Arabidopsis Mutants Lacking Long Chain Base Phosphate Lyase Are Fumonisin-sensitive and Accumulate Trihydroxy-18:1 Long Chain Base Phosphate*

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The sphingolipid long chain bases (LCBs) and their phosphorylated derivatives (LCB-Ps) are important signaling molecules in eukaryotic organisms. The cellular levels of LCB-Ps are tightly controlled by the coordinated action of the LCB kinase activity responsible for their synthesis and the LCB-P phosphatase and lyase activities responsible for their catabolism. Although recent studies have implicated LCB-Ps as regulatory molecules in plant development and growth, much less is known about their metabolism and function in plants. To investigate the functions of LCB-Ps in plants, we have undertaken the identification and characterization of Arabidopsis genes that encode the enzymes of LCB-P metabolism. In this study the Arabidopsis At1g27980 gene was shown to encode the only detectable LCB-P lyase activity in Arabidopsis. The LCB-P lyase activity was characterized, and mutant plant lines lacking the lyase were generated and analyzed. Whereas in other organisms loss of LCB-P lyase activity is associated with accumulation of high levels of LCB/LCB-Ps and developmental abnormalities, the sphingolipid profiles of the mutant plants were remarkably similar to those of wild-type plants, and no developmental abnormalities were observed. Thus, these studies indicate that the lyase plays a minor role in maintenance of sphingolipid metabolism during normal plant development and growth. However, a clear role for the lyase was revealed upon perturbation of sphingolipid synthesis by treatment with the inhibitor of ceramide synthase, fumonisin B1.

Sphingolipids are ubiquitous membrane components that are critical for normal membrane function. Sphingolipid metabolites, including sphingoid long chain bases (LCBs), phosphorylated LCBs (LCB-Ps), and ceramides, also function as signaling molecules in eukaryotic cells (1–4). For example, sphingosine 1-phosphate, a key sphingolipid second messenger, regulates proliferation, invasiveness, and programmed cell death. These effects of LCB-Ps have been observed in organisms as diverse as yeast and humans (5). Although far less is known about sphingolipid functions in plants (6, 7), recent studies indicate that sphingolipid-derived metabolites also act as signaling molecules in plants. For example, sphingosine 1-phosphate and more recently phytosphingosine 1-phosphate have been implicated in abscisic acid-dependent guard cell closure through a G-protein-mediated pathway (8–10). In addition, disruption of a ceramide kinase gene has been found to cause enhanced apoptosis in the Arabidopsis ACD5 mutant, suggesting that ceramides regulate programmed cell death in plants (11). Similarly, inhibition of the ceramide synthase step of sphingolipid biosynthesis by the fungal toxins fumonisin and Alternaria alternata f.sp. lycopersici toxin has also been shown to promote programmed cell death (12–15).

Despite their possible importance as signaling molecules, the synthesis and metabolism of LCB-Ps in plants and the role of LCB-Ps in plant physiology remain largely unknown. It is clear that the formation of LCB-Ps is catalyzed by the LCB kinases and that once formed the LCB-Ps can be either dephosphorylated back to LCBs by LCB-P phosphatases or cleaved at the C2–3 bond by LCB-P lyases to yield a long chain aldehyde and an alcohol (6, 16). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: LCBs, long chain bases; LCB-Ps, long chain base phosphates; DHS-P, dihydrosphinganine 1-phosphate; PHS-P, phyto-

sphingosine 1-phosphate; Sc, prefix designating a Saccharomyces cerevisiae gene or gene product; At, prefix designating an Arabidopsis thaliana gene or gene product; DHS, dihydrosphinganine; PHS, phytosphingosine; SPH, sphingosine; WT, wild type; ER, endoplasmic reticulum; PLP, pyridoxal 5′-phosphate; HA, hemagglutinin; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; BisTris, 2-(bis-[2-hydroxy-ethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; Fmoc, N-(9-fluorenyl) methoxycarbonyl; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.
that are optimized for characterizing the activities of the putative proteins.

In this study, we report that the *Arabidopsis* At1g27980 gene, encoding a protein with 42% identity to yeast Dpl1p, exhibits LCB-P lyase activity. Expression of At1g27980 (AtDPL1) in yeast was shown to rescue the phenotypes associated with the yeast dpl1Δ knock-out mutation, and the expressed protein was found to degrade several structurally diverse LCB-P species. We further demonstrated that this gene encodes the only lyase activity in *Arabidopsis* and that the enzyme resides in the endoplasmic reticulum (ER). Despite the absence of lyase activity, homozygous *Atdpl1* knock-out plants were indistinguishable from wild-type plants under typical growth conditions. Furthermore, although loss of LCB-P lyase activity in other organisms resulted in accumulation of high levels of LCB/LCB-P, the sphingolipid profiles of the leaves from the mutant and wild-type plants were remarkably similar, the only difference being that the mutant plants accumulated t18:1 LCB-P.

**EXPERIMENTAL PROCEDURES**

*Yeast Growth*—Standard yeast media were prepared, and yeast were cultured according to established procedures (19). Yeast strains used in this study are listed in Table 1.

