Disruption of long-chain base hydroxylation alters growth and impacts sphingolipid synthesis in *Physcomitrella patens*

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**Abstract**
Sphingolipids have roles as membrane structural components and as bioactive molecules in plants. In *Physcomitrella patens*, 4-hydroxysphinganine (phytosphingosine, t18:0) is the predominant sphingolipid long-chain base (LCB). To assess the functional significance of t18:0, CRISPR-Cas9 mutagenesis was used to generate mutant lines lacking the sole SPHINGOID BASE HYDROXYLASE (*SBH*) gene encoding the hydroxylase responsible for converting sphinganine (d18:0) to t18:0. Total sphingolipid content in *sbh* protonemata was 2.4-fold higher than in wild-type. Modest changes in glycosyl inositolphosphorylceramide (GIPC) glycosylation patterns occurred. Sphingolipidomic analyses of mutants lacking t18:0 indicated modest alterations in acyl-chain pairing with d18:0 in GIPCs and ceramides, but dramatic alterations in acyl-chain pairing in glucosylceramides, in which 4,8-sphingadienine (d18:2) was the principal LCB. A striking accumulation of free and phosphorylated LCBs accompanied loss of the hydroxylase. The *sbh* lines exhibited altered morphology, including smaller chloronemal cell size, irregular cell shape, reduced gametophore size, and increased pigmentation. In the presence of the synthetic trihydroxy LCB t17:0, the endogenous sphingolipid content of *sbh* lines decreased to wild-type levels, and the mutants exhibited phenotypes more similar to wild-type plants. These results demonstrate the importance of sphingolipid content and composition to *Physcomitrella* growth. They also illuminate similarities in regulating sphingolipid content but differences in regulating sphingolipid species composition between the bryophyte *P. patens* and angiosperm *A. thaliana.*

**KEYWORDS**
C-4 hydroxylase, CRISPR-Cas9, glycosyl inositolphosphorylceramide, long-chain base, Physcomitrella, sphingolipid

**Abbreviations:** Cer, ceramide; CRISPR-Cas9, clustered regularly interspersed short palindromic repeats-crisper associated protein 9 complex; d18:0, sphinganine; d18:1, sphingenine; d18:2, 4-8-sphingadienine; d20:0, eicosasphinganine; FA, fatty acid; FAH, fatty acid hydroxylase; GIPC, glycosyl inositolphosphorylceramide; GlcCer, glucosylceramide; hCer, hydroxyceramide; hFA, α-hydroxy fatty acid; LCB, long-chain base; LCBP, phosphorylated long-chain base; LOH, lag one homolog; nhGlcCer, nonhydroxyglucosylceramide; ORM, orosomucoid; PCD, programmed cell death; SBD, sphingolipid base; C-4 hydroxylase; SLD, sphingoid LCB Δ8 desaturase; SPT, serine palmitoyltransferase; t18:0, 4-hydroxysphinganine (phytosphingosine); t18:1, 4-hydroxysphingine; TC, transformation control; WT, wild-type.

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Sphingolipids are a widespread, structurally diverse, and functionally important group of molecules involved in many critical plant processes. They contribute to the integrity of major plant cellular membranes, especially the plasma membrane, in which they account for as much as 40% of constituent lipids (Lynch & Dunn, 2004; Markham et al., 2013; Sperling et al., 2005). Sphingolipids populate the microdomains of plant plasma membranes (Borner et al., 2005; Cacas et al., 2016; Lefebvre et al., 2007) and can interact with plant cell wall components (Voxeur & Fry, 2014). In addition to serving as membrane structural components, sphingolipids work within the signaling pathways involved in the plant response to biotic stressors, including the jasmonate, salicylic acid, and ethylene signaling pathways, as well as programmed cell death (PCD) (Huby et al., 2020). Abiotic stress response pathways also involve sphingolipids, most notably abscisic acid-mediated stomatal closure but also those initiated by salt or oxidative stress (Coursol et al., 2003, 2005; Huby et al., 2020; Ng et al., 2001). This wide functional range found for plant sphingolipids mirrors their structural heterogeneity and invites an understanding of sphingolipid function based upon structure.

Sphingolipids are composed of a sphingoid long-chain base (LCB), a normal chain or α-hydroxy fatty acid (FA or hFA, respectively), and a polar headgroup. Considering only ceramides (Cers), that is, an LCB having an amide-linked FA or hFA, there are >288 different molecular species theoretically possible in plants (Markham et al., 2013). Including complex sphingolipids, those having a Cer with a polar headgroup, more than 200 different molecular species have been reported in the leaves of Arabidopsis (Markham et al., 2006) and 300 in the seeds (Tellier et al., 2014).

One driver of this sphingolipid diversity is the existence of three potential mechanisms for the modification of the LCB: C-4 hydroxylation, Δ⁴ desaturation, and Δ⁸ desaturation. In many plant tissues, trihydroxy LCBs are most abundant, a result of C-4 hydroxylation. The formation of 4-hydroxysphinganine (t18:0) from sphinganine (d18:0) is catalyzed by a C-4 hydroxylase (Figure 1) active in the endoplasmic reticulum (Chen et al., 2008; Wright et al., 2003). In Arabidopsis, where trihydroxy LCBs account for approximately 90% of total LCB content in leaves, there are two sphingoid base hydroxylase genes, SBH1 and SBH2. Experiments linking sphingolipid structure with function and metabolism focused on this common modification in plants: they demonstrated that in sbh1sbh2 double mutants the elimination of trihydroxy LCBs leads to a dwarfed phenotype that never progresses to the reproductive growth stage and exhibits necrotic legions on its leaves (Chen et al., 2008). Accompanying these changes in phenotype, Chen et al. (2008) observed a 2.5-fold increase in total sphingolipids and a >60-fold increase in free and phosphorylated LCB (LCBP) content. Based on this dysregulation of sphingolipid content, a model was proposed for regulating plant sphingolipid synthesis in which trihydroxy LCBs, whether free or components of complex sphingolipids, modulate the synthesis of d18:0 (Chen et al., 2008). In addition to this alteration in sphingolipid content, detailed sphingolipid profiling provided insight into the use of trihydroxy- and dihydroxy LCBs by different ceramide synthase enzymes exhibiting different fatty acyl-CoA preferences. It was proposed that two separate classes of ceramide synthase are active in Arabidopsis and have distinct preferences for substrates (Chen et al., 2008). Subsequent characterization of three ceramide synthases (LOHs) present in Arabidopsis have confirmed that LOH2 exhibits a preference for C16 fatty acyl-CoA and dihydroxy LCBs as substrates, whereas LOH1 and LOH3 exhibit preferences for very long-chain fatty acyl-CoAs and trihydroxy LCBs as substrates (Luttgeharm et al., 2016; Markham et al., 2011; Ternes et al., 2011).

