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Functional Characterization of a Higher Plant Sphingolipid $\Delta 4$ -Desaturase: Defining the Role of Sphingosine and Sphingosine-1-Phosphate in *Arabidopsis*^{1[W][OA]}

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The role of $\Delta 4$ -unsaturated sphingolipid long-chain bases such as sphingosine was investigated in *Arabidopsis* (*Arabidopsis thaliana*). Identification and functional characterization of the sole *Arabidopsis* ortholog of the sphingolipid $\Delta 4$ -desaturase was achieved by heterologous expression in *Pichia pastoris*. A *P. pastoris* mutant disrupted in the endogenous sphingolipid $\Delta 4$ -desaturase gene was unable to synthesize glucosylceramides. Synthesis of glucosylceramides was restored by the expression of *Arabidopsis* gene At4g04930, and these sphingolipids were shown to contain $\Delta 4$ -unsaturated long-chain bases, confirming that this open reading frame encodes the sphingolipid $\Delta 4$ -desaturase. At4g04930 has a very restricted expression pattern, transcripts only being detected in pollen and floral tissues. *Arabidopsis* insertion mutants disrupted in the sphingolipid $\Delta 4$ -desaturase At4g04930 were isolated and found to be phenotypically normal. Sphingolipidomic profiling of a T-DNA insertion mutant indicated the absence of $\Delta 4$ -unsaturated sphingolipids in floral tissue, also resulting in the reduced accumulation of glucosylceramides. No difference in the response to drought or water loss was observed between wild-type plants and insertion mutants disrupted in the sphingolipid $\Delta 4$ -desaturase At4g04930, nor was any difference observed in stomatal closure after treatment with abscisic acid. No differences in pollen viability between wild-type plants and insertion mutants were detected. Based on these observations, it seems unlikely that $\Delta 4$ -unsaturated sphingolipids and their metabolites such as sphingosine-1-phosphate play a significant role in *Arabidopsis* growth and development. However, $\Delta 4$ -unsaturated ceramides may play a previously unrecognized role in the channeling of substrates for the synthesis of glucosylceramides.

Sphingolipids are ubiquitous membrane lipids and have been shown to be essential in many different eukaryotes (Dunn et al., 2004). It is widely considered that sphingolipids play an important role in membrane structure and organization and that they are enriched in plasma membrane microdomains or so-called "lipid

rafts" (Borner et al., 2005). Such properties are likely to be derived from the differences in structure (and hence biophysical properties) between sphingolipids and phosphoglycerolipids. Sphingolipids comprise a fatty acid (usually a very long chain of C₂₂₋₂₆, but it can also be C₁₆) N-linked to a long-chain base (LCB), usually with the addition of a polar head group to generate the mature lipid (Sperling and Heinz, 2003; Sperling et al., 2004). There is considerable diversity in both the LCB and the fatty acid components between eukaryotes, although the functional significance of this has not yet been fully explored. In addition to structural roles, sphingolipids and their metabolites also act as second messengers (Spiegel and Milstien, 2003). In animal cells, the phosphorylated sphingolipid metabolite sphingosine-1-phosphate (S-1-P) acts as a potent messenger, modulating a range of processes such as proliferation and apoptosis (Saba and Hla, 2004). A number of roles for phosphorylated long chain bases (LCB-1-P) have been observed in fungi, *Drosophila*, and *Caenorhabditis elegans* (Oskouian and Saba, 2004). In higher plants, a role for S-1-P in calcium-mediated stomatal closure has been reported for *Commelina communis* (Ng et al., 2001) and *Arabidopsis* (*Arabidopsis thaliana*;

¹ This work was supported by the Deutsche Forschungsgemeinschaft (grant no. SP 967/1-1 to P.S. and S.Z.) and the Biotechnology and Biological Sciences Research Council (to Rothamsted Research).

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.129411

Coursol et al., 2003; for review, see Brownlee, 2001; Worrall et al., 2003). More recently, a similar role for phytosphingosine (4-hydroxysphinganine)-1-P has been observed in *Arabidopsis* (Coursol et al., 2005).