*Construction of the AtDPL1, HA-AtDPL1, and ScDPL1 Yeast Expression Plasmids*—The AtDPL1 cDNA (U09463) in the pUNI51 vector was provided by *Arabidopsis* Biological Resource Center (Ohio State University). The cDNA was subcloned into the pADH yeast expression plasmid using the Gateway cloning system (Invitrogen) according to the manufacturer’s instructions. Briefly, the EcoRV restriction fragment carrying the Gateway Reading Frame Cassette A (Invitrogen) was ligated into the pADH1 plasmid at the HindIII site that had been blunt-ended with DNA polymerase I to create the destination plasmid. The AtDPL1 cDNA from the pUNI51 vector was ligated between the EcoRI and Ncol site of pENTR™ 4 (Invitrogen). *In vitro* recombination between the plasmids was done according to the manufacturer’s instructions to generate the plasmid with AtDPL1 fused to the ADH1 promoter. An Xhol-ended AtDPL1 PCR fragment was amplified and ligated

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**FIGURE 1.** LCB/LCB-P synthesis and catabolism. The enzymes involved in the synthesis and degradation of the LCB-Ps are shown.
concentrations of membranes and lysates were determined
from the supernatant was used to assay lyase activity. Protein
and resuspended in membrane extraction buffer (50 mM
Myces cerevisiae DPL1

The supernatant was transferred to a microcen-
tesulfonyl fluoride, 5 mM EDTA, 0.1 mM bestatin, 0.015 mM
pepstatin A, 0.015 mM E-64, 2.344

Microsomal Membrane Protein Preparation—Yeast cells
grown on selective minimal media were pelleted by centrifugation
and resuspended in membrane extraction buffer (50 mM
Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM
phenylmethylsulfonyl fluoride, 1.15 M 4-(2-aminoethyl)ben-
zenesulfonyl fluoride, 5 mM EDTA, 0.1 mM bestatin, 0.015 mM
pepsatin A, 0.015 mM E-64, 2.344 μM leupeptin, and 2 μM/ml
aprotinin). Cells were broken with glass beads by alternating 0.5
min of vortex mixing with 1 min of incubation on ice (eight
cycles). Unbroken cells and debris were removed by centrifugation
at 8000 × g, 4 °C, and the supernatant was centrifuged at 100,000 × g in a TLA 100.3 rotor (Beckman) for 30 min at 4 °C
to provide the membrane and cytosolic fractions. The pellet
was washed twice with membrane extraction buffer and finally
resuspended in the same buffer containing 33% glycerol. Ara-
bidopsis microsomal membranes were prepared as described
previously for corn (17). Leaf lysates were prepared by homog-
enizing four leaves in 200 μl of TEGM buffer (50 mM Tris-HCl,
PH 7.5, 1 mM EGTA, 1 mM β-mercaptoethanol, and plant pro-
tease inhibitor mixture from Sigma) using an ice-cold mortar
and pestle. The resulting liquid was transferred to a microcen-
trifuge tube and centrifuged at 8000 rpm for 30 s to pellet the
debris. The supernatant was used to assay lyase activity. Protein
centrations of membranes and lysates were determined using Bradford reagent (Pierce).

Characterization of Membrane Association of Proteins—One
volume of membrane extraction buffer containing 1 M NaCl, 0.2
M Na2CO3, 5 M urea, 0.4% Nonidet P-40, or 2% Triton X-100
was added to a membrane fraction containing 100 μg of protein.
Following incubation at room temperature for 1 h, the samples
were centrifuged at 100,000 × g in a TLA 100.3 rotor (Beckman) for 30 min at 4 °C. The resulting pellets and super-
natants were resuspended in SDS sample buffer for analysis as
described below.

Protease Protection Assay—Spheroplast preparation and protease
treatment were done as described previously with minor modifications (21). The dpl1 Δsur2Δ mutant cells
expressing HA-AtDPL1 were grown on minimal media. Cells
(140 A600 units) were harvested by centrifugation, washed with
10 mM NaN3, resuspended at 100A600/ml in 10 mM NaN3, 250
M β-mercaptoethanol, and incubated on ice for 10 min. Spheroplasts were prepared by addition of 70 μg of oxalyticase
(Enzogenetics, Corvallis, OR) in an equal volume of oxalyticase
buffer (2.8 mM sorbitol, 100 mM potassium phosphate, pH 7.5, 10 mM NaNO3) and incubation at 30 °C for 1 h. Spheroplasts were
layered on a cushion of 2 M sorbitol, collected by centrifugation
at 500 × g for 5 min at 4 °C in a Sorvall SS-34 rotor, and resus-
pended in lysis buffer (0.1 M sorbitol, 50 mM potassium acetate,
20 mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol). Following homogenization, intact cells and debris were removed by cen-
trifugation at 500 × g for 5 min, and membrane fractions were
prepared as described above. Membrane protein (40 μg) was
added to 40 μl of 250 mM sorbitol, 50 mM potassium acetate, 20
mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol for proteinase
K treatment or to a 100 mM NH4HCO3 solution, pH 7.5, for trypsin treatment. Protease was added (0.3 mg/ml proteinase K
or 0.0625% trypsin) with or without 0.4% Triton X-100; samples were incubated on ice for 15 min, and reactions were stopped by
addition of phenylmethylsulfonyl fluoride to a final concentra-
tion of 1 mM. An equal volume of 10% trichloroacetic acid was
added; samples were incubated on ice for 20 min, and the pre-
cipitated proteins were collected by centrifugation. The pellets
were washed with 200 μl of −20 °C acetone and resuspended in
1× SDS sample buffer, and the proteins were resolved by SDS-
PAGE and analyzed by immunoblotting.

Generation of the Anti-AtDPL1 Antibodies—AtDPL1 was
expressed in Escherichia coli using the pET-28a vector (Novag-
en). For this purpose, a PCR fragment that encoded amino
acids 58 to the end of AtDPL1 (thereby eliminating the N-ter-
minal membrane-spanning domain) was generated using
primers. The PCR product was digested with HindIII and XhoI
and ligated into the pRS316 plasmid (20).

TABLE 1

| Strain              | Genotype                          |
|---------------------|-----------------------------------|
| Wild type           | Mata ura3 met15 his3 leu2Δ         |
| dpl1Δ               | Mata lys2 ura3 met15 his3 leu2Δ    |
| dpl1Δsur2Δ          | Mata ura3 met15 his3 leu2Δ        |
| dpl1Δch3Δ/pScDPL1*  | Mata lys2 u3 trp1Δ leu2Δ dpl1 Δ   |
| dpl1Δch3Δ/pAtDPL1   | Mata lys2 u3 trp1Δ leu2Δ dpl1 Δ   |
| dpl1Δsur2Δ/pScSUR2* | Mata lys2 u3 trp1Δ leu2Δ dpl1 Δ   |
| dpl1Δsur2Δ/pAtDPL1  | Mata lys2 u3 trp1Δ leu2Δ dpl1 Δ   |

* Sc designates the S. cerevisiae DPL1 and SUR2 genes.