To examine these aspects of sphingolipid function and metabolism in a plant with a lineage distant from the angiosperm Arabidopsis, we used CRISPR/Cas9 mutagenesis to knockout the single SBH-encoding gene in the moss Physcomitrella patens. Although published
studies of lipids in Physcomitrella are limited (Gomann et al., 2021; Resemann et al., 2019), the bryophyte was selected for its genetic resources (Rensing et al., 2020) and ease of manipulation. Here, we show that Physcomitrella has very high proportions of glycosyl inositolphosphorylceramide (GIPC) species containing the trihydroxy LCB, t18:0. Physcomitrella sbh mutants lacking t18:0 exhibited changes in growth phenotype and increased total sphingolipid content, and both features were complemented by the exogenously supplied trihydroxy LCB, t17:0, indicating a role for trihydroxy LCB in regulating sphingolipid synthesis as well as influencing plant morphology. Comparison with Arabidopsis reveals differences in sphingolipid species profiles, but similarities in the regulation of sphingolipid synthesis between angiosperms and bryophytes.

2 | MATERIALS AND METHODS

2.1 | Moss culture

The Gransden ecotype of Physcomitrella patens was provided by the Bezanilla lab and grown at 25 °C under a 16 h/8 h light/dark cycle (80 μmol m⁻² s⁻¹) on PpNH₄ medium supplemented with 0.7% agar and overlaid with cellophane disks (https://sites.dartmouth.edu/bezanillalab/moss-methods/). For complementation experiments, wild-type (WT) and mutant lines were grown as above on PpNH₄ medium containing 10 μM t17:0 (Avanti Polar Lipids) added as a 500X methanolic stock when pouring plates.

2.2 | CRISPR-Cas9 mutagenesis: Protospacer design and vector creation

The CRISPR-Cas9 vector system and procedures developed by Mallett et al. (2019) were followed. For protospacer design, the CRISPOR software (crispor.tefor.net) (Haeussler et al., 2016) was used to select protospacer sequences based on the P. patens Phytozome 11 genome. Two constructs were created, each identical except for their protospacer sequence: one matched the 514th to the 533rd bp of the sense strand of the SBH genomic sequence including the 5’ UTR oriented from 5’ to 3’ and the other with a protospacer matching the 1639th to the 1658th bp of the sense strand oriented from 5’ to 3’ (Table S1A). The annealed pairs of oligos (purchased from IDT; Table S1B) were ligated (using a sticky end ligase kit from NEB) into the BsaI-digested entry vector pENTRPpU6P-sgRNA-L1L2 (a gift from Magdalena Bezanilla; Addgene plasmid # 113735; RRID:Addgene_113735) containing Cas-9 and hygromycin resistance (Mallett et al., 2019). The expression vectors used for moss transformation were isolated from transformed E. coli DH5α (NEB) grown on LB-ampicillin and verified by Sanger sequencing (Retrogen).

2.3 | Transformation

The polyethylene glycol-based protocol described by Augustine et al. (2011) was used to transform moss protoplasts generated by Driselase treatment (Bezanilla et al., 2005). After transformation, protoplasts were plated on cellophane disks atop PRMB (PpNH₄ medium containing 8.5% mannitol and 10 mM CaCl₂) to regenerate. After 4 days, the cellophane disks were transferred onto PpNH₄ medium containing 15 μg ml⁻¹ of hygromycin to select for transformants. After 7 days on selective media, the cellophane disks were transferred to PpNH₄ medium to allow for healthy growth (Mallett et al., 2019). Each individual surviving plantlet was later transferred to a separate plate for continued growth and propagation. To note, the media used for protoplast regeneration and selection also contained 5 μM t18:0 to ensure survival of transformants lacking C-4 hydroxylase activity.

2.4 | Mutant screening and genotyping

Small tissue fragments of recovered transformants were screened by HPLC analysis of total LCBs (described below) to identify lines having high proportions of d18:0 (Figure S1). Seven transformants having high proportions of d18:0, four hygromycin-resistant transformants having WT HPLC profiles, and WT moss were genotyped. For genotyping, regions (approx. 700 to 900 bp) surrounding the sequences targeted by the protospacers were amplified using PCR (primers listed in Table S2) and sequenced (Retrogen).

2.5 | Analysis of LCB composition and quantification of total LCB

Tissue (approx. 1 mg lyophilized or <10 mg fresh tissue) was collected from moss cultures after 7 days of propagation for protonema and after 35 days for gametophores. Samples were hydrolyzed in 1.0 ml of 0.5 N HCl/methanol overnight at 65 °C in tightly capped glass tubes to liberate LCBs from complex sphingolipids. For quantitative analysis, 20 μl of 20 μM eicosaposphinganine (d20:0) in methanol (internal standard, equivalent to 400 pmol d20:0) was added to the tube before hydrolysis. To extract the LCBs, 0.8 ml of 1 N KOH and 1.0 ml of chloroform were added to the cooled sample, and, following centrifugation, the lower phase was transferred to an autosampler vial and the sample was evaporated to dryness. For derivatization, the LCBs were dissolved in 100 μl methanol and reacted with 50 μl of o-phthalaldehyde reagent (Wright et al., 2003) for 30 min prior to diluting with 300 μl of methanol. The derivatized
LCBs were separated by HPLC (Agilent 1100 series quaternary pump and fluorescence detector) using a 250 $\times$ 4 mm Luna C18 reversed phase column (Phenomenex) and an isocratic mobile phase typically consisting of 80% methanol/12% acetonitrile/8% phosphate buffer (5 mM potassium phosphate adjusted to pH 7.0) at a flow rate of 1.5 ml/min. Fluorescence was excited at 230 nm and detected at 455 nm. Values for LCB composition and total LCB quantity are expressed as mean $\pm$ SD.

### 2.6 Quantification of free LCB

The amount of free LCB in moss tissue was quantified following extraction and mild alkaline hydrolysis (Wright et al., 2003): approximately 1 mg of lyophilized moss tissue, along with 20 $\mu$l of d20:0 internal standard, was extracted using 1.0 ml of methanol and 0.5 ml of chloroform. Following addition of 0.5 ml of chloroform, 0.8 ml of 0.1 N NH$_4$OH and 50 $\mu$l of 1% NaCl, the sample was centrifuged and the lower phase was transferred to a separate tube. Mild alkaline hydrolysis to degrade glycerolipids was performed by adding 0.8 ml of 0.4 N KOH/methanol and reacting for 60 min at room temperature. To effect phase separation, 0.7 ml of H$_2$O was added. The lower phase was transferred, evaporated, and the LCBs derivatized and analyzed as above.

