Original Resear	Volume - 11 Issue - 08 August - 2021 PRINT ISSN No. 2249 - 555X DOI : 10.36106/ijar
and Of Replice	Biochemistry A STUDY OF ORM EXPRESSION CHANGES FUMONISIN B1-INDUCED PERTURBATIONS OF SPHINGOLIPID HOMEOSTASIS AND DIFFERENTIALLY TOUCHES CERAMIDE SYNTHASE ACTIVITIES
Dr. Anand Shanker Singh	Associate Professor, Department of Chemistry, Chinmaya Degree College, BHEL, Haridwar

Singh	
Dr. G. Radhika	MBBS, MD (Biochemistry), HOD , Department of Biochemistry, Sri .Venkateshwara Medical College and Hospital , Pondicherry.
Dr. Ankita Singh*	DNB, DGO, MBBS. *Corresponding Author
Dr. Debarshi Jana	Young Scientist, IPGMER and SSKM Hospital, Kolkata, WB.
APSTDACT Sphingolinid synthesis is tightly regulated in subgryates. This regulation in plants ensures sufficient sphingolinids to	

ABSTRACI Sphingolipid synthesis is tightly regulated in eukaryotes. This regulation in plants ensures sufficient sp support growth, while limiting accumulation of sphingolipid metabolites that induce programmed cell death (PCD). Serine palmitoyltransfersase (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 longchain 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.

KEYWORDS : Serine palmitoyltransfersase, Sphingolipid synthesis, Ceramide Synthase Activities.

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.¹ 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.² 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.³

Furthermore, sphingolipids function through their bioactive longchain base (LCB) and ceramide metabolites to initiate programmedcell death (PCD), important for mediating plant pathogen resistance through the hypersensitive response (HR).

Sphingolipid biosynthesis is highly regulated in all eukaryotes. In plants, maintenance of sphingolipid homeostasis is vital to ensure sufficient sphingolipids for growth while restricting the accumulation of PCD-inducing ceramides and long-chain bases (LCBs), until required for processes such as pathogen-triggered HR.⁴ Serine palmitoyltransferase (SPT), which catalyzes the first step in LCB synthesis, is generally believed to be the primary control point for sphingolipid homeostasis. SPT synthesizes LCBs, unique components of sphingolipids, by catalyzing a pyridoxal phosphate-dependent condensation of serine and palmitoyl (16:0)-CoA in plants. Similar to other eukaryotes, the Arabidopsis SPT is a heterodimer consisting of LCB1 and LCB2 subunits. 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. 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.

Evidence from yeast and mammalian research points to a more critical role for proteins termed ORMs in sphingolipid homeostatic regulation.⁵ The Saccharomyces cerevisiae Orm1p and Orm2p negatively regulate SPT through reversible phosphorylation of these

polypeptides in response to intracellular sphingolipid levels.5 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. In this sphingolipid homeostatic regulatory mechanism, the S. cerevisiae ORM1p and ORM2p are phosphorylated at their N-termini by Ypk1, a TORC2dependent protein kinase.5 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.6

RNAi suppression of ORM genes in rice has been shown to affect pollen viability, 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.7 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.

METHODS

Yeast Growth and Expression Plasmids. Yeast (Saccharomyces cerevisiae) strain TDY9113 (Mat a tsc3 Δ :NAT lcb1 Δ :KAN ura3 leu2 lys2 trp1 Δ) 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 15mM 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 pADH1.⁴ 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 re-ligation. The deletion was confirmed by sequencing.

Immunoprecipitation. Immunoprecipitation was conducted as described with minor modifications. Microsomal membrane proteins were prepared from yeast cells expressing FLAG-tagged At LCB1 and Myc-tagged AtLCB2a with HA-tagged AtsSPTa and HA-tagged AtORM1 or AtORM2.⁴ Microsomal membrane proteins were solubilized in 1.5% digitonin at 4°C for 2.5 h and incubated with Flagbeads (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-FLAG (GenScript; 1:5000 dilution).

SPT Assay. Plant microsomes were prepared and SPT activity was assayed as described except that 50 μ M palmitoyl-CoA and 20 μ M BSA were used for the Arabidopsis microsomal SPT assays. SPT activity was measured using [3H] Ser and palmitoyl-CoA.⁴

Yeast Complementation. Synthetic complete (SC) media was used to grow yeast (BY4741). SC media was supplemented with 15 mM phytosphingosine (PHS) and 0.1% tergitol. Yeast knockout mutants, Dorml and Dorm2, 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. 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.