Western Blotting—Proteins were separated by SDS-PAGE
using a 4—12% BisTris NuPAGE gel system (Invitrogen)
according to the manufacturer’s instructions and were trans-
ferrered to nitrocellulose. The blots were blocked in 0.1 M Tris,
pH 7.5, 0.15 M NaCl, 0.1% Tween 20, 8% dry milk and were
incubated with anti-HA mouse monoclonal antibody (1:1000)
conjugated with horseradish peroxidase (Roche Applied Sci-

Arabidopsis LCB-P Lyase
ence). AtDPL1p and Kar2p were detected using rabbit polyclonal antibodies (anti-AtDPL1p at 1:500 and anti-Kar2p at 1:10,000) followed by horseradish peroxidase-conjugated goat anti-rabbit (1:3000, Bio-Rad). Bound antibodies were detected using the ECL-Plus Western blotting detection system (Amer sham Biosciences).

**Extraction of LCBs and LCB-Ps from Yeast Cells**—LCBs and LCB-Ps were extracted from yeast cells essentially as described (22). Cells (15 A\textsubscript{600}) were incubated in 10% cold trichloroacetic acid for 30 min on ice and then washed three times with water. Pelleted cells were resuspended in 500 μl of ethanol:ether:water:pyridine:ammonium hydroxide (15:5:15:1:0.018, v/v) and were incubated at 65 °C for 30 min with frequent mixing. The supernatant containing the LCBs and LCB-Ps was separated from other cellular debris by centrifugation at 14,000 rpm for 3 min and transferred to a new tube. Samples were dried using a stream of nitrogen gas, resuspended in 300 μl of MeOH, 190 mM triethylamine (2:0.3, v/v), and 240 μl were transferred to an HPLC vial containing 60 μl of AccQ reagent (Waters) and allowed to react overnight. Esters from aminophospholipids were deacylated by adding 24 μl of 1 M KOH in methanol and incubating at 37 °C for 30 min. The samples were neutralized by addition of 24 μl of 1 M acetic acid in methanol. Insoluble materials were removed by brief centrifugation, and the supernatant was transferred to a new HPLC vial for chromatographic analysis.

**Analysis of AccQ-derivatized LCB and LCB-Ps by HPLC**—HPLC analysis was performed using an HP Series II 1090 liquid chromatograph with HP Chemstation coupled to an Agilent 1100 series fluorescence detector. The derivatized LCBs and LCB-Ps were fractionated and detected as described (22) with minor modifications. The derivatized LCB and LCB-P samples were fractionated by reverse-phase HPLC analysis on a 0.46 × 25-cm C18 column (4 μm) (GraceVydac, CA). Elution was carried out isocratically with Solvent A composed of acetonitrile:methanol:water:acetic acid:triethylamine (480:320:165:30:4, v/v) for 60 min at a flow rate of 1.5 ml/min. Between runs the column was washed by changing the solvent from 100% Solvent A to 100% Solvent B (acetonitrile:methanol, 60:40, v/v) in a 1-min linear gradient to a flow rate of 1 ml/min. The column was continuously washed isocratically with Solvent B for 6 min at the same flow rate before changing to Solvent A in a 1-min linear gradient to 1.5 ml/min and 8 min isocratically with Solvent A at the same flow rate. The AccQ-derivatized LCB and LCB-P peaks were identified using the fluorescence detector that was set at 244 nm excitation and 398 nm emission.

**Immunofluorescence Microscopy**—Indirect immunofluorescence on whole fixed yeast cells was performed as described (23). Spheroplasts prepared from cells expressing HA-AtDPL1 were fixed on poly-l-lysine-coated glass slides and permeabilized using 0.05% saponin in phosphate-buffered saline containing 0.1% bovine serum albumin. Following incubation with either anti-HA mouse monoclonal antibody (1:350) or anti-Kar2p rabbit polyclonal antibody (1:350) for 1 h, Cy3-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Sigma; 1:5000) were added. The stained cells were visualized with an Olympus IX70 inverted fluorescence microscope.

**Disruption of the DPL1, LCB3, and SUR2 Genes**—A BamHI/Xhol-ended PCR fragment extending from 415 bp upstream of the start codon of DPL1 to 924 bp past the stop codon (see above) was ligated into pUC19. The resulting plasmid was digested with SnaBI to release a 975-bp fragment of the coding region that was replaced with a SnaBI-ended TRP1 fragment. The plasmid was digested using BamHI and PstI to liberate a fragment that was used to disrupt DPL1. The lcb3::KAN disrupting allele was PCR-amplified using genomic DNA prepared from the yeast knock-out collection (Invitrogen) using primers that annealed 427 bp upstream from the start codon and 370 bp downstream from the stop codon. The sur2::NAT disrupting allele was constructed by substituting an Xhol-ended nourseothricin resistance marker fragment into the Xhol site of the pUC19-based plasmid that was used for constructing the SUR2::TRP1 disrupting allele (24). The dpl1Δ::TRP1lcb3Δ::KAN double mutant was generated by crossing a dpl1Δ::TRP1/pRS316-pScDPL1 haploid mutant with an lcb3Δ::KAN haploid. The resulting diploid was sporulated, and following tetrad dissection, the products of meiosis that were the dpl1Δ::TRP1 lcb3Δ::KAN double mutants were identified as tetrads containing prototrophs that were kanamycin-resistant.