### 2.7 Sphingolipid profiling and GIPC identification

Sphingolipids were extracted as described (Markham & Jaworski, 2007). Briefly, Physcomitrella tissue was lyophilized and 30 mg of dried tissue was homogenized and extracted with isopropanol/heptane/water (55:20:25 v/v/v). Internal standards for the different sphingolipid classes were added. The supernatants were dried and de-esterified with methylvamine in ethanol/water (70:30 v/v). The lipid extract was re-suspended in tetrahydrofuran/methanol/water (5:2:5 v/v/v) containing 0.1% formic acid. Sphingolipid species were analyzed using a Shimadzu Prominence ultra-performance liquid chromatography system and a 4000 QTRAP mass spectrometer (AB SCIEX). Sphingolipids were separated on a Zorbax Eclipse Plus narrow bore C18 column, 2.1 $\times$ 100 mm, 1.8-$\mu$m particle size (Agilent) at 40 $^\circ$C and a flow rate of 0.2 ml/min. For discovery of the glycosylation patterns of GIPC types, LC/MS/MS precursor scans were performed by scanning for precursors of 666.6 m/z for WT and 650.6 m/z for sbh (the t18:0_h24:0 and d18:0_h24:0 ceramide backbone fragments produced from WT and sbh GIPCs), in conjunction with chromatographic separation using the standard GIPC gradient conditions and instrument settings described previously (Kimberlin et al., 2013; Luttgeharm et al., 2015). The t18:0_h24:0 and d18:0_h24:0 backbones were chosen due to their respective abundances in the hydroxyceramide (hCer) and GIPC profiles of WT and sbh mutants. Data analysis and quantification were performed using the software Analyst 1.5 and Multiquant 2.1 as described (Davis et al., 2020; Kimberlin et al., 2013; Markham & Jaworski, 2007).

### 2.8 Phenotypic characterization and cell size measurements

Colonies started from protoplast preparations were used rather than tissue fragments produced by routine propagation techniques. Week-old tissue was used to generate protoplasts (as above) and 100 $\mu$l of liquid plating medium (PpNH$_4$ containing 8.5% mannitol and 10-mM CaCl$_2$) containing roughly 60,000 protoplasts was added to six wells of a 12-well plate for each of the following lines: WT, TC, sbh-1, sbh-2a, and sbh-3 (Table S3). For each group of six wells, three contained PRMB with 10 $\mu$M t17:0, and three did not. After 4 days each cellophane disk was transferred to a small petri dish with PpNH$_4$ medium, with or without 10 $\mu$M t17:0. One cellophane disk of each line was removed for imaging (and tissue harvesting for LCB analysis) at 8, 14, and 35 days following protoplast formation. Samples were imaged using a Zeiss Stemi dissecting microscope and a Zeiss PrimoStar compound microscope, both equipped with Excels color digital 1080p cameras with displays. Chloronema and caulonema cell dimensions (length and width) were measured from calibrated images using ImageJ. Apical cells were not included in these measurements. The areas of gametophore phylloid cells were determined by tracing their outline using ImageJ. Confidence intervals for means and standard deviations of cell dimensional measurements were calculated by bootstrapping with 1000 resampled parameters.

### 3 RESULTS AND DISCUSSION

#### 3.1 Initial characterization of sphingolipids in Physcomitrella

Initial studies of Physcomitrella sphingolipids used direct hydrolysis of tissue to liberate LCBs for quantification of sphingolipid content and analysis of LCB composition. Quantitative analysis of fluorescent o-phthalaldehyde derivatives of LCBs following direct hydrolysis of protenemata including internal standard (d20:0) yielded a total sphingolipid content of 426 $\pm$ 48.3 pmol mg$^{-1}$ FW. Use of a traditional chloroform/methanol extraction method (based on Bligh & Dyer, 1959) resulted in recovery of 115 $\pm$ 8.66 pmol mg$^{-1}$ FW, or 27% of the total sphingolipid content obtained by direct tissue hydrolysis. This difference is attributed to the prevalence of polar sphingolipids, especially GIPCs, that do not readily partition into low polarity solvents during extraction (Markham et al., 2006). By way of comparison, the sphingolipid content reported here as well as the estimated proportion of putative GIPCs (>70% of total sphingolipid) are both greater than the respective values reported for Arabidopsis leaf tissue (Markham et al., 2006).

HPLC analysis of the fluorescent derivatives of total LCBs liberated by hydrolysis demonstrated the prevalence of t18:0, accounting for >90% of total LCB content in protenemata and >80% of the total in gametophores. While Physcomitrella sphingolipids contained abundant t18:0, isomers of 4-hydroxyphosphine (t18:1), the dominant LCBs in Arabidopsis leaf sphingolipids, were absent. Plant GIPCs...
contain almost exclusively trihydroxy LCBs (Markham et al., 2013) so the high proportions of t18:0 in both protonemata and gametophores are consistent with the high proportions of non-extracted polar sphingolipids, presumably GIPCs.

### 3.2 Generation of mutants using CRISPR-Cas9

To assess the consequences of eliminating t18:0 in Physcomitrella we used CRISPR-Cas9 mutagenesis to disrupt the sole C-4 LCB hydroxylase (Pp3c23_17650) having significant sequence homology to the two C-4 LCB hydroxylases in Arabidopsis (Chen et al., 2008). The LCB profiles of all plantlets recovered after transformation and hygromycin selection were obtained by HPLC and evaluated for alterations in the t18:0/d18:0 ratio. Using two distinct protospacers to target different regions of the moss SPHINGOID BASE HYDROXYLASE (SBH) gene, a total of seven mutants were recovered, all with similar LCB profiles containing low levels of t18:0 and dramatically elevated levels of d18:0 (Figure S1). At this stage of selection t18:0 was included in all media to ensure survival, so variable but small amounts of t18:0 were present in the HPLC profiles of mutants. The mutant lines were designated sbh-1 through sbh-5, with those having the same disruption (based on genotyping; Table S3) designated sbh-2a, -2b, and -2c. All seven lines exhibited the same phenotypes (including LCB profiles), as described below. Several moss plants recovered following transformation and hygromycin selection, so designated transformation controls (TC): they exhibited genotypes and phenotypes (including LCB profiles) indistinguishable from WT plants. Following recovery of transformants, all sbh and TC lines (along with WT) were transferred to medium with and without 10 μM t17:0. It was not known whether mutants unable to form t18:0 could survive, so the synthetic C17 trihydroxy LCB was provided to maintain viability while being distinguishable from t18:0 by HPLC. However, we found that mutants survived and grew in the absence of trihydroxy base, even following multiple transfers and propagation over months. Nevertheless, lines were grown on medium with and without t17:0 for the purposes of this study. Colonies of WT, TC, and sbh lines at

![Figure 2](image-url)  
**Figure 2.** Representative profiles of total LCBs from WT and sbh protonemata and gametophores. Profiles were obtained by HPLC of fluorescent o-phthalaldehyde derivatives of LCBs liberated by acid hydrolysis. Profiles for WT protonemata (a), TC gametophores (b), sbh-1 protonemata (c), and sbh-1 gametophores (d) are shown. Each labeled peak is identified in the key shown in (d). The LCBs were identified based on retention times and co-chromatography with known standards. Peak 2, “unknown,” corresponds to an unidentified o-phthalaldehyde derivative that does not appear to be a free LCB. Note that t17:0 (peak 1) and its anhydro form (peak 7) are not present in these samples as they are not produced by *P. patens* and t17:0 was not supplied. Also note that (a) and (b) are typical of both WT and TC samples and (c) and (d) are typical of sbh lines.
8, 14, and 35 days after protoplast formation were used when comparing LCB profiles and morphology to ensure all plants were at comparable growth stages.

