is sphingolipid regulation.

In the fourth chapter we describe a potential tool for measuring SPT activity in planta. The LCB1C133W mutation found in humans is conserved in Arabidopsis as plants overexpressing AtLCB1C144W produce the aberrant LCB, DoxSA. It is currently unclear if the complete replacement of native AtLCB1 with AtLCB1C144W will result in viable plants. If it does we will have a system to easily measure SPT activity and will be able to test the impacts of various stressors or genetic mutations on SPT activity in planta.
5.2 Works Cited


APPENDIX A – Supplemental Material from Chapter Two

Supplemental material, including figures and tables from chapter two can be accessed from the journal Plant Cell.

A

\[
\text{CH}_3(\text{CH}_2)_14\text{C} \cdot \text{S} \cdot \text{CoA} \quad \text{Serine} \quad \text{Palmitoyl-CoA} \quad \text{Palmitoyltransferase} \quad \text{LBC1/ LBC2} \quad \text{CO}_2 + \text{CoA-SH} \quad \text{3-Ketosphinganine} \quad \text{3-Ketosphinganine Reductase} \quad \text{OH} \quad \text{CH}_3(\text{CH}_2)_14\text{CH} \cdot \text{CH}_2\text{OH} \quad \text{Dihydrosphingosine}
\]

B

- Sphinganine (d18:0)
- Phytosphingosine (t18:0)
- C20-sphinganine (d20:0)
- C20-phytosphingosine (120:0)

C

\[
\text{LCB Kinase} \quad \text{LCB Phosphatase}
\]
Supplemental Figure 1. Long-chain base synthesis, structures, and phosphorylation/dephosphorylation reactions. (A) Synthesis of long-chain bases via sequential serine palmitoyltransferase and 3-ketosphinganine reductase activities. (B) Common LCBs in plants include sphinganine (d18:0) and phytosphingosine (t18:0), where the d=dihydroxy and t=trihydroxy. The first number indicates the number of carbons which is typically 18, whereas the second number represents the number double bonds which is typically 0, 1, or 2. The unusual C20 LCBs were found in the OE AtssSPTb M19V mutant. (C) LCBs can be phosphorylated by LCB kinase and dephosphorylated by LCB phosphatases. Phosphorylation is considered the first step towards LCB degradation.
Supplemental Figure 2. Phylogenetic analysis of ssSPTs from diverse eukaryotes. Phylogenetic analysis of ssSPTs was done using the neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. All phylogenetic analysis was done using MEGA5.
Supplemental Figure 3. Comparison of Arabidopsis SPT activity +/- AtssSPTa or b with myristoyl (14:0)-, palmitoyl (16:0)-, and stearoyl (18:0)-CoA. SPT activity was measured in yeast microsomes expressing AtLCB1/LCB2a or AtLCB1/LCB2b with or without AtssSPTa or AtssSPTb, to assess their ability to enhance AtSPT activity. SPT activity was measured using $[^3]$H serine and acyl-CoA of the indicated chain lengths (C14, C16 and C18). Activities in heterodimers (No. 1 and 4) were barely detected. Both AtssSPTs greatly increase AtSPT activity, most notably with palmitoyl CoA (C16) as substrate. The different isozymes show slightly different SPT activity enhancement on different SPT heterodimers, e.g., the LCB1-2a heterodimer (No. 2 and 3) is more readily activated than the LCB1-2b heterodimer (No. 5 and 6).
Supplemental Figure 4. RT-PCR showing complete loss of AtssSPTb transcript in sssptb-1 -/- and sssptb-2 -/-.