**Characterization of T-DNA Insertion Mutants**—Salk lines 020151 and 093662 (25), each containing a T-DNA insertion in the Arabidopsis Biological Resource Center. Mutant and wild-type (Col-0) plants were grown in Conviron growth chambers maintained at 22 °C, 13,200 lux (245 μmol/m² s), on a 16-h light/8-h dark cycle. Homozygous mutant plants were identified by PCR using the gene-specific primers 5'-GGGGTTTATCATGGGACCTCCTTCAAA-3' (forward) and 5'-ATGCCGTCTTTCATGCCCCAATAA-3' (reverse), and the T-DNA insert primer used was from the Salk website, designated LB_6313 (5'-TCAAACAGGATTTTCGTCGCTGCT-3'). DNA was extracted from leaves, and PCR was performed using a REDExtract-N-Amp Plant PCR kit (Sigma) following the manufacturer’s instructions. For analysis of DPL1 transcript, RNA was isolated from freshly cut Arabidopsis leaf tissue using a Spectrum™ Plant Total RNA kit (Sigma) following the manufacturer’s instructions. Reverse transcription of mRNA to cDNA and PCR of the cDNA was accomplished using reagents from a real time One-step PCR kit, version 2.0 (Takara). Primers used for DPL1 were 5'-CAAGCTGTGGCCGCTGTGAACTCGGCTTCTGTTAACA-3' (forward) and 5'-CTGTACTAGTTCTCCCCCTAGGACCGCAACC-3' (reverse). Primer sequences for β-tubulin (provided by Charles Dietrich) were 5'-GTGAACTTCATCTCGCCATCTGCAATATGTTCACTGCAATG-3' (forward) and 5'-CTTCCGTCCTGCAATTCCCTTTCCTTATTCCT-3' (reverse). Response of wild-type and mutant plants to fumonisin B1 was performed as follows: 1.6 ml of sterile fertilizer solution (Peter’s Professional) was pipetted into a 47-mm Petri dish with absorbent pad in the absence or presence of 1.0 μM fumonisin B1 (Biomol). Surface-sterilized seeds collected from homozygous mutant, or wild-type plants were spread on the top of each pad, ~30 seeds per plate. Dishes were sealed with parafilm and held in the growth chamber. Seeds were allowed to germinate, and the seedlings were observed regularly for signs of growth or injury. Other protocols for examining plant responses to environmental conditions were taken from Weigel and Glazebrook (26).
In Vitro LCB-P Lyase Assay—Lyase activity was assayed as described previously (27) with several important differences, most significant being the fluorescence detection of the derivatized aldehyde product. A typical assay contained 50 μM DHS-P (Avanti, Alabaster, AL), 100 mM phosphate buffer (pH 7.4), 0.32 mg/ml BSA, 200 μM pyridoxal phosphate, 1 mM dithiothreitol, 1 mM EDTA, 25 mM NaF, and 20–200 μg of protein (microsomal membrane or leaf lysate) in a total volume of 200 μl. Note that DHS-P was first added to assay tubes and the solvent evaporated before adding aqueous components and sonicating to resuspend the lipid. After adding the enzyme source to tubes containing all other assay components and holding on ice, the reaction was started by transferring the tubes to a 30 °C water bath and was allowed to proceed for 40 or 60 min. The reaction was stopped by adding 400 μl of methanol with mixing. The aldehyde product was extracted by adding 800 μl of hexane, mixing well, and centrifuging to separate phases. A 600-μl aliquot of the upper phase was transferred to a new tube and washed with 400 μl of methanol:water (1:1, v/v), and then 400 μl of the washed hexane was transferred to an HPLC vial insert. After evaporating solvent under nitrogen, the aldehyde product was derivatized by the addition of 40 μl of acetonitrile, 5 μl of 1.75 N acetic acid in methanol, and 5 μl of 2 mM 9-fluorenylmethoxycarbonyl hydrazine (Fmoc-hydrazine; Molecular Probes, Eugene, OR) in acetonitrile followed by heating the capped vial at 65 °C for 10 min. After cooling and dilution with 100 μl of acetonitrile, the Fmoc-aldehyde derivatives were analyzed by HPLC using a 250 × 4-mm Luna C18 column (Phenomenex, Torrence, CA) and an isocratic mobile phase consisting of methanol:acetonitrile:water (80:18:2, v/v) at a flow rate of 1 ml/min. The fluorescent derivatives were detected using excitation and emission wavelengths of 266 and 310 nm, respectively. Reactions stopped by addition of methanol prior to incubation at 30 °C or lacking sphinganine 1-phosphate were used as controls. In some instances, cis-11 hexadecenal (Aldrich) was added as an internal standard prior to extraction. However, its lability during storage and the presence of contaminating aldehyde species (including hexadecenal) limited its utility.

32P-Labeled LCB-Ps were prepared using a purified Arabidopsis LCB kinase4 and 32P-labeled ATP (Amersham Biosciences). The kinase reaction was stopped by addition of 800 μl of chloroform:methanol:HCl (100:200:1, v/v), and the LCB-Ps were extracted from the reaction mixture by sequential addition of 250 μl of CHCl3 followed by addition of 250 μl of 2 M KCl and phase separation. The organic phase (400 μl) was transferred into a new tube, and residual ATP in the organic phase was removed by adding 400 μl of the chloroform:methanol:HCl followed by addition of 250 μl of CHCl3 and 250 μl of 2 M KCl. After phase separation, the organic phase was again transferred into a new tube and dried under nitrogen. The 32P-labeled LCB-Ps were resuspended in 200 μl of lyase reaction buffer (see above). The LCB-Ps (40 μl) were added to a 160-μl reaction mixture containing 135 μg of microsomal protein and incubated at 30 °C for 60 min. The reaction was stopped by addition of 800 μl of chloroform:methanol:HCl (100:200:1, v/v), and the LCB-Ps were extracted from the reaction mixture as described above. The labeled LCB-Ps were resolved using silica Gel GHL (w/PA zone) TLC plates (Analytech, Inc.) with 1-butanol:acetic acidwater (3:1:1, v/v) as the mobile phase.