3.3 | Sphingolipid analyses of mutants

Analysis of the LCB profiles by HPLC following methanolic acid hydrolysis of protonemata and gametophores and extraction of liberated LCBs demonstrated that all sbh mutant lines contained essentially undetectable amounts of t18:0, and both sbh protonemata and gametophores were highly enriched in d18:0 (Figures 2 and 3). These results indicate that Physcomitrella possesses a single gene encoding LCB C-4 hydroxylase activity, and confirm previous observations using RNAi to transiently downregulate SBH expression (Essman, 2013). Very minor amounts of unsaturated dihydroxy LCBs were present in both WT and sbh mutants. The lack of a pronounced increase in unsaturated dihydroxy LCB content in sbh mutants was unexpected, especially given the abundance of d18:0 (or d18:0-containing species) potentially available to serve as substrate for desaturation. In Arabidopsis sbh1sbh2 mutants devoid of t18:1 isomers, (E)-8-sphingenine (d18:1(8E)) was the most abundant LCB (Chen et al., 2008). Two sphingolipid LCB Δ8 desaturases in Arabidopsis, encoded by SLD1 and SLD2 (Chen et al., 2012), share 50% and 51% homology, respectively, with the P. patens Pp3c25_1720 peptide product (using the Needleman-Wunsch algorithm). Our results suggest that differences exist between the substrate selectivity, catalytic activity and/or expression of the putative moss SLD and the Arabidopsis orthologs.

The difference in LCB profiles of sbh mutants and WT was accompanied by a difference in the total LCB content. Quantification of total LCB content by addition of an internal standard (d20:0) prior to hydrolysis of a pre-weighed amount of lyophilized tissue indicated that sbh protonemata contained approximately 2.4-fold more sphingolipid than WT (11.0 ± 1.64 nmol mg⁻¹ DW for sbh vs. 4.60 ± 0.60 nmol mg⁻¹ DW for WT), which corresponded to a 200-fold increase in total d18:0 content. This alteration in content is similar to the reported 2.5-fold increase in total LCB content in Arabidopsis sbh1sbh2 mutants (Chen et al., 2008). Although the LCB compositions of sbh and WT gametophores were determined, quantification of the LCB content of gametophores was not feasible due to the difficulty in collecting sufficient material for accurate weight determination following lyophilization.

The consequences of trihydroxy LCB depletion on complex sphingolipid composition were evaluated in two ways; analyses of GIPC glycosylation patterns and molecular species profiling of the major sphingolipid classes. Identification of Physcomitrella GIPC types, based on glycosylation patterns, was carried out as previously described (Kimberlin et al., 2013; Luttgeharm et al., 2015). ESI-MS/MS-based precursor scans used product ion 666.60 m/z for WT GIPCs having t18:0_h24:0 and product ion 650.6 m/z for sbh GIPCs having d18:0_h24:0 (Figure 4). These two ceramide backbones were chosen for scanning due to their respective abundances in WT and sbh GIPCs. Based on multiple reaction monitoring, the four most abundant types identified in WT and sbh protonemata included those having two saccharide units (Series A), HexN-GlcA-IPC, Hex-GlcA-IPC, and HexNAc-GlcA-IPC, and one having three saccharide units (series B), Hex-Hex-GlcA-IPC. Three others, Hex-HexN-GlcA-IPC, Hex-HexNAc-GlcA-IPC (Series B) and Pent-Hex-Hex-GlcA-IPC (Series C) were also detected in both WT and sbh GIPCs. Overall, the GIPC content increased approximately two-fold in sbh lines, commensurate with the increase in total sphingolipid content (above); but some GIPC types increased disproportionately, with Hex-GlcA-IPC, Pent-Hex-GlcA-IPC, and Hex-HexNAc-GlcA-IPC (Series B) increasing by approximately three-fold, four-fold, and 11-fold, respectively (Figure 4). Performing precursor m/z 650.6 scanning of WT GIPCs in order to detect the glycosylation patterns of the small fraction of WT GIPCs possessing the d18:0_h24:0 ceramide backbone demonstrated a pattern qualitatively similar to that of sbh GIPCs (Figure S2).
the GIPC glycosylation patterns found here are generally similar to those reported previously for P. patens (Cacas et al., 2013), our results clearly identified hexosamine as a constituent of GIPC headgroups in Physcomitrella. This novel finding was a result of an improvement in chromatographic separation of hexosamine-containing GIPCs and hexose-containing GIPCs that differ by just one mass unit (Figures 4 and S2). The major GIPC types present in Arabidopsis leaf and pollen (Luttgeharm et al., 2015) also include members of Series A reported here.

Sphingolipidomic analyses of WT and sbh protonemata using LC/MS/MS (Markham & Jaworski, 2007) revealed striking differences in the molecular species (pairing of LCB and FA/hFA) of the main sphingolipid classes and in the free LCB and LCBP profiles (Figure 5). Collectively, free LCB and LCBP concentrations were >50-fold higher in sbh mutants than in WT. Specifically, free d18:0 was increased by >50-fold and d18:0-P was increased by >380-fold (Figure 5a,b). The minor amounts of t18:0 and t18:0-P present in WT were absent in mutants. The free d18:1 present in WT was also absent in sbh samples. This increase in free LCB content may reflect an imbalance in LCB synthesis and Cer synthesis in sbh mutants: the 2.4-fold elevation in total sphingolipid content in mutants (above) is consistent with elevated LCB synthesis, but the more dramatic increase in free d18:0 suggests that ceramide synthase (LOH) capacity is insufficient to acylate the abundant LCBs to form Cers. The 150-fold increase in total LCBP content suggests that LCBP kinase capacity is sufficient to respond to the elevated level of LCB substrate, although the activities of LCBP phosphatase and LCBP lyase must also be considered for catabolic mediation of sphingolipid homeostasis. The increases in both LCB and LCBP content in sbh lines may influence apparent viability (see below), given the evidence that LCBs and LCBPs modulate PCD in Arabidopsis (Alden et al., 2011; Luttgeharm et al., 2016; Shi et al., 2007).