(A) Col-0 cDNA amplified with AtssSPTb primers yielded a PCR product of ~85bp. (B) Col-0 cDNA amplified with control gene (PP2AA3) primers yielded a PCR product of ~75bp. (C) sssptb-1 cDNA amplified with ssSPTb primers yielded no PCR product. (D) sssptb-1 cDNA amplified with PP2AA3 primers yielded a PCR product of ~75bp. sssptb-2 cDNA amplified with AtssSPTb primers (E) yielded no PCR product, but yielded a PCR product of ~75bp when amplified with PP2AA3 primers (F).
Supplemental Figure 5. Pollen isolated from *sssptb-1* and *sssptb-2* homozygous mutants displayed high levels of viability. No defective pollen was found in these mutants and no growth phenotype was observed.
Supplemental Figure 6. PCR-based genotyping of the *ssspta-1* plants identified only heterozygous mutants. (A) Col-0 genomic DNA amplified with 104888Lp/Rp gene specific primers yielded a ~1.1 kbp band representing the wild-type copy of *AtssSPTa*. (B) Col-0 genomic DNA amplified with 104888Rp/LBb1.3 T-DNA specific primers yielded no PCR product demonstrating that Col-0 lacks the T-DNA insertion. (C) SALK_104888 genomic DNA amplified with 104888Lp/Rp gene specific primers yielded a PCR product of the predicted size for the wild-type allele. (D) SALK_104888 genomic DNA amplified with 104888Rp/LBb1.3 yielded a PCR product of ~600 bp demonstrating that the T-DNA insertion is present in one allele of the *AtssSPTa* gene. Several generations of PCR-based genotyping revealed a roughly 50:50 wild type: heterozygous mutant distribution. No homozygous *ssspta-1* mutants were identified in the original SALK_104888 mutant population.
Supplemental Figure 7. Siliques of heterozygous sspta-1 mutants show normal ovule development. Siliques from 3-week-old Col-0 plants (A) and sspta-1 mutants (B) were isolated, dissected, and checked for aborted ovules. Three siliques from a total of 59 Col-0 plants were checked for aborted ovules and three siliques from a total of 53 heterozygous sspta-1 mutants were checked for aborted ovules. No significant difference between Col-0 and sspta-1 mutants were observed.
Supplemental Figure 8. Homozygous ssspta-1 mutants are recoverable by complementation with a wild-type copy of *AtssSPTa*. (A) Col-0 genomic DNA amplified with genomic *AtssSPTa* F/R primers yielded a PCR product of ~3kbp indicating a wild-type genomic copy of *AtssSPTa*. (B) Transgenic plants expressing genomic *AtssSPTa* in the *ssspta-1/-* background amplified with genomic *AtssSPTa*F/R primers yielded no PCR product, indicating a homozygous *ssspta-1* T-DNA insertion mutant. (C) Col-0 genomic DNA amplified with the control gene *AtADS1*F/R primers yielded a PCR product of ~3kbp. (D) Transgenic plants expressing genomic *AtssSPTa* in the *ssspta-1/-* background amplified with the control gene *AtADS1*F/R primers also yielded at PCR product of ~3kbp.
Supplemental Figure 9. RT-PCR showing complete loss of AtssSPTa transcript in ssspta-1 /- complemented with an AtssSPTb cDNA linked to the AtssSPTa promoter. (A) Col-0 cDNA amplified with AtssSPTa specific primers yielded a PCR product of ~75bp. (B) Col-0 cDNA amplified with AtssSPTb primers yielded a PCR product of ~85bp. (C) Col-0 cDNA amplified with the control gene primers (PP2AA3) yielded a PCR product of ~75 bp. cDNA from a homozygous AtssSPTa-1 mutant expressing the pAtssSPTa::cAtssSPTb complementation construct amplified with AtssSPTa (D), AtssSPTb (E), and PP2AA3 primers (F). No PCR product was detected for AtssSPTa (D).
Supplemental Figure 10. AtssSPTa expression levels in over-expression and RNAi lines. qPCR analysis of overexpressing AtssSPTa mutants shows increased AtssSPTa transcript. Silencing AtssSPTa by RNAi leads to a lower amount of AtssSPTa transcript.
Supplemental Figure 11. Sphingolipidomic analysis of ceramides from Col-0 and the AtssSPTb M19V mutant.
Supplemental Figure 12. ssSPTb M19V OE mutant has increased LCB and LCB(P) content when grown on FB1 and displays an fbs phenotype. (A) Increased expression of ssSPTb M19V impacts accumulation of cytotoxic free long-chain base and long-chain base phosphate [LCB(P)] levels in response to FB1 treatment. Wild-type plants (wt) show increased LCB and LCB(P) levels when treated with FB1. Compared to WT, ssSPTb M19V over-expression (OE) plants display strong sensitivity to FB1 and show elevated LCB and LCB(P) levels. Plants were grown on LS plates +/- FB1. (B) Seeds were sown on LS-agar plates supplemented with FB1 at 0.3 μM and 0.5 μM as indicated, which distinguish between the FB1-resistant (fbr) and FB1-sensitive (fbs) phenotypes, respectively (Stone et al., 2000). Wild-type (Col-0) is extremely sensitive to FB1 at 0.5 μM FB1, and less affected at 0.3 μM FB1. Overexpression of ssSPTb M19V causes an fbs phenotype.
Supplemental Figure 13: Amino acid sequence alignment of selected ssSPTs.
### Supplemental Table 1: Oligonucleotides used in these studies.

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Supplemental Table 2: MRM Q1/Q3 transitions (m/z) for t20:0 LCB-containing sphingolipid species used to monitor t20:0 sphingolipids by LC-ESI-MS/MS.