Subcellular Localization of AtDPL1 in Plants—The ER marker construct, CSP-YFP-HDEL, was made by PCR amplification with the primer oligonucleotides as follows: 5′-ATAT-GGCAGCAGCAACTGGAAGCTATTGTAACTTCTTTTCTC-3′ (italic letters represent Ascl site, and the underlined sequence represents basic chitinase signal peptide) and 5′-ATGCTTAAATTATAGGCTCATGC-TGTGACAGCTGTCATGCGGAGA (italic letters represent PacI site, and the underlined sequence represents ER retention signal HDEL) using the plant expression vector pCAMBIA (CAMBIA, Canberra, Australia) as a template. The resulting PCR product containing the signal peptide of basic chitinase (45) and ER retention signal (HDEL) was subcloned into vector pMDC32 at the Ascl and PacI sites (46) to generate pMDC32-CSP-YFP-HDEL.

For construction of the pMDC32DPL1-CFP, the CFP gene was amplified by PCR with primers 5′-ATAT-GGCAGCAGCAACTGGAAGCTATTGTAACTTCTTTTCTC-3′ (italic letters represent Ascl site and the underlined sequence represents NcoI site) and 5′-ATGCTTAAATTATAGGCTCATGC-TGTGACAGCTGTCATGCGGAGA (italic letters represent PacI site) using the Cerulean variant form of CFP (provided by Dr. David Piston) as a template. The product was inserted into the Gateway cassette in the plasmid PMDC32 to generate pMDC32CFP. The At-DPL1 cDNA was then amplified using primers 5′-ATAT-GGCAGCAGCAACTGGAAGCTATTGTAACTTCTTTTCTC-3′ (italic letters represent Ascl site) and 5′-ATGCTTAAATTATAGGCTCATGC-TGTGACAGCTGTCATGCGGAGA (italic letters represent PacI site) and the product was cloned into the Ascl and NcoI sites of pMDC32CFP to generate pMDC32DPL1-CFP.

For subcellular localization of AtDPL1, tobacco leaves (Nicotiana benthamiana) were co-infiltrated with Agrobacterium tumefaciens GV3101 harboring the plasmids AtDPL1-CFP or CSP-YFP-HDEL as described previously with slight modifications (47). Agrobacterium cultures (A600 = 1.0) were collected by centrifugation and resuspended in buffer containing 10 mM MES, pH 5.7, 10 mM MgCl2 to A600 = 1.0. Acetosyringone was then added to the agrobacterium solution at 100 μM. The resuspended cells were incubated at room temperature for 3–4 h before infiltration. The leaves were observed 36–72 h after infiltration. CFP and YFP fluorescence were observed using a confocal laser scanning microscope (Carl Zeiss LSM 510). The filter sets for CFP were excitation 458 nm, emission 480–520 nm, and for YFP excitation 514 nm, emission 535–590 nm, respectively. Images were processed by LSM510 Browser and Photoshop 6.0.

Sphingolipid Profile Analysis of Wild-type and AtDpl1 Mutant Lines—The sphingolipid profile of wild-type and Atdpl1 mutant plants was performed by reversed-phase high performance liquid chromatography coupled to electrospray ionization-tandem mass spectrometry detection as described (28).
The Arabidopsis At1g27980 Gene Substitutes for Yeast DPL1—A BLAST homology search using the yeast Dpl1p protein against the translated Arabidopsis genome identified a single Arabidopsis gene (At1g27980) predicted to encode an LCB-P lyase (Fig. 2A). The amino acid sequence of this putative AtDPL1 protein is 42% identical and 62% similar to yeast Dpl1p. To investigate whether the candidate gene indeed encodes a LCB-P lyase, it was expressed and characterized in S. cerevisiae. Two different plasmids were constructed, one encoding the untagged AtDPL1 protein and the other an N-terminal triple HA-tagged protein (see "Experimental Procedures"). An HA-
tagged membrane-associated protein of the predicted molecular mass (63.3 kDa) was detected in microsomes prepared from cells harboring the plasmid encoding the HA-tagged protein (Fig. 2B). Localization of the AtDPL1 protein to the membrane is discussed further below.

The ability of the candidate *Arabidopsis* LCB-P lyase to substitute for the yeast Dpl1p protein was investigated. Although yeast lacking Dpl1p are viable, several phenotypes have been reported for the *dpl1Δ* knock-out mutant. For example, the mutant displays elevated levels of LCB-Ps and is hypersensitive to LCBs provided in the growth medium (16, 29). Furthermore, elimination of the LCB-P phosphatase, Lcb3p, along with the Dpl1p lyase is lethal (30, 31). The synthetic lethality of the *dpl1Δlcb3Δ* double mutant is clearly because of elevated LCB-Ps as it is suppressed by deletion of the *LCB4* gene, which encodes the kinase responsible for the synthesis of the majority of the LCB-Ps in yeast (30, 31). Expression of *AtDPL1* reversed the sensitivity of the *dpl1Δ* mutant to DHS, PHS, and SPH, suggesting that it was able to degrade the phosphorylated forms of these LCBs (data not shown). Furthermore, AtDPL1 suppressed the lethality of the *dpl1Δlcb3Δ* double mutant, i.e. introduction of a plasmid expressing either untagged or HA-tagged AtDPL1 (but not the empty plasmid) resulted in the ability to lose the *URA3*-marked *DPL1*-rescuing plasmid and thus to grow in the presence of 5-fluoroorotic acid (Fig. 2C). These results provide evidence that the *AtDPL1* gene encodes an active LCB-P lyase.