The GIPC species of sbh mutants exhibited hFA profiles very similar to those of WT, despite the approximate >2-fold increase in GIPC

| Symbol | GIPC Structure | M+H m/z for sbh (d18:0_h24:0) | M+H m/z for WT (t18:0_h24:0) | sbh/WT ratio (MRM est.) |
|--------|----------------|-------------------------------|-------------------------------|-------------------------|
| A, A'  | IPC            | 910.9                         | 927.0                         | nd                      |
| B, B'  | GlcA-IPC       | 1087.0                        | 1103.0                        | nd                      |
| C, C'  | HexN-GlcA-IPC  | 1248.0                        | 1263.9                        | 2.1                     |
| D, D'  | Hex-GlcA-IPC   | 1249.0                        | 1264.9                        | 3.0                     |
| E, E'  | HexNAc-GlcA-IPC| 1290.0                        | 1305.9                        | 2.2                     |
| F, F'  | Hex-HexN-GlcA-IPC| 1410.0                      | 1425.9                        | 11.3                    |
| G, G'  | Hex-Hex-GlcA-IPC| 1411.0                      | 1426.9                        | 2.2                     |
| H, H'  | Hex-HexNAc-GlcA-IPC| 1452.1                  | 1468.1                        | nd                      |
| I, I'  | Pent-Hex-Hex-GlcA-IPC| 1543.5                  | 1559.1                        | 3.9                     |
FIGURE 5  Measurements of free LCBs and LCBPs and molecular species compositions (pairing of FA/hFA and LCB) of sphingolipid classes in extracts from WT (a, c, e, g, i, k) and sbh (b, d, f, h, j, l) protonemata determined by HPLC-ESI-MS/MS. The profiles are shown for LCB/LCBPs (a, b), hex-GlcA-IPC, one of the major types of GIPC (c, d), ceramide (Cer; e, f), hydroxy FA-containing ceramide (hCer; g, h), glucosylceramide (GlcCer; i, j) and GlcCer having nonhydroxy FA (nhGlcCer; k, l) of WT and the sbh-3 mutant line. Note the differences in the y-axes (nmol g⁻¹ DW) when comparing the WT and sbh profiles of the different sphingolipid classes. Results shown as mean ± SD (n = 3). The molecular species profiles shown here for WT and sbh Hex-GlcA-IPC were mirrored in the other GIPC types. Results obtained were similar for WT and TC lines, and similar for all four sbh lines analyzed.
Paralleling changes in GIPC species, both Cer and hCer species of sbh mutants exhibited FA and hFA profiles similar to those of WT, in spite of t18:0 being replaced by d18:0 (Figure 5e–h). Like GIPCs, very minor amounts of d18:1-containing species of Cer and hCer were present in sbh mutants. In both Cers and hCers, species having C24 acyl chains were the major constituents, with lesser amounts of C22- and C20-containing species, while species with shorter (especially C16) acyl chains were negligible in both WT and sbh mutants. This preponderance of C20 and longer acyl chains contrasts with the prevalence of C16 acyl chains in Cers and hCers of Arabidopsis mutants, and suggests differences in the substrate specificities or expression of the Arabidopsis LOHs and their orthologs present in Physcomitrella. While the Cer and hCer species profiles were similar for WT and sbh, the total Cer content was 15-fold greater in sbh mutants than in WT, whereas hCer content was similar in both. This increase in Cer content but not hCer content (a trend also seen in nhGlcCers and GlcCers) may reflect limited FA C-2 hydroxylase activity, resulting in an accumulation of Cers. In Arabidopsis, evidence indicates that FAH1 hydroxylates Cer-associated very long-chain FA, while FAH2 hydroxylates Cer-associated C16 FA (Mitchell & Martin, 1997; Nagano et al., 2012). In Physcomitrella, a single homolog for AtFAH1 and AtFAH2 (encoded by Pp3c3_15570) is predicted to exist, based on sequence homology. There is evidence that PCD is promoted in Arabidopsis by elevated Cer content (Liang et al., 2003; Townley et al., 2005), so this increase in Cer content may contribute to the apparent diminished tissue viability observed in sbh mutants (see below).

The GlcCer and nhGlcCer species differed drastically from the other complex sphingolipids in lacking t18:0 and containing >90% 4,8-sphingadienine (d18:2) in both sbh and WT (Figure 5i–l). This is consistent with the idea that the combination of Δ4 and Δ8 desaturation destines a Cer for incorporation into a GlcCer (Michaelson et al., 2009). In WT GlcCers, d18:2_h20:0 was the prevalent species, but the sbh mutants accumulated appreciable amounts of GlcCer containing d18:2 paired with h22:0, h24:0, and h24:1. For nhGlcCers the change was more pronounced: the predominant species in WT nhGlcCer was d18:2_20:0, but in sbh, d18:2_24:0 was the major species, and species pairing d18:2 to 22:0 and 24:1 were similar in abundance to d18:2_20:0. Thus, the elimination of t18:0 in sbh mutants led to increased glycosylation of hCer and Cer species with longer acyl chains. This implies that the lack of t18:0 has an indirect effect on the acyl chain profiles, given that WT GlcCers and nhGlcCers contain d18:2 and not t18:0. As observed for Cers (above), the total nhGlcCer content was >8-fold greater in sbh mutants than in WT, whereas GlcCer content was not significantly different. As mentioned above, this increase in the content of nhGlcCer but not GlcCer may reflect limited FA C-2 hydroxylase activity, resulting in an accumulation of Cers and nhGlcCers in sbh mutants overproducing LCBs as observed in Arabidopsis mutants defective in serine palmitoyltransferase (SPT) regulation (Gonzalez-Solis et al., 2020).