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 μ mol/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.⁸

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). A green LED light and a Red 2 camera filter were used to screen seeds and identify transformed seed that contained DsRed.

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 μ g) 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 \pm SD. SYBR green was used as the fluorophore in a qPCR supermix (Qiagen). QuantiTect (Qiagen) primer sets P5-P7 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.

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, 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. 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 1ml/ml Triton X-100 and 1mg/ml 5-bromo-4-chloro-3-indolyl-g-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

INDIAN JOURNAL OF APPLIED RESEARCH

tissue were taken with an Olympus AX70 optical microscope.

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. 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.

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.

Sensitivity was determined by plant growth rate and germination at the varying FB1 concentrations. Plants were grown for two weeks on FB1 before analysis.

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.⁹

Ceramide Synthase Assays. Ceramide synthase assays were performed on microsomal protein derived from hydroponically grown root. The reactions were performed as described previously using $10 \Box g$ of microsomal protein. 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.

Sphingolipid Analysis. Sphingolipids were extracted from 2-15 mg of lyophilized seedling tissue as described previously.⁴

RESULTS AND 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. 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 Nterminal 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. 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. 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.10 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 ORMs 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.7 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. 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.^{4,11} 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. 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. 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. 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 beside 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. 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. 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.¹ Longer-chain ceramides produced by the class II enzymes are essential and appear to be vital to cell viability. 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.

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. 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.

CONCLUSION

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.

REFERENCES

- Sperling P, Franke S, Luthje S, Heinz E (2005). Are glucocerebrosides the predominant sphingolipids in plant plasma membranes? Plant Physiol Biochem 43: 1031-1038.
- Spining on provide and passing memorances is rainer prystor biochemists. 1031-1038. Cacas JL, Bure C, Grosjean K, Gerbeau-Pissot P, Lherminier J, Rombouts Y, Maes E, Bossard C, Gronnier J, Furt F, Fouillen L, Germain V, Bayer E, Cluzet S, Robert F, Schmitter JM, Deleu M, Lins L, Simon-Plas F, Mongrand S (2015). Re-visiting plant plasma membrane lipids in tobacco: a focus on sphingolipids. Plant Physiol. Bach L, Gissot L, Marion J, Tellier F, Moreau P, Satiat-Jeunemaitre B, Palauqui JC,
- 3 Nahier JA, Faue JD (2011). Very-long-chain fatty acids are required for cell plate formation during cytokinesis in Arabidopsis thaliana. J Cell Sci 124: 3223-3234. 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. Plant Cell 25: 4627-4639
- Han S, Lone MA, Schneiter R, Chang A (2010). Orm1 and Orm2 are conserved 5 endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. Proc Natl Acad Sci U S A 107: 5851-5856.
- Hjelmqvist L, Tuson M, Marfany G, Herrero E, Balcells S, Gonzalez-Duarte R (2002). ORMDL proteins are a conserved new family of endoplasmic reticulum membrane proteins. Genome Biology 3: RESEARCH0027. Chueasiri C, Chunthong K, Pitnjam K, Chakhonkaen S, Sangarwut N, Sangsawang K, Suksangpanomrung M, Michaelson LV, Napier JA, Muangprom A (2014). Rice
- 7. ORMDL controls sphingolipid homeostasis affecting fertility resulting from abnormal
- Ortim De Control spinningen più noncosassi ancering returny resulting non aotorna pollen development. PIOS one 9: e106386.
 Conn SJ, Hocking B, Dayod M, Xu B, Athman A, Henderson S, Aukett L, Conn V, Shearer MK, Fuentes S, Tyerman SD, Gilliham M (2013). Protocol: optimising hydroponic growth systems for nutritional and physiological analysis of Arabidopsis thaliana and other plants. Plant Methods 9:4 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD,
- 9. Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985). Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76-85.
- 10 Roelants FM, Breslow DK, Muir A, Weissman JS, Thorner J (2011). Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 108: 19222-19227.
- Chen M, Han G, Dietrich CR, Dunn TM, Cahoon EB (2006). The essential nature of sphingolipids in plants as revealed by the functional identification and characterization 11 of the Arabidopsis LCB1 subunit of serine palmitoyltransferase. The Plant cell 18: 3576-3593
- Luttgeharm KD, Cahoon EB, Markham JE (2015). A mass spectrometry-based method for the assay of ceramide synthase substrate specificity. Anal Biochem 478: 96-101

55