### The AtDPL1 Protein Has LCB-P Lyase Activity

To further establish that the heterologously expressed AtDPL1 protein is a LCB-P lyase, its *in vivo* and *in vitro* activities were investigated. Wild-type yeast have low levels of free LCBs, with C-18-PHS being the only LCB present at appreciable levels (Fig. 3A). Of note, there are undetectable levels of LCB-Ps in wild-type yeast (Fig. 3A). As has been reported previously (32), the *dpl1Δ* mutant displays a 2–3-fold increase in the level of C18-PHS and an even larger (more than 10-fold) increase in the levels of C16-DHS and C16-PHS in comparison with wild type (Fig. 3A). Furthermore, lack of Dpl1p results in accumulation of LCB-Ps, including C18-PHS-P and C16-DHS-P (Fig. 3A). Thus, it is clear that Dpl1p plays a significant role in maintaining the low steady state levels of the LCBs/LCB-Ps (16, 31, 32). Expression of *AtDPL1* in the *dpl1Δ* mutant reduced the LCB/LCB-P levels in the mutant, restoring LCB/LCB-P levels to those observed in wild-type yeast (Fig. 3A). Consistent with its ability to reduce sensitivity to both DHS and PHS, the levels of both PHS-P and DHS-P were diminished by AtDPL1.
Because the HPLC peaks corresponding to C18-PHS-P and C16-DHS-P were not well resolved, the activity of AtDPL1 toward C16-DHS-P was further addressed using a dpl1Δ sur2Δ double mutant. Sur2p catalyzes the hydroxylation that converts DHS to PHS (24), and thus the sur2Δ mutant synthesizes no PHS (Fig. 1). Compared with wild type, the sur2Δ mutant accumulated very high levels of C18-DHS as well as high levels of C20-DHS (Fig. 3B, note the different scale compared with Fig. 3A). Because the LCB-Ps that accumulated in the dpl1Δ sur2Δ mutant were exclusively DPHs, the ability of AtDPL1 to degrade DPHs (both C16 and C18) could be clearly demonstrated (Fig. 3B).

Two different assays for measuring in vitro LCB-P lyase activity were conducted. In the first, microsomal membranes isolated from dpl1Δ mutant cells harboring the control plasmid or the AtDPL1 expression plasmid were assayed for lyase activity. DHS-1-P was used as substrate, and formation of the product (hexadecanal) was monitored by HPLC with fluorescence detection following derivatization (Fig. 3C). The strain expressing AtDPL1 exhibited lyase activity, whereas the strain carrying the empty plasmid did not, complementing the in vivo results discussed above.

In the second assay, microsomal membranes were prepared from dpl1Δlcb3Δ mutant cells expressing either the AtDPL1 gene or an AtSUR2 gene, which also rescued the lethality of the dpl1Δlcb3Δ double mutant (discussed below). These microsomes were incubated with [γ-32P]ATP with a purified Arabidopsis LCB kinase (see “Experimental Procedures”). Following incubation with the microsomes, the LCB-Ps were extracted and analyzed by thin layer chromatography. As shown in Fig. 3D, right panel, the microsomes prepared from the dpl1Δlcb3Δ double mutant that was expressing AtDPL1 degraded a wide range of LCB-Ps, including DHS-P (d18:0-P), PHS-P (t18:0-P), SPH-P (d18:14-P), as well as the plant-specific LCB-Ps that contain a double bond at C8 (d18:2 Δ8,14-P and t18:1 Δ8-P). Importantly, the microsomes from the AtSUr2-rescued the dpl1Δlcb3Δ double mutant (Fig. 3D, left panel), as well as boiled microsomes from the AtDPL1-rescued mutant (Fig. 3D, top of right panel), had no LCB-P lyase or phosphatase activity thus confirming that the disappearance of the LCB-Ps was because of degradation by the heterologously expressed AtDPL1. The ability of AtDPL1 to rescue the lethality of the dpl1Δlcb3Δ double mutant and to restore LCB-P lyase activity, both in vivo and in vitro, demonstrates that the At1g27980 gene encodes an LCB-P lyase.

AtDPL1 is an Integral Membrane Protein—As mentioned above, AtDPL1 fractionated with the yeast membranes. Moreover, efficient solubilization required membrane-disrupting detergents (Fig. 4A), indicating that AtDPL1 is an integral membrane protein. The yeast Dpl1p protein localizes to the ER (33). Immunofluorescence localization studies of the heterologously expressed HA-AtDPL1 also revealed perinuclear and peripheral ER staining characteristic of ER-localized proteins (Fig. 4B). For comparison, the immunofluorescence of the well-characterized ER-localized Kar2p protein is shown (Fig. 4B).

To confirm that AtDPL1 is also localized to the ER membrane in planta, tobacco leaves were co-transformed with the ER-targeted chitinase signal peptide-YFP-HDEL fusion construct and CFP-tagged AtDPL1p, both under control of the strong constitutive 35S cauliflower mosaic virus promoter. Transformed leaves were examined for CFP and YFP fluorescence using a confocal laser-scanning microscope. Transient expression of CFP-AtDPL1p in the epidermal cells of tobacco leaves showed a staining pattern that is typical of resident ER proteins, including the co-transfected ER-targeted chitinase signal peptide (Fig. 4C). These results clearly show that AtDPL1 resides in the ER.

Although various algorithms for predicting membrane-spanning segments suggested different topologies for the protein, several programs predicted the presence of a membrane-spanning domain between residues 30 and 50 of the AtDPL1 protein. Furthermore, Dpl1p orthologs from different species all have a hydrophobic domain near their N termini that is of sufficient length to span the membrane (Fig. 2A). To test for the presence of an N-terminal membrane-spanning domain in AtDPL1, protease protection studies using right-side-out vesicles were conducted. An HA-tagged N-terminal fragment of AtDPL1 was resistant to protease cleavage in intact vesicles, but this fragment was completely degraded when the vesicles were disrupted with detergent (Fig. 4D). The sizes of the protected fragments were consistent with cleavage at the first potential recognition site after residue 50, i.e. valine 52 for proteinase K to yield a protected fragment of 9773 Da and at lysine 56 for tryps in to yield a slightly larger protected fragment of 9970 Da. As expected, the luminal ER Kar2p protein was protected from protease cleavage in the intact right-side-out vesicles. These results confirm the presence of a membrane-spanning domain between residues 30 and 50 of AtDPL1 and also reveal that the N terminus of the protein resides in the lumen of the ER.
and senescent leaf tissue of the Atdpl1-1 mutant and a greater than 10-fold reduction of the AtDPL1 protein in microsomes from the Atdpl1-2 mutant (Fig. 5D). It is interesting to note that AtDPL1 can be detected at low levels in senescent tissues from the Atdpl1-2 mutant. This is consistent with the microarray data that revealed a 4-fold induction of AtDPL1 expression during senescence (34).