Previously, using TLC to separate sphingolipid classes of polar lipid extracts and quantifying LCB content in each fraction by HPLC following hydrolysis, the total GlcCer content of Physcomitrella was estimated to be <5% of total sphingolipid (Domínguez, 2017). That d18:2 was found only in GlcCers and nhGlcCers, and was the predominant LCB in both (accounting for >90% of LCB), allows us to estimate the total GlcCer content in Physcomitrella by different means. First, a minor HPLC peak (0.5–2% of total LCB) was observed for d18:2 in sbh samples devoid of t18:0 (Figures 2c,d and 3), thus indicating a total GlcCer content of approximately 2% of total sphingolipid. Second, comparing the total GlcCer content (approx. 136 pmol mg⁻¹ DW; Figure 5) to the total sphingolipid content (4.6 nmol mg⁻¹ DW; above and Figure 6), it is calculated that GlcCers account for approximately 3% of total sphingolipid in WT moss. Taken together, these results confirm the earlier estimation, and indicate that GlcCers are minor sphingolipids in Physcomitrella, in contrast to the much higher GlcCer content reported in angiosperms (Markham et al., 2006; Sperling et al., 2005).
3.4 | Morphological phenotypes in sbh mutants

All the sbh mutant lines displayed the same growth phenotype, distinct from that of WT or TC lines. The mutants exhibited a more stunted growth form than WT moss, with more and smaller branching of protonephal filaments (Figure 7a–d). In addition to this stubby growth pattern, sbh chloronemal cells often were oddly shaped, being bulbous, dumbbell shaped, or oval, rather than the smooth cylindrical shape common to WT. Quantitative differences were observed as well: measurements of non-apical chloronemal cell lengths at 8, 14, and 35 days of growth from protoplasts indicated sbh mutants had mean cell lengths that were significantly shorter than WT at all three time points (Figure 7e). Measurements of the lengths of caulonemal cells also present in the protonemata of sbh mutants and WT did not differ significantly at 14 and 35 days (Table S4) suggesting that caulonemal cell growth is not as dependent upon t18:0 or a functional C-4 hydroxylase.
The gametophores of 
mutants were much smaller than their wild type counterparts (Figure 8). Although they appeared to have numerous phylloids, the gametophores were dwarfed (Figure 8a). Measurements indicate the mean area of gametophore phylloid cells of 35 day old mutants were less than half that of WT (Figure 8b). Thus, the small overall size of the gametophores derives at least in part from the reduction in constituent cell size. Although mutants were much smaller, they appeared to be produced in greater numbers in the mutant lines. This density of gametophores can be visualized by the comparison of backlit photos (Figure 8c). While this was difficult to quantify, as it was near impossible to see all the small buds that develop into gametophores, there appeared to be many more gametophores in the lines than in WT. The appearance of so many densely growing dwarfed gametophores raises questions about the relationship between LCB profile and the regulation of

FIGURE 8  The size, morphology and density of WT and mutants after 35 days of growth from protoplasts. (a) Images of representative WT and mutants individual gametophores grown on PpNH4 medium show differences in size and pigmentation. The three images are all of the same scale (note scale bars). (b) The mean phylloid cell areas of WT and mutants gametophores grown on PpNH4 medium or PpNH4 medium supplemented with 10 μM t17:0 ("+ t17:0"). Results shown as mean cell area (n = 86 to 158). Error bars indicate 95% confidence intervals calculated by bootstrapping with 1000 resampled parameters, and each mean differs significantly from every other (p < .0005). (c) Backlit images of representative WT and mutants colonies grown on PpNH4 medium show differences in gametophore density. The WT and mutants images are of the same scale (note scale bars).

FIGURE 9  Red-brown pigment is prevalent in the cell walls of mutants but not WT protonema. Cell wall pellets were obtained by solvent extraction of tissue with chloroform:methanol, 1:2 v/v and washing once with methanol.
gametophore budding, including a possible connection between sphingolipids and cytokinins, some of which are known to induce budding (von Schwartzenberg et al., 2007). In contrast to the pronounced differences in gametophore size and phylloid cell size, the rhizoid cells of sbh and WT moss had modest differences in mean cell lengths, that is, 134.2 μm (95% CI, 130.2–138.6) for sbh as compared to 147.1 μm (95% CI, 139.9–154.3) for WT (Table S4). This suggests that rhizoidal cell growth—as with caulonemal cell growth—is not as dependent upon t18:0 or a functional C-4 hydroxylase.

The sbh mutants differed from WT in color, having a darker, more red/brown hue. This color difference was apparent when observing the protonema (Figure 7a–d), the gametophore (Figure 8a) and the macroscopic plant colony mass (Figure 8c). The red/brown pigment appeared to localize to the cell wall in the protonemata.
solvent extraction this color persisted in the wall material of sbh samples (Figure 9), indicating that it might be an insoluble phenolic component of the cell wall. Significant color was observed in the bases of the dwarfed gametophores, which contained red pigmented inclusions in cells as well as pigmented cell walls (Figure 10). These inclusions rarely occurred in WT gametophores but were quite common in the mutants. The red/brown cell wall deposits and cell inclusions appear to be related to a constitutive plant defense response triggered by the absence of trihydroxy LCBs or the increase in total LCB content. We also noted the presence of protonemal fragments and cell debris in older mutant cultures but not in WT cultures of the same age. While we did not quantify differences in viability, and we cannot state whether our qualitative observations reflect enhanced necrosis or PCD in sbh mutants, it was reported that Arabidopsis C-4 hydroxylase mutants exhibit enhanced PCD (Chen et al., 2008). The involvement of sphingolipids in PCD (Alden et al., 2011; Liang et al., 2009; Shi et al., 2007) and in the response to pathogens (Huby et al., 2020; Wang et al., 2008) by angiosperms may extend to bryophytes as well.

Another, more subtle difference between sbh and WT colonies was observed: sbh protonemata often appeared drier than WT, which typically retain a large amount of moisture on the plant surface. This apparent surface dryness, while variable, may reflect differences in cell wall composition, which could influence the ability of water to adhere to the surface of the moss. This observation suggests that alterations in interactions between GIPCs and wall components (Voxeur & Fry, 2014) and/or effects on secretory processes resulting from changes in sphingolipid species (Markham et al., 2011) may impact cell wall composition and properties.

3.5 | Complementation by exogenous t17:0

The ability of Physcomitrella to take up and incorporate exogenous LCBs was previously demonstrated by monitoring the hydroxylation of supplied LCBs, d17:0 and d20:0, the incorporation of exogenous LCBs into complex sphingolipids, and the ability of specific LCBs to rescue moss colonies treated with otherwise lethal concentrations of myriocin, an inhibitor of LCB synthesis (Guerra, 2017). Initially, t17:0 was provided in the medium to compensate for the lack of trihydroxy LCB and, potentially, to ensure survival of sbh mutants. Analyses of the LCB profiles of WT and sbh mutants indicated significant uptake of t17:0 by the protonema, but only minor uptake by gametophores (Figures 11 and 12). This difference may be a consequence of the contact the tissues had with the medium: while protonemal filaments were in intimate contact with the cellophane-topped medium containing t17:0, the gametophores typically bud from protonema and develop above the medium. This observation further suggests that the ability of protonemal cells/tissues to transport LCBs from the medium to the gametophores is limited at best.

It was likely that a portion of the t17:0 detected by HPLC was not taken up and incorporated but was bound to the cell wall or free in the traces of liquid included during harvesting of the tissue for lyophilization. We analyzed free LCB content of protonemata as previously described (Wright et al., 2003) and determined that the free t17:0 content was approximately 40% of the total t17:0 in WT and 26% of the total t17:0 in sbh mutants. Although these are approximations and can vary between harvests, they confirm that much of the t17:0 was actually incorporated into more complex sphingolipid species.