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Figure S1. Gene expression levels for ORM1 (At1g01230) and ORM2 (At5g42000) in certain tissue types (Schmid et al., 2005). ORM1 is more highly expressed in tissues outside floral organs and seems predominately expressed in developing seed. ORM2 appears more highly expressed in floral tissues.
Figure S2. Genotyping of T-DNA knockout mutants. The gene structures for three T-DNA mutant lines are displayed in (A). Introns are shown as white boxes, exons are shown as black boxes, and the UTR regions are shown as black lines. Homozygous T-DNA mutants can be identified for SALK_046054, GK-143A01, and SAIL_1286_D09 lines (B). T-DNA insertion mutant alleles were confirmed through PCR based genotyping. Genotyping was done using two primer pairs, one pair with two gene specific primers and another pair with one T-DNA specific primer and one gene specific primer, these primers can be found in Supplementary Table 1. These homozygous mutant lines still contain full length transcript as confirmed through RT-PCR using full length cDNA primer pairs found in Supplementary Table 1 (C).
Figure S3. SPT activity from root microsomes. ORM RNAi suppression lines show increased SPT activity when compared to wild type. ORM overexpression lines do not show significantly reduced SPT activity when compared to wild type.
Figure S4. Sphingolipid profile of ORM overexpressing lines that display the dwarf phenotype. Ceramide profiles are shown in (A), glucosylceramide profiles are shown in (B), and GIPC profiles are shown in (C).
Figure S5. Ceramide profile for Col-0 and ORM mutant lines grown on LS ±FB₁.
Figure S6. Glucosylceramide (GlcCer) profile for Col-0 and ORM mutant lines grown on LS ±FB₁.
Figure S7. Glycosylinositolphosphorylceramide (GIPC) profile for Col-0 and ORM mutant lines grown on LS ±FB$_1$. 
Figure S8. qPCR analysis of ORM RNAi lines. RNA was extracted from leaf tissue of 3 week old plants. As shown, both ORM1 and ORM2 transcripts were suppressed.
Figure S9. FB$_1$ sensitivity of an ORM overexpressing line that displays the dwarf phenotype. While the plants are dwarfed when compared to Col-0, they display the same FB$_1$ resistance as other lines.
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<tr>
<td>P10</td>
<td>ORM2cDNAF: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P11</td>
<td>ORM2cDNAR: TCATGGTCTCCATTGATCC</td>
</tr>
<tr>
<td>P12</td>
<td>ORM1promoterF: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P13</td>
<td>ORM1promoterR: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P14</td>
<td>ORM2promoterF: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P15</td>
<td>ORM2promoterR: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P16</td>
<td>ORM1yfpF: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P17</td>
<td>ORM1yfpR: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P18</td>
<td>ORM2yfpF: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P19</td>
<td>ORM2yfpR: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P20</td>
<td>SALK_046054L: TAGTCCGTTACACATCACCCCGG</td>
</tr>
<tr>
<td>P21</td>
<td>SALK_046054R: GTGGCTCGGTGTCCTCGTTG</td>
</tr>
<tr>
<td>P22</td>
<td>G_X_143A01L: TGCTATTCAAGATCCCATGG</td>
</tr>
<tr>
<td>P23</td>
<td>G_X_143A01R: TCATGGTCTCCATTGATCC</td>
</tr>
<tr>
<td>P24</td>
<td>SAIL_1286_D09L: TCATGTTGACATGCAAGACCTCC</td>
</tr>
<tr>
<td>P25</td>
<td>SAIL_1286_D09R: GGATTGAGAAGAATGCGGAG</td>
</tr>
<tr>
<td>P26</td>
<td>Lbb1.3: ATTTCCGAAATCCCTCCCAATC</td>
</tr>
<tr>
<td>P27</td>
<td>SAIL F-DNA: GCCCTTTAATGACCAGACCATC</td>
</tr>
<tr>
<td>P28</td>
<td>GAB F-DNA: CCTCTAGTTGAGAATGCGG</td>
</tr>
<tr>
<td>P29</td>
<td>ORM1OE F: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P30</td>
<td>ORM1OE R: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P31</td>
<td>ORM2OE F: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P32</td>
<td>ORM2OE R: ATGCCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P33</td>
<td>ORM1RNAiF: TAATCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P34</td>
<td>ORM1RNAiR: TATAGATGCTACGCGGTAAGCAGCTGATGCGTA</td>
</tr>
<tr>
<td>P35</td>
<td>ORM2RNAiF: TAATCTCGAGTATGCAAGACCTCCCTCC</td>
</tr>
<tr>
<td>P36</td>
<td>ORM2RNAiR: TATAGATGCTACGCGGTAAGCAGCTGATGCGTA</td>
</tr>
<tr>
<td>P37</td>
<td>plINTF: GAATTCAATCAGACTCATTGAGACCCAGAAGAGAGAGACCTCC</td>
</tr>
<tr>
<td>P38</td>
<td>plINTR: TCTAGAAGGCGGCGAATTACCAGACCTCCAC</td>
</tr>
</tbody>
</table>

**Supplemental Table 1: Oligonucleotides used in these studies.**