A number of the *Arabidopsis* genes involved in the biosynthesis of plant sphingolipids have recently been identified and functionally characterized (predominantly by complementation of *Saccharomyces cerevisiae* mutants and reverse genetics in *Arabidopsis*; reviewed by Sperling and Heinz, 2003; Dunn et al., 2004; Sperling et al., 2004). Such studies have experimentally confirmed the essential nature of sphingolipids in *Arabidopsis* (Chen et al., 2006; Dietrich et al., 2008; Teng et al., 2008) and also indicated the contribution of sphingolipids to many aspects of plant growth, development, and stress responses (Liang et al., 2003; Dietrich et al., 2005; Zheng et al., 2005; Ryan et al., 2007).

As part of our efforts to understand the functional consequences of sphingolipid heterogeneity, we have examined the role of sphing-4-ene (commonly known as sphingosine; d18:1 Δ 4⁵) in plants and fungi. Sphingosine is synthesized via the Δ 4-trans-desaturation of sphinganine (d18:0; also known as dihydrosphingosine), although this desaturation occurs on *N*-acylated LCBs (i.e. using the dihydroceramide as the substrate, rather than the free dihydrosphingosine LCB). In animal cells, free sphingosine is only generated through the catabolism of sphingolipids (Spiegel and Milstien, 2003; Saba and Hla, 2004). For this reason, the dihydrosphingosine Δ 4-desaturase is also known as the dihydroceramide Δ 4-desaturase (Michel et al., 1997). However, in this article, we will refer to this activity as the sphingolipid Δ 4-desaturase, since this broader term avoids confusion regarding the substrate of this enzyme (which remains to be defined in many organisms). The first examples of the sphingolipid Δ 4-desaturase were isolated by Ternes et al. (2002), who functionally identified candidate desaturases by heterologous expression in *S. cerevisiae sur2* Δ mutants. Yeast cells lacking the SUR2 sphingolipid hydroxylase are unable to synthesize C4-hydroxylated LCBs such as phytosphingosine and thus prevent competition between SUR2 and the heterologous sphingolipid Δ 4-desaturase for their common substrate (sphinganine; Sperling et al., 2001). This approach identified active sphingolipid Δ 4-desaturases from human (MLD), mouse (mDES-1), *Drosophila* (DES-1), and *Candida albicans*, and a similar study identified the same activity from *Schizosaccharomyces pombe* (Garton et al., 2003). These enzymes defined a new class of desaturases (Napier et al., 2002; Hashimoto et al., 2008), with apparent higher plant orthologs present in the *Arabidopsis* and rice (*Oryza sativa*) genome sequences and other plant EST collections. However, attempts to express a tomato (*Solanum lycopersicum*) ortholog of the sphingolipid Δ 4-desaturase in *S. cerevisiae sur2* Δ mutants did not result in any detectable activity (Ternes et al., 2002).

In animals, sphingosine (the direct precursor of S-1-P; Fig. 1A) is the most abundant LCB (Pyne and Pyne,

2000). However, sphingosine is a very minor component of most higher plant sphingolipids (Imai et al., 1997; Sullards et al., 2000; Dunn et al., 2004; Lynch and Dunn, 2004; Markham et al., 2006), with the predominant LCB modifications being C4-hydroxylation, Δ 8-desaturation, or both, yielding phytosphingosine, sphing-8-ene, or 4-hydroxysphing-8-ene, respectively (Fig. 1A). While Δ 4-unsaturated sphingolipids are abundant in some plant species (such as soybean [*Glycine max*]), these predominantly occur in conjunction with Δ 8-unsaturation, in the form of sphinga-4,8-dienine (Markham et al., 2006). In order to better define the role of sphingosine and S-1-P in *Arabidopsis*, we have functionally characterized the single ortholog of the sphingolipid Δ 4-desaturase. Insertional mutagenesis of the *Arabidopsis* sphingolipid Δ 4-desaturase does not result in any phenotypic alterations to growth or development, likely indicating a very limited role for S-1-P and other Δ 4-unsaturated LCBs and their phosphorylated metabolites in this organism.

RESULTS AND DISCUSSION

Functional Identification of a Sphingolipid Δ 4-Desaturase Candidate Gene of *Arabidopsis* by Heterologous Expression in *Pichia pastoris*

Orthologs of the sphingolipid Δ 4-desaturases identified by Ternes et al. (2002) are easily identifiable in eukaryotic genome sequences, forming a distinct clade from other microsomal lipid desaturases (Hashimoto et al., 2008). *Arabidopsis* contains a single ortholog of the sphingolipid Δ 4-desaturase, located in proximity to the centromere of chromosome IV. This gene (At4g04930; T1J1.1) is annotated as showing similarity to the *Drosophila* DES-1 gene characterized by Ternes et al. (2002) and is made up of two exons split by a single intron. At4g04930 represented the only plausible detectable candidate for the sphingolipid Δ 4-desaturase in the *Arabidopsis* genome (Fig. 1B). Therefore, functional characterization of the open reading frame (ORF) encoded by At4g04930 was attempted by heterologous expression in *S. cerevisiae sur2* Δ mutants, similar to that described previously (Ternes et al., 2002; Garton et al., 2003). However, despite numerous attempts, no activity was detected in association with the expression of the *Arabidopsis* ORF, in agreement with previous attempts to characterize plant orthologs of this enzyme (Ternes et al., 2002; our unpublished observations). This lack of activity in *S. cerevisiae sur2* Δ mutants most likely related to the sphingolipid substrate required by the plant desaturase, rather than to inactivity of the recombinant enzyme per se. Given that *S. cerevisiae* lacks some common classes of sphingolipids (such as glucosylceramides [GlcCers]) and also does not normally synthesize sphingosine, this is perhaps unsurprising, although it is important to note that this host was able to support sphingosine synthesis by other nonplant sphingolipid Δ 4-desaturases (Ternes et al., 2002; Garton

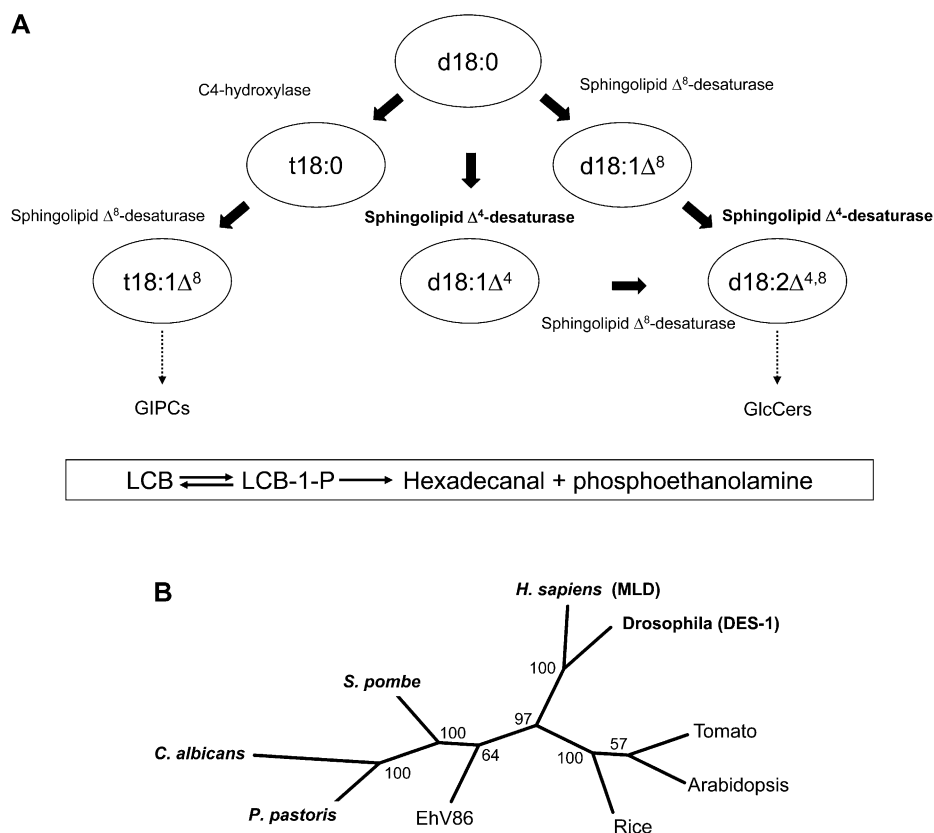


Figure 1. Sphingolipid biosynthesis and the role of the sphingolipid Δ^4 -desaturase. **A**, A schematic representation of sphingolipid LCB modification in higher plants. Note that the actual substrates in terms of free LCBs versus *N*-acylated LCBs are currently undefined, as is the order of desaturation reactions to generate $d18:2\Delta^{4,8}$. Standard sphingolipid LCB nomenclature is used (e.g. $d18:1\Delta^8$ indicates that this is a dihydroxy- C_{18} LCB with a double bond at the Δ^8 position; $t18:0$ indicates that this is a trihydroxy- C_{18} LCB with no double bonds). The sphingolipid class in which different LCBs are enriched is indicated (broken arrows), although it should be noted that other LCBs occur in these classes. Also shown (boxed) is the enzymatic conversion of LCBs to their phosphorylated form LCB-1-P (via LCB kinases), the dephosphorylation of LCB-1-Ps back to LCBs (via LCB-1-P phosphatases), and also the breakdown of LCB-1-Ps via the LCB-1-P lyase to generate phosphoethanolamine and hexadecanal. Free LCBs can be generated either by de novo synthesis or ceramidase-mediated release from sphingolipids (the latter is the predominant route in animals). **B**, Phylogenetic tree of sphingolipid Δ^4 -desaturase orthologs from different organisms. Functionally characterized examples are from *P. pastoris* (AY700778), *C. albicans* (XM_716863), *S. pombe* (NM_001022326), *H. sapiens* (AF002668; MLD), and *Drosophila* (AF466379; DES-1). Higher plant orthologs from Arabidopsis (NM_116731) and tomato (AF466378) have previously proved intractable to heterologous characterization. An unusual virally encoded ortholog is also included for comparison from *Emiliana huxleyi* virus 86 (EhV86; AJ890364).

et al., 2003). These observations also serve to highlight our partial understanding of sphingolipid biosynthesis even in model systems.

In view of the failure of *S. cerevisiae* to serve as a suitable expression host for the functional characterization of higher plant sphingolipid Δ^4 -desaturases, an alternative system was developed. In common with many fungi, the methylotrophic yeast *P. pastoris* synthesizes GlcCers that contain Δ^4 -unsaturated LCBs, predominantly in the form of 9-methyl-sphinga-4,8-dienine. The three *P. pastoris* enzymes (sphingolipid C9-methyltransferase and sphingolipid Δ^4 - and Δ^8 -desaturases) responsible for the synthesis of this LCB were recently identified and characterized by heterologous expression in *S. cerevisiae* (Ternes et al., 2006). In this study, we deleted the intrinsic *P. pastoris* sphingo-

lipid Δ^4 -desaturase gene to establish a mutant strain suitable as an expression host to characterize heterologously expressed sphingolipid Δ^4 -desaturase candidate genes. The sphingolipid Δ^4 -desaturase knockout strain did not contain Δ^4 -unsaturated LCB (data not shown) and, surprisingly, was completely devoid of GlcCers (Fig. 2A). Taking advantage of this potential screen, the Arabidopsis At4g04930 ORF was expressed in the sphingolipid Δ^4 -desaturase-disrupted strains of *P. pastoris*. As can be seen in Figure 2A, heterologous expression of the Arabidopsis ORF restores the levels of GlcCers to that observed in wild-type *P. pastoris*, consistent with Arabidopsis ORF At4g04930 encoding a functional sphingolipid Δ^4 -desaturase.

Additional confirmation of this activity was provided by the analysis of LCBs isolated from the GlcCers

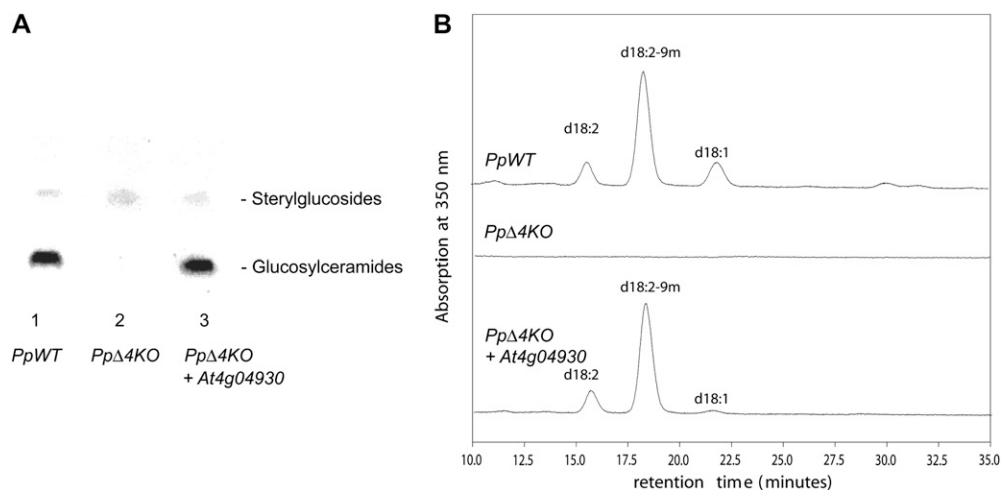


Figure 2. Functional characterization of Arabidopsis gene At4g04930 as a sphingolipid $\Delta 4$ -desaturase. A, Expression of Arabidopsis At4g04930 ORF in a *P. pastoris* mutant lacking sphingolipid $\Delta 4$ -desaturase activity restores the synthesis of GlcCer. The *P. pastoris* mutant disrupted in the endogenous sphingolipid $\Delta 4$ -desaturase AY700778 was transformed with either empty expression vector or vector containing the Arabidopsis ORF. Sphingolipids were extracted, separated by thin-layer chromatography, and stained by spraying with α -naphthol/sulfuric acid and subsequent heating to 160°C. The presence of GlcCer is clearly visible in *P. pastoris* mutants complemented with the Arabidopsis ORF (Pp $\Delta 4$ KO + At4g04930) but absent in mutants transformed with the empty vector (Pp $\Delta 4$ KO). For comparison, wild-type *P. pastoris* cells transformed with the empty vector are also accumulating GlcCer (PpWT). B, GlcCer from *P. pastoris* cells was isolated and subjected to sphingolipid LCB analysis via deacylation and derivatization with 1-fluoro-2,4-dinitrobenzene. LCBs were fractionated by HPLC and detected by A_{350} . GlcCers from wild-type *P. pastoris* transformed with the empty vector (top trace) and from the *P. pastoris* sphingolipid $\Delta 4$ -desaturase mutant transformed with Arabidopsis ORF At4g04930 (bottom trace) both contain $\Delta 4$ -unsaturated LCBs (predominantly in the form of the C9-methyl-sphinga-4,8-dienine). The *P. pastoris* mutant transformed with the empty vector does not contain any GlcCers (middle trace).

of *P. pastoris* knockout mutant cells expressing the Arabidopsis ORF. As can be seen in Figure 2B, the LCB profiles of GlcCers isolated from wild-type *P. pastoris* and the *P. pastoris* knockout mutant disrupted in the sphingolipid $\Delta 4$ -desaturase but expressing the plant ORF are similar, specifically with respect to the synthesis of the predominant 9-methyl-branched diene. As noted above, the *P. pastoris* knockout mutant completely lacks GlcCers (Fig. 2). On the basis of these data, we conclude that the Arabidopsis gene At4g04930 encodes the sphingolipid $\Delta 4$ -desaturase responsible for the synthesis of $\Delta 4$ -unsaturated LCBs such as sphingosine and sphinga-4,8-dienine. Our data also indicate the utility of *P. pastoris* as a new system for the identification of LCB-modifying enzymes that are not tractable using the *S. cerevisiae* system. The requirement for $\Delta 4$ -unsaturated LCBs to initiate the synthesis of GlcCers in *P. pastoris* also implies that this modification is the first committed step for the biosynthesis of this class of sphingolipids in this organism, although it is equally clear that the absence of GlcCers is not essential for the normal growth of this yeast.

Expression of the Sphingolipid $\Delta 4$ -Desaturase Gene in Arabidopsis Is Restricted to Floral Tissues

Having identified the sole Arabidopsis ortholog of the sphingolipid $\Delta 4$ -desaturase, we wished to determine the role of this enzyme in planta. Specifically, we

wished to determine if ablating this enzyme activity resulted in reduced levels of $\Delta 4$ -unsaturated LCBs such as sphingosine and what the functional consequences of this might be at the phenotypic level. Surprisingly, the tissue-specific expression pattern of At4g04930 is extremely restricted, as judged by analyses of microarray data sets, with high levels reported for pollen, low transcript levels being detected in floral tissue, and transcript being virtually absent in all other tissues (Supplemental Table S1). Northern-blot analysis of total RNA from Arabidopsis leaves failed to display a signal when probed with At4g04930, even after prolonged exposure (data not shown). However, such analysis cannot exclude the possibility that At4g04930 has a limited cell-specific expression pattern that is restricted to a few cell types, although it is interesting that this gene is apparently not expressed in guard cells or modulated by abscisic acid (ABA), according to transcriptomic analyses carried out by the Schroeder laboratory and available through the Arabidopsis eFP browser (Winter et al., 2007).

Identification of Insertional Mutants of Arabidopsis Disrupted in the Sphingolipid $\Delta 4$ -Desaturase Gene

To better define the role of the sphingolipid $\Delta 4$ -desaturase At4g04930 in sphingolipid metabolism in Arabidopsis, a reverse genetic approach was taken. Insertional mutants of At4g04930 were identified by

searching the SALK (Alonso et al., 2003) and RIKEN (Kuromori et al., 2006) databases, and two suitable alleles were identified and further characterized (Supplemental Fig. S1). The first allele (RIKEN 15-1202-1) was generated as a result of the insertion of a modified *Ds* transposon (Kuromori et al., 2006) after the 10th residue of exon 1. The second allele (SALK_107761.42.15.x) resulted from a T-DNA insertion after 25 residues of the second exon. In both cases, the insertions were predicted to generate ORFs that lacked the essential His box motifs required for desaturase function (Hashimoto et al., 2008). Segregating T3 seeds for these two insertion mutants were obtained from BioResource Center-RIKEN and the Nottingham Arabidopsis Stock Centre, respectively, and used to isolate homozygous lines for these disruptions (via genomic PCR using primers designed against At4g04930 and the insertion cassette). Since the RIKEN *Ds* transposon mutants were generated in the Nössen (No-0) ecotype, we also identified and retained wild-type null lines that lacked insertions in the target gene to serve as control material for

subsequent analysis. As a further confirmation of the mutant status of these two insertion alleles, quantitative (Q)-PCR was carried out on RNA isolated from developing flowers (Supplemental Fig. S1). As shown, transcripts for At4g04930 are detected in wild-type material (Columbia [Col-0] and No-0) but not in the insertion mutants, in agreement with the genomic PCR analysis. Therefore, we conclude that these two different insertion mutants have both resulted in the disruption of the synthesis and/or accumulation of transcripts derived from gene At4g04930, which encodes the sole sphingolipid $\Delta 4$ -desaturase in Arabidopsis.

Sphingolipidomic Analyses of Arabidopsis Insertion Mutants

Recent advances in mass spectrometry-based approaches to measuring sphingolipids have provided powerful tools to yield quantitative and qualitative information on Arabidopsis sphingolipid composition. Using the HPLC-electrospray ionization-tandem

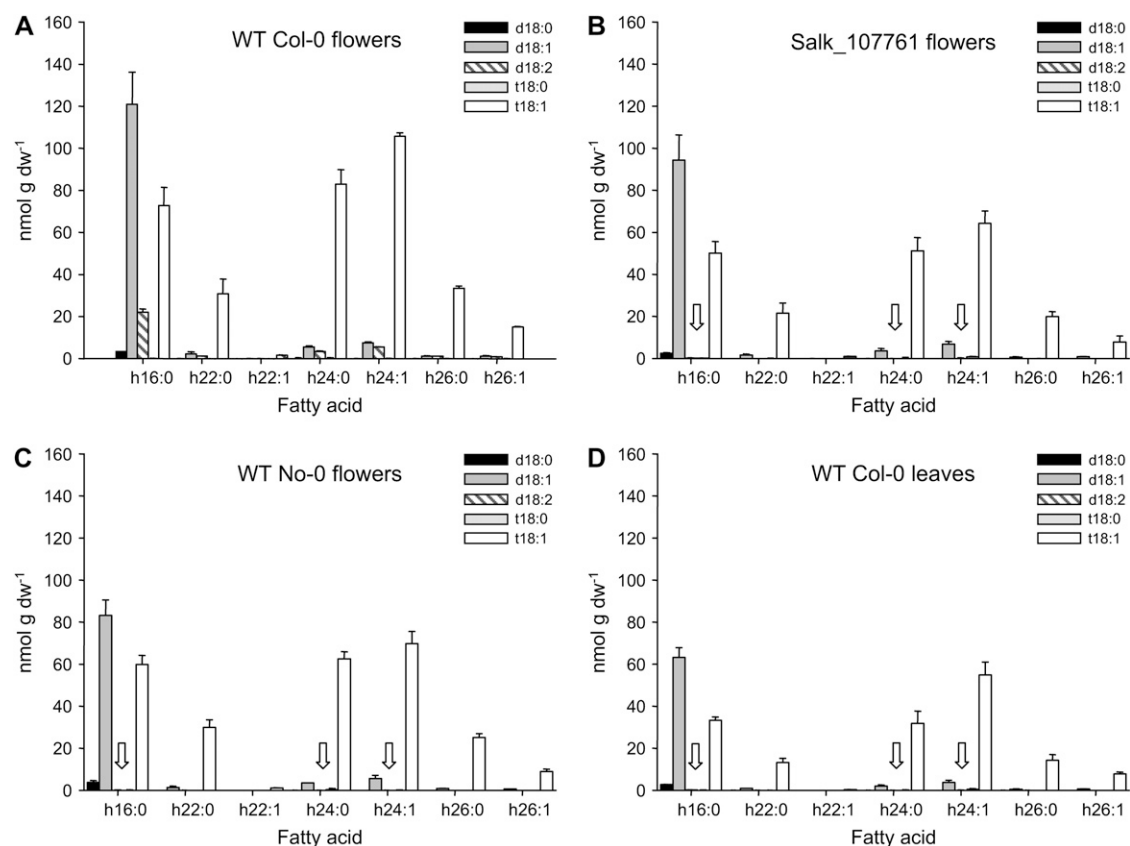


Figure 3. Molecular species composition of GlcCer of wild-type (WT) and sphingolipid $\Delta 4$ -desaturase mutant plants. Sphingolipids were extracted from wild-type Col-0 flowers (A), SALK_107761 flowers (B), wild-type No-0 flowers (C), and wild-type Col-0 leaves (D), and the amount of sphingolipid in the extract was determined by LC-MS/MS as described previously (Markham and Jaworski, 2007). Only GlcCers were found to contain appreciable amounts of d18:2 LCBs (A) with no d18:2 detectable in the other sphingolipid classes (data not shown; Markham et al., 2006). GlcCers containing d18:2 were undetectable (white arrows) in the SALK mutant line (B) and also absent from wild-type Col-0 leaf tissue (D). No d18:2 GlcCers were detected in floral tissues in wild-type No-0 (C) from which the RIKEN transposon mutant was isolated (Supplemental Table S2). Bars show averages ($n = 5$), and error bars show SD. dw, Dry weight.

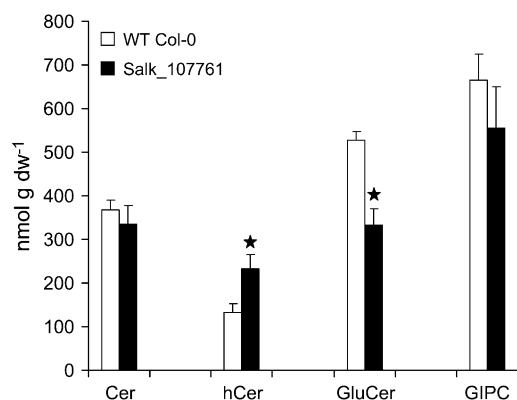


Figure 4. Spingolipid content of wild-type (WT) and spingolipid $\Delta 4$ desaturase mutant lines. Spingolipids were extracted from floral tissue (three samples for wild-type Col-0 and six samples for SALK_107761), and the amount of spingolipid in the extract was determined by LC-MS/MS as described previously (Markham and Jaworski, 2007). Total amounts of each class of spingolipid were calculated compared with added internal standards. Significant differences ($P < 0.05$) between the samples were determined by Student's t test and are indicated by stars. dw, Dry weight.

mass spectrometry (MS/MS) protocol developed by Markham and Jaworski (2007), spingolipidomic profiles were obtained from leaf and floral tissue of wild-type and insertion mutant alleles of the spingolipid $\Delta 4$ -desaturase At4g04930. Profiling of rosette leaf material was carried out, but comparison of the mutants with their respective wild-type backgrounds (SALK_107761.42.15.x versus Col-0; RIKEN 15-1202-1 versus No-0) failed to reveal any significant alterations to different spingolipid classes (ceramide, hydroxyl-ceramide, GluCer, glycosyl inositol phosphoryl ceramides [GIPCs]; Supplemental Data Set S1). Intriguingly, the levels of two phosphorylated LCBs, t18:0-1-P and t18:1 Δ 8-1-P, were reduced in the leaf samples of SALK_107761.42.15.x compared with Col-0 (Supplemental Data Set S2), although the reason for these changes is not obvious. No $\Delta 4$ -unsaturated spingolipids or spingolipid metabolites were detected in these leaf samples, indicating the absence of sphingosine and sphinga-4,8-dienine in all samples, confirming previous spingolipid analyses (Sullards et al., 2000; Markham et al., 2006) and in agreement with our observations that At4g04930 transcripts are not detected in leaf tissue.

In view of the microarray expression pattern and our own Q-PCR data (Supplemental Fig. S1), spingolipidomic profiling was also carried out on floral material (unopened flower buds) that expresses the spingolipid $\Delta 4$ -desaturase at moderate levels. In these samples, the effect of insertional inactivation of At4g04930 was observed, specifically in the SALK insertion allele (Fig. 3, B and C). Comparison between this line and wild-type Col-0 (Fig. 3A) indicated the complete loss of $\Delta 4$ -unsaturated LCBs in GluCers (in the form of sphinga-4,8-dienine N -linked to a hydroxyl- C_{16} fatty

acid), confirming our assignment of this gene as the spingolipid $\Delta 4$ -desaturase. Note that although the regioisomer LCB d18:1 Δ 8 is present in Arabidopsis floral and leaf tissues (Fig. 3D), sphingosine is not detected (Supplemental Fig. S2; Supplemental Table S2). Thus, d18:1 LCBs (Fig. 3) represent the $\Delta 8$ -unsaturated form rather than the $\Delta 4$ -unsaturated isomer.

Interestingly, the loss of $\Delta 4$ -unsaturated LCB in GluCer (which represent less than 5% of the LCBs present in this class of spingolipid) resulted in significant reduction in the total levels of GluCer between the wild type (528 nmol g⁻¹ dry weight) and SALK_107761 (334 nmol g⁻¹ dry weight; Fig. 4). This was mirrored by an increase in the hydroxyl-ceramide levels in these same tissues (wild-type, 132 nmol g⁻¹ dry weight) compared with SALK_107761 (232 nmol g⁻¹ dry weight; Fig. 4). Given the unexpected nature of this perturbation to spingolipid biosynthesis, we also determined by LCB analysis the levels of neutral spingolipids (predominantly GluCers) in a chloroform-soluble extract of floral tissues. This confirmed the decrease in these spingolipids (322.1 nmol g⁻¹ GluCers in the wild type, 248.2 nmol g⁻¹ GluCers in SALK_107761, corresponding to 77% of the wild-type level), although this method appears to underestimate the levels of these lipids. The decrease in GluCers observed in SALK_107761 is partially analogous to the situation in the *P. pastoris* mutant lacking spingolipid $\Delta 4$ -desaturase activity and consequently devoid of GluCer. Our data, therefore, indicate a previously unsuspected role for $\Delta 4$ -unsaturated LCBs in the channeling of substrate for the synthesis of GluCers in both plants and fungi. It is perhaps relevant that in some plant species that do accumulate $\Delta 4$ -unsaturated spingolipids, such as tomato, there are also markedly high levels of GluCers (Markham et al., 2006). Interestingly, a recent study has shown that disruption of microsomal fatty acid elongation in the Arabidopsis *pas2-1* mutant results in greatly reduced levels of GluCers but not GIPCs (Bach et al., 2008).

No other significant differences were observed in ceramides or GIPCs isolated from this same floral material. Very low levels of the free LCB sphinga-4,8-dienine were detected in the Col-0 wild type, but these were absent in the SALK insertion mutant (indicating that these LCBs are most likely derived from GluCers; Supplemental Data Set S2). Thus, these data confirm that $\Delta 4$ -unsaturated LCBs (either in spingolipids or as free LCBs) are absent in Arabidopsis Col-0 lines in which the spingolipid $\Delta 4$ -desaturase At4g04930 has been insertional inactivated. Moreover, this also indicates that this gene represents the sole spingolipid $\Delta 4$ -desaturating activity in Arabidopsis, since no compensatory synthesis was detected in the knockout mutant.

In the case of the RIKEN *Ds* insertion allele 15-1202-1, similar profiling of spingolipids from developing flower buds indicated the complete absence of $\Delta 4$ -unsaturated LCBs in both the wild type (No-0) and mutant, even in GluCers (Supplemental Data Set S1).