Quantitative analyses of LCB content demonstrated that sbh protonemata had approximately 2.4-fold greater sphingolipid content than WT, but providing t17:0 decreased endogenous LCB (sphingolipid) content.

**FIGURE 12** The total LCB compositions of protonemata and gametophores of WT (including TC) and sbh lines grown on PpNH4 medium supplemented with 10 μM t17:0. The results, obtained by HPLC as in Figure 11, are shown as the mean percentage of the total LCB content ± SD for protonemata (n = 11 and 14) and gametophores (n = 2 and 3)
content in sbh protonema, returning it to WT levels (Figure 6). To note, a decrease in endogenous LCB content in WT protonema provided t17:0 also occurred. The 2.4-fold increase in sbh moss is comparable to the 2.5-fold increase in LCB content in Arabidopsis sbh1sbh2 double mutants (Chen et al., 2008). Based upon their observations, it was proposed that LCB synthesis, catalyzed by SPT, is regulated by sphingolipids containing a trihydroxy LCB, and that depletion of trihydroxy LCBs allows a less restrained production of d18:0. Our results demonstrating an increase in total LCB content in the absence of t18:0 are consistent with this model. Further, the decrease in endogenous LCB content to WT levels caused by exogenous t17:0, as well as the decrease in endogenous LCB content in WT protonemata provided exogenous t17:0, reinforces this model of regulation. The consistency between an angiosperm and a bryophyte indicates that the mechanisms controlling LCB synthesis may be conserved throughout embryophytes.

Inclusion of 10 μM t17:0 in the medium was found to complement many, but not all, of the phenotypic changes associated with the lack of C-4 hydroxylase activity. Protonemal filaments of sbh mutants grown on medium with t17:0 did not appear as stunted or pigmented as mutants grown on medium lacking the LCB, and the chloronemal cells had a more cylindrical shape and appeared similar in size to the WT (Figure 13). Actual measurement of chloronemal cell lengths at different times following growth initiation from protoplasts on medium including t17:0 confirmed this observation (Figure 7e); the mean chloronemal cell length of sbh mutants grown for 14 or 35 days on medium with t17:0 was significantly greater than that of mutants grown in the absence of t17:0. At 35 days, the chloronemal cell lengths of sbh grown on t17:0 did not differ significantly from WT grown on medium with or without t17:0, indicating that exogenously supplied t17:0 complemented the lack of C-4 hydroxylase activity. Interestingly, WT moss grown 8 days and 14 days on medium with t17:0 had slightly but significantly shorter chloronemal cell lengths than WT on medium without t17:0, but by 35 days of growth any effect of t17:0 on WT cell length was negligible (Figures 7e and S3).

The gametophores of sbh mutants grown on medium with t17:0 exhibited a significantly larger mean phylloid cell area than those grown on medium without t17:0 (Figure 8b). Even with this increase, from 564 μm² to 714 μm², the mean cell area of sbh mutants complemented with t17:0 did not approach that of WT phylloids with or without t17:0 (Figure 8b). The t17:0 in the medium thus only partially complemented sbh phylloid cell area but, as noted above, the t17:0 content in gametophores was much less than that of protonema (Figure 12). The gametophores of sbh lines grown on medium with t17:0 appeared only marginally larger than those grown on medium without t17:0, and many appeared to retain significant red/brown pigment (Figure 14a,b). However, there was variability, with some gametophores closer in size and appearance to the wild type gametophores and others resembling the mutants grown on medium without t17:0 (Figure 14c). This variability may be related to the amount of contact between an individual gametophore and the medium, impacting uptake of t17:0. The gametophores of WT grown on medium with t17:0 had a significantly smaller mean phylloid cell area than WT

![Figure 13](image_url) The effects of exogenously supplied t17:0 on sbh protonema morphology. Two different mutant lines, sbh-2a (a–b) and sbh-1 (c–d) were grown for 14 days on PpNH₄ medium (a, c) or on PpNH₄ medium supplemented with 10 μM t17:0 (b, d). The scale (note scale bar) is the same for all images.
grown on medium without it (Figures 8b and S3). This mild growth inhibition paralleled the inhibitory effects of t17:0 on WT chloronemal cell length during early growth from protoplasts (Figure 7e). The cause of this growth inhibition of WT, but not sbh, tissues by the exogenous LCB is unclear, but possibly the excess of trihydroxy LCBs experienced by WT inhibited growth because it inhibited sphingolipid synthesis.

While morphological differences were related to the LCB composition and content, the nature of the causal relationship is less obvious. For example, it can be asked whether the morphological effects are attributable to a lack of t18:0 or to an overabundance of d18:0 in the sphingolipids of the mutants. Three lines of evidence point to the morphological changes being the result of a lack of the trihydroxy LCB. First, the addition of t17:0 completely reversed the reduction in mean chloronemal cell length in sbh mutants, despite increasing total LCB content. Second, while the amount of d18:0 was reduced in mutants grown on medium with t17:0, it was still 80-fold greater than the d18:0 content present in WT moss: if excess d18:0 was responsible for the mutant phenotype, the observed recovery of the WT phenotype by t17:0 would not be expected. Third, in the presence of t17:0 the LCB profiles of the sbh gametophores had a very low t17:0 content, and their morphology recovered only incompletely, suggesting their small size and enhanced pigmentation result from the lack of trihydroxy LCB incorporation, even when grown on medium with t17:0.

This report, demonstrating the sphingolipid composition of Physcomitrella and the importance of trihydroxy LCBs in a bryophyte, allows comparisons with Arabidopsis and points to future areas of study. The loss of t18:0 reduced the size of cells and tissues, enhanced pigmentation typically observed in response to biotic and abiotic stresses, and increased tissue lethality. Despite the evolutionary distance and gross morphological differences between Physcomitrella and Arabidopsis, the changes accompanying loss of trihydroxy LCBs in the bryophyte roughly parallel those reported in the angiosperm (Chen et al., 2008). More detailed investigations of these morphological alterations in sbh mutants, including changes in expression of genes involved in pathogen response and PCD, would provide further insight into sphingolipid function in bryophytes.