Despite the apparent lack of lyase activity, the homozygous Atdpl1-1 and Atdpl1-2 plants were indistinguishable from wild-type plants under typical growth conditions. As well, the mutants responded similarly as did the wild-type plants to heat stress (40 °C for 6 h), in assays of water loss from excised leaves, and growth at different pH and salinity (results not shown). As discussed above (Fig. 3A), yeast mutants lacking Dpl1p accumulate much higher levels of free LCB/LCB-Ps than do the wild-type yeast. Therefore, it was of interest to determine whether the mutant plants lacking AtDPL1 displayed altered LCB/LCB-P profiles. The LCB/LCB-Ps from wild-type and the Atdpl1 mutant plants were extracted and analyzed using recently described methods capable of analyzing >160 distinct species of sphingolipids (28, 35). The only significant alteration observed was an increase in the levels of trihydroxy-C18:1-P (Fig. 6A).

To investigate a potential role of AtDPL1 in the turnover of LCB/LCB-Ps other than t18:1-P, we took advantage of the observation that inhibition of ceramide synthase (sphinganine N-acyltransferase) with fumonisin B1 leads to an accumulation of free LCB (17) and LCB-P5 in plant tissues and is lethal. Thus, we investigated whether the mutants lacking the LCB-P lyase would accumulate higher levels of LCB and LCB-P and display increased sensitivity to fumonisin B1. Consistent with a role for AtDPL1 in turnover of the accumulated LCB/LCB-Ps, both Atdpl1 mutant lines exhibited hypersensitivity to fumonisin B1 (Fig. 6B and C), i.e. compared with wild-type seedlings, which were also sensitive to the inhibitor, and both mutant lines exhibited less growth and greater bleaching and death. Moreover, the two mutant lines responded similarly to fumonisin, supporting the contention that the disruption of AtDPL1 is responsible for the hypersensitive phenotype.

The role of AtDPL1 in the maintenance of LCB/LCB-P levels was investigated further by growing wild-type and mutant plants hydroponically in a solution supplemented with 1 μM fumonisin B1 for 24 h and then analyzing free LCB/LCB-Ps from the pooled roots of the treated plants. As expected, fumonisin treatment increased LCB/LCB-P levels in the wild-type plants, especially d18:0/d18:0-P and t18:0/t18:0-P, but the

FIGURE 4. AtDPL1 is an integral ER membrane protein with an N-terminal membrane-spanning domain. A, membrane protein (100 μg) prepared from cells expressing HA-AtDPL1 was incubated with 0.5 M NaCl, 0.1 M Na2CO3, 2.5 M urea, 0.2% Nonidet P-40, or 1% Triton X-100 at room temperature for 1 h. Following centrifugation, the proteins from the supernatants (S) and pellets (P) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-HA. B, immunofluorescence localization of HA-AtDPL1 in yeast cells using the anti-HA antibody revealed that HA-AtDPL1 localizes to the ER membrane. Immunofluorescence localization of the well characterized ER luminal Kar2p protein is shown for comparison. C, fluorescence microscopic localization of the AtDPL-CFP (panel A) and the ER marker CSP-YFP-HDEL (panel B) in tobacco cells. Co-localization of AtDPL1 with the CSP-YFP-HDEL ER marker protein (panel C) along with the differential phase (panel D) are also shown. D, N-terminal fragment of AtDPL1 is inaccessible to both proteinase K (left panel) and trypsin (right panel) in right-side-out vesicles. The size of the protected fragment is consistent with protease cleavage just C-terminal to the predicted membrane-spanning domain (residues 30–50, see Fig. 2A) and indicates that the N terminus of AtDPL1 is in the ER lumen.

5 D. V. Lynch, unpublished data.
accumulation was 4-fold higher in the Atdpl1-1 mutant plants (data not shown). Thus, although the mutant plants appeared similar to wild-type plants under typical growth conditions, AtDPL1 plays an important role in normalizing the elevated LCB/LCB-P levels that accompany this inhibitor-induced metabolic disruption.

**DISCUSSION**

In this study, the *Arabidopsis AtDPL1* (*At1g27980*) gene was demonstrated to encode a functional LCB-P lyase that can degrade a wide range of LCB-Ps. The characterization of AtDPL1 expressed in yeast was facilitated by our finding that the expression of an *AtSUR2* gene (*At1g14290*), as well as increased expression of yeast Sur2p, suppressed the lethality of the *dpl1Δlcb3Δ* yeast mutant.4 This allowed us to conclusively demonstrate that all LCB-P turnover in microsomes from the AtDPL1-rescued *dpl1Δlcb3Δ* yeast mutant was due to AtDPL1 because LCB-Ps added to microsomes prepared from the Sur2p-rescued *dpl1Δlcb3Δ* yeast mutant were completely stable (Fig. 3D).

AtDPL1 is the only ortholog of Dpl1p in *Arabidopsis*, and two mutants with T-DNA insertions in *AtDPL1* were found to lack LCB-P lyase activity. The *AtDPL1* gene is ubiquitously expressed in all tissues throughout the development of the plant (34, 36), and its expression is increased in senescence. Despite its ubiquitous expression and the fact that cleavage of LCB-Ps by LCB-P lyases is the only known pathway for the degradation of sphingoid bases, the *Atdpl1* mutant plants were remarkably similar to wild type under a variety of growth conditions. That AtDPL1 is apparently not essential for plant development and growth is perhaps surprising in view of recent studies that have implicated LCB-P lyase in stress response in yeast (30) and in the development of several different species, including slime mold, *Caenorhabditis elegans*, Zebrafish, and mouse (37, 38).

