Investigation of Pathways for Complex Sphingolipid Biosynthesis in *Arabidopsis thaliana* (L.) Heynh

By

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ARABIDOPSIS THALIANA CONTAINS THREE CERAMIDE SYNTHASE ISOFORMS EACH WITH DISTINCT SUBSTRATE SPECIFICITY THAT MEDIATE SPHINGOLIPID COMPOSITION, PLANT GROWTH, AND MYCOTOXIN RESISTANCE

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Sphingolipids are essential components of eukaryote membranes. The ceramide backbone of complex sphingolipids is composed of an 18 carbon Long Chain Base (LCB) bound to a 16-26 carbon fatty acid (FA) through an amide linkage. Ceramides are synthesized *de novo* from a free LCB and fatty acyl coA by ceramide synthase (sphingosine N-acyl transferase, EC 2.3.1.24) which can be inhibited by the fungal mycotoxin Fumonisin B_1. *Arabidopsis thaliana* contains three ceramide synthases denoted *LOH1*, *LOH2*, and *LOH3* that have previously been hypothesized to have unique substrate preferences that control the final sphingolipid composition, different susceptibilities to Fumonisin, and different influences plant growth/development. This dissertation works to answers to these questions as well as identify novel complex sphingolipid biosynthetic pathways. Through the use of *in vitro* assays it was found that LOH1 and LOH3 prefer LCBs witth hydroxyls at the C1, C2, and C4 positions (trihydroxy) and C20-24 saturated FA while LOH2 prefers LCBs with hydroxyls at the C1 and C2 positions (dihydroxy) and C16 saturated fatty acids. None of the isoforms

were able to use ω9 desaturated acyl CoAs which are abundant in the final sphingolipid profile. Surprisingly LOH2 showed the highest level of activity with C4 unsaturated LCBs which are not commonly found in leaf. Each isoform was also overexpressed *in planta* to determine the effects ceramide composition has on plant growth. Overexpression of *LOH1* or *LOH3* led to an increase in biomass while overexpression of *LOH2* resulted in a dwarf phenotype. Both the *in vitro* assays and *in planta* overexpression found LOH1 to the most susceptible to FB_1 inhibition. In addition to ceramide synthesis a novel Δ8 LCB desaturase from castor bean was identified which required the presence of a Δ4 double bond for activity. The presence of Δ4,8 unsaturated LCBs was found to result in increased glucoscylceramide levels as revealed by LCB feeding experiments and pollen sphingolipid profiling. Therefore, it is hypothesized that the presence of a Δ4 unsaturation targets LCBs through a LOH2-like ceramide synthase for subsequent Δ8 desaturation and glucosylceramide synthesis.

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CHAPTER 1

INTRODUCTION

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1.1 INTRODUCTION

Sphingolipids were originally identified in the late 19th century by Johann Thudichum as an "enigmatic" major lipidic component of the brain (Thudichum 1884). Since this discovery, 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 stresses. Since the late 1990s, plant sphingolipids have become an increasing research focus. Driving this heightened interest is the realization that sphingolipids are among the most abundant endomembrane lipids in plant cells and that they contribute not only to membrane structure and function that underlies abiotic and biotic stress resistance, but also to the regulation of cellular processes (Dunn et al. 2004). Recent advances in plant sphingolipid research have been spurred by development and application of advanced mass spectrometry methods that enable the rapid and quantitative measurement of molecular species of specific sphingolipid classes (Markham and Jaworski 2007). Coupling of these methods with the characterization of Arabidopsis mutants and transgenics have resulted in advances in our fundamental understanding of plant sphingolipid metabolism.

The backbone of complex sphingolipids, the ceramide, is composed of a long chain base bound to a fatty acid through an amide linkage (Dunn et al. 2004). Ceramide

synthesis has been recognized as a key branching point in sphingolipid metabolism with the ceramide long chain base/fatty acid composition hypothesized to play a key role in determining the final complex sphingolipid formed (Markham et al. 2011; Chen et al. 2008). In mammals it has been found that different ceramide synthases have distinct substrate preferences allowing the organism to control ceramide composition (Venkataraman et al. 2002a; Laviad et al. 2008; Mizutani et al. 2006, 2005; Riebeling et al. 2003). Through the use of mutants, evidence suggests that plant ceramide synthases also have distinct substrate preferences (Markham et al. 2011; Ternes et al. 2011; Chen et al. 2008), though this has yet to be determined through the use of *in vitro* assays. This dissertation describes the characterization of the Arabidopsis ceramide synthases and the effects that ceramide composition has on plant growth/development, fungal mycotoxin resistance, and complex sphingolipid synthesis.

1.2 SPHINGOLIPID STRUCTURE

Sphingolipids consist of hydrophobic ceramide backbones that are typically linked to polar sugar residues to form amphipathic lipid components of membranes (Lynch and Dunn 2004; Chen et al. 2010). 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. In plants, LCBs typically have chain lengths of 18 carbon atoms and can contain double bonds in the Δ4 or Δ8 positions (Figure 1.1A). 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. Following its initial synthesis, a LCB has two hydroxyl groups at the C-1 and C-3 carbons (Lynch and Dunn 2004; Chen et al. 2010). These LCBs are referred to as dihydroxy LCBs. A third

hydroxyl group can be enzymatically added at the C-4 carbon to form a trihydroxy LCB. In the short-hand nomenclature, a dihydroxy LCB with 18 carbons and one double bond is referred to as "d18:1", and a trihydroxy LCB with 18 carbons and one double bond is referred to as "t18:1". LCBs can be phosphorylated at the C-1 position to form LCB-phosphates (LCB-P). Free LCBs and their phosphorylated forms are typically in low abundance in plant cells (Markham and Jaworski 2007; Markham et al. 2006). Instead, the majority of LCBs are found linked to fatty acids in ceramides (Figure 1.1B). The chain-lengths of plant ceramide fatty acids range from 16 to 26 carbon atoms, the majority of which contain an enzymatically added hydroxyl group at the C-2 or α-position (Lynch and Dunn 2004; Chen et al. 2010). vAnalogous to the diacylglycerol backbone of glycerolipids, ceramides serve as the hydrophobic component of complex sphingolipids. 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 the glucosylceramide (GlcCer) with a single glucose residue and comprises approximately one-third of the glycosphingolipids of Arabidopsis leaves (Markham and Jaworski 2007; Markham et al. 2006) (Figure 1.1C). The most abundant glycosphingolipid in plants contains an inositol phosphate bound to the ceramide with up to seven additional hexose and pentose residues (Figure 1.1C) (Cacas et al. 2013). These molecules are referred to as glycosyl inositolphosphoceramides or GIPCs and comprise approximately two-thirds of the glycosphingolipids of Arabidopsis leaves (Markham and Jaworski 2007; Markham et al. 2006). 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 elucidated (Markham et al. 2013; Bure et al. 2011).