Another similarity between the two plant species is the accumulation of total sphingolipid in the sbh mutants, reflecting a dysregulation of LCB synthesis (Figure 15). The ability of Physcomitrella to incorporate exogenous LCBs proved advantageous in demonstrating the complementation of sbh mutants by t17:0 such that they exhibited WT levels of endogenous sphingolipid content and, presumably, LCB synthesis. In Arabidopsis the proteins orosomucoid 1 (ORM1) and ORM2 impact LCB synthesis by interacting with SPT (Gonzalez-Solis et al., 2020; Kimberlin et al., 2016). The restoration of WT concentrations of total sphingolipids in the mutants by t17:0 supplementation provides direct evidence that dihydroxy LCBs or dihydroxy LCB-containing sphingolipids (e.g., ceramides), in contrast to trihydroxy LCB counterparts, do not effectively regulate SPT, as previously hypothesized based on results from the Arabidopsis sbh1sbh2 mutant (Chen et al., 2008). It is likely that this regulation by trihydroxy LCBs or sphingolipids is mediated through ORM to restrain SPT activity. This regulation may arise from direct binding of the trihydroxy LCBs or sphingolipids to ORM, as recently shown for ceramide-conferred regulation of mammalian SPT via an ORM ortholog (Davis et al., 2019). By extension, angiosperm and moss ORMs may have low affinity for dihydroxy LCB-containing ceramides, resulting in defective SPT regulation in mutants. Studies of the predicted moss ORM orthologs (Pp3c11_20050 and Pp3c7_7910) may shed light on functional

FIGURE 14 The effects of exogenously supplied t17:0 on sbh gametophore morphology. Backlit images of sbh-3 gametophores grown for 35 days on PpNH4 medium (a), or grown on PpNH4 medium supplemented with 10 μM t17:0 (b). Mutant gametophores grown on medium supplemented with t17:0 appear more variable in size and pigmentation (c), possibly reflecting differences in contact with t17:0 in the medium. Note scale bars.
commonalities and the detailed mechanism of SPT regulation by trihydroxy LCBs.

Sphingolipid species having C20, C22, and C24 acyl chains were predominant while sphingolipid species having shorter (especially C16) acyl chains were negligible in both WT and sbh mutants of Physcomitrella. This contrasts with the sphingolipids of Arabidopsis in which dramatic increases in C16-containing species of Cer, hCer, GlcCer, and GIPC were observed in the absence of trihydroxy LCBs (Chen et al., 2008). The prevalence of C16-containing species in Arabidopsis mutants but not in moss sbh lines strongly suggests that differences in the properties and/or expression of Cer synthases exist between the two plants species. The putative moss ortholog of LOH1 and LOH3 (Class II) Cer synthases (Pp3c4_17010) presumably acylates t18:0 using very long chain acyl-CoA, but it may also exhibit activity using dihydroxy LCB as substrate; or, the putative Class I synthase (LOH2) ortholog (Pp3c23_10940), that in angiosperms selectively acylates dihydroxy LCB using 16:0-CoA (Markham et al., 2011; Ternes et al., 2011), may instead use longer acyl-CoAs, such as 20:0-CoA, as substrate in Physcomitrella. Differences may exist in the regulation of the orthologs of LOH2 and LOH1/3 between bryophytes and angiosperms: ORM proteins in Arabidopsis are not discernible from our results with Physcomitrella. Future studies of LOHs in Physcomitrella comparable to previous investigations in Arabidopsis (Markham et al., 2011; Ternes et al., 2011) are needed to define their roles in establishing moss sphingolipid species profiles.

The classes of sphingolipids, and their respective LCB profiles, present in WT Physcomitrella are not unlike those of other plants: for example, GIPCs were characterized by a prevalence of t18:0 paired with very long chain (>C20) hFAs, whereas GlcCers had

**FIGURE 15** A model displaying the synthesis and routing of ceramide species in WT and sbh Physcomitrella. In WT (top), SBH converts 90–95% of d18:0 to t18:0 that is routed to ceramides having C22 and C24 acyl chains and converted to glucosyl inositolphosphorylceramides (GIPC) types. Minor amounts of d18:0 are routed to ceramides and to glucosylceramides (GlcCers) highly enriched in d18:2 paired with C20 nonhydroxy and hydroxy acyl chains. Homologs of ceramide synthase (CS or LOH) may exhibit substrate specificity and so contribute to the routing of dihydroxy and trihydroxy LCB species. Serine palmitoyltransferase (SPT) activity is negatively regulated by t18:0 or species containing it (dashed red line) so total sphingolipid synthesis is restrained. In mutants (bottom), loss of SBH results in d18:0 converted to ceramides with d18:0 and C20 and longer acyl chains. The vast majority of d18:0-containing ceramides are converted to GIPCs, but those used to form GlcCers have d18:2 paired with a more heterogeneous mix of acyl chains than WT. Lack of trihydroxy LCB results in unrestrained SPT activity (note arrow widths) and total sphingolipid synthesis, but exogenous t17:0 can inhibit SPT activity (dashed red line). KSR, 3-ketosphinganine reductase
predominantly d18:2 paired with C20 acyl chains. This typical “routing” of specific ceramide species to either GIPCs or GlcCers seen in WT moss was altered in sbh mutants, in that dihydroxy LCB paired with C20, C22, and C24 acyl chains were prevalent in both GIPCs and GlcCers (Figure 15). One unexpected finding was the low GlcCer content in Physcomitrella, estimated to be approximately 3% as compared to approximately 30% in many angiosperms (Markham et al., 2006; Sperling et al., 2005). However, it has been shown that Arabidopsis lines with artificial miRNA-suppressed expression of the GlcCer synthase were viable and fertile with as little as 2% of WT lines with artificial miRNA-suppressed expression of the GlcCer synthase were viable and fertile with as little as 2% of WT GlcCer levels (Msanne et al., 2015). This suggests that plants and mosses have considerable plasticity in the amount of GlcCer needed to support growth and development.

4 CONCLUSIONS

The results presented here demonstrate the importance of trihydroxy LCB-containing sphingolipids that are prevalent in Physcomitrella. The combination of SBH mutagenesis and t17:0 supplementation provides strong evidence that WT morphology, growth, and development specifically require trihydroxy LCBs. Lack of trihydroxy LCBs in sbh mutants leads to dysregulation of LCB synthesis and alterations in the amounts and types of different sphingolipid molecular species and GIPC glycosylation that also may influence the mutant phenotype. While this report demonstrates the importance of trihydroxy LCB-containing species in growth and morphology of the haploid gametophyte stage of Physcomitrella, the Gransden ecotype used in this study fails to reach sexual maturity (sporophyte development and spore formation), so we could not evaluate the consequences of SBH disruption on these developmental processes. Further studies in both the gametophyte and sporophyte stages are needed to gain a full understanding of sphingolipid metabolism and function in bryophytes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ARS conducted most aspects of moss transformation, characterization of moss morphology, and manuscript preparation including analysis and figure preparation. WOM performed protospacer design and generation and verification of expression vectors. REC conducted sphingolipidomic analyses and related statistics and figure preparations. EBC contributed to sphingolipidomic analyses and manuscript preparation. DVL conceived experimental question, conducted aspects of lipid analysis and manuscript preparation.

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