The sphingolipid profiles of the leaves from *Atdpl1* mutant plants were also surprisingly similar to those from wild-type plants, again in contrast to the situation in other organisms. For example, as discussed above, the yeast *dpl1Δ* mutants accumulate much higher levels of free LCB/LCB-Ps than do wild type. This is not the case for *Arabidopsis* plants during normal growth; surprisingly, t18:1-P, thought to derive from the catabolism of complex lipids, is the only LCB-P species whose level is altered in the *Atdpl1* mutants. The unaltered levels of the other LCB-Ps cannot be explained by compensatory activities of the LCB kinases or LCB-P phosphatases in the mutants because the amplification was performed for 32 cycles. Product migration on gels confirmed the expected product size of 1036 bp. C, *in vitro* lyase assays of leaf lysates prepared from WT, *Atdpl1-1* (020151), and *Atdpl1-2* (093662) plants and detection of hexadecanal product by HPLC. For each plant type, results of the assay reaction without added DHS-P (sphinganine-1-P) is shown on top, and the assay with DHS-P added is shown on the bottom. Assays contained 170 μg of leaf protein for WT and 185 μg of leaf protein for mutants and proceeded for 40 min prior to termination. Note that all 40-min incubations resulted in the formation of Fmoc hydrazine-reactive compounds that are not products of the LCB-P lyase reaction. Only WT lyase incubated in the presence of DHS-P demonstrated a product peak above background controls. D, 50 μg of protein from lysates prepared from young leaves and senescent leaves of WT, *Atdpl1-1*, and *Atdpl1-2* were analyzed by immunoblotting. The AtDPL1 protein was detected using an anti-AtDPL1p antibody.
unphosphorylated LCBs are also not elevated. Similarly, increased partitioning of the LCBs into ceramides or complex sphingolipids does not account for the unaltered LCB/LCB-P levels because these sphingolipids are not elevated in the Atdp1 mutants. Thus, it may be that turnover of the LCB-Ps plays a minor role in maintenance of LCB/LCB-P homeostasis in plants, at least in expanding tissues.

Alternatively, it is possible that de novo sphingoid base synthesis is suppressed in the Atdp1 mutants and that sphingolipid levels are maintained by increased recycling of the LCBs. In fact, several studies have suggested an inverse relationship between cellular LCB-P levels and de novo LCB synthesis, possibly because of direct inhibition of serine palmitoyltransferase by LCB-P. For example, studies by van Echten-Deckert et al. (39) showed a direct correlation between the intracellular concentration of the phosphorylated derivatives of sphingosine analogs and their inhibitory effect on serine palmitoyltransferase. Furthermore, overexpression of human sphingosine 1-phosphate lyase in mammalian cells led to increased de novo sphingolipid synthesis (40). It will therefore be interesting to compare the rates of de novo LCB synthesis in the wild-type and Atdp1 mutant plants.

The AtDPL1 protein localizes to the ER membrane in both tobacco and yeast cells. The protein is tethered to the yeast ER membrane by an N-terminal membrane-spanning domain that is located between amino acids 50 and 80, with the N terminus in the lumen of the ER. Although it is possible that the protein has a different topology in planta, this is unlikely because the mammalian orthologs have also been reported to localize to the ER and to have an N-terminal membrane-spanning domain followed by a large hydrophilic domain containing the active site that faces the cytosol (41, 42). The LCB-P lyases are PLP-dependent enzymes, having a lysine residue in the active site that forms an internal Schiff base with PLP. The conserved PLP-binding domain is present in AtDPL1, with Lys-349 being predicted to form the Schiff base with PLP (Fig. 2A).

It is interesting that the lyase resides in the ER where de novo LCB synthesis is occurring rather than in the organelles (tonoplast and plasma membrane) where complex sphingolipids are expected to be degraded. This would be consistent with the aforementioned possibility that LCB-Ps, which are relatively polar, act as a signal to relay the status of complex sphingolipid availability in the late secretory organelles to serine palmitoyltransferase activity in the ER membrane. This is also consistent with the specific accumulation of t18:1-P, presumably derived from the catabolism of complex sphingolipids, which contain predominantly t18:1 as LCB. In this regard, it will be interesting to determine the intracellular localization of the LCB kinases. It is also interesting that one of the putative LCB kinases (AtLCBK2, At4g21535), like AtDPL1, is highly expressed in senescent tissues. The long chain aldehyde product of the lyase can be oxidized to palmitate by an ER-localized enzyme. Fumonisin treatment led to a dramatic accumulation of LCB and LCB-P in plant tissues, and the lack of lyase activity in the mutant lines enhanced the accumulation and exacer-

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FIGURE 6. Phenotypes of mutant plants. A, LCB/LCB-P profiles from leaf tissues of WT, Atdp1-1, and Atdp1-2 plants. B, effects of sphingolipid inhibitors on seedling growth. Photographs of WT, Atdp1-1 (020151), and Atdp1-2 (093662) were taken 8 days following germination on fertilizer (control), or fertilizer plus 1 μM fumonisin B₁. C, response of WT and mutants to fumonisin B₁. Seedlings of WT, Atdp1-1 (020151), and Atdp1-2 (093662) germinated for 8 days on fertilizer, 1 μM fumonisin B₁ (FB₁) were scored as follows: ++++, healthy robust seedling growth; ++, healthy appearing but smaller plants or having fewer leaves; +, stunted/shriveled plants with evidence of chlorophyll bleaching; −, very stunted plants with severe bleaching. The percentage of total seedlings in each category is shown. Although the only control shown is indicated as WT seedlings on fertilizer, both mutants were also germinated on fertilizer and both scored at least 94% "+++," essentially identical to WT plants.

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6 B. Grass and D. V. Lynch, unpublished data.
Addendum—While this manuscript was in revision, another report on the At1g27980 gene appeared (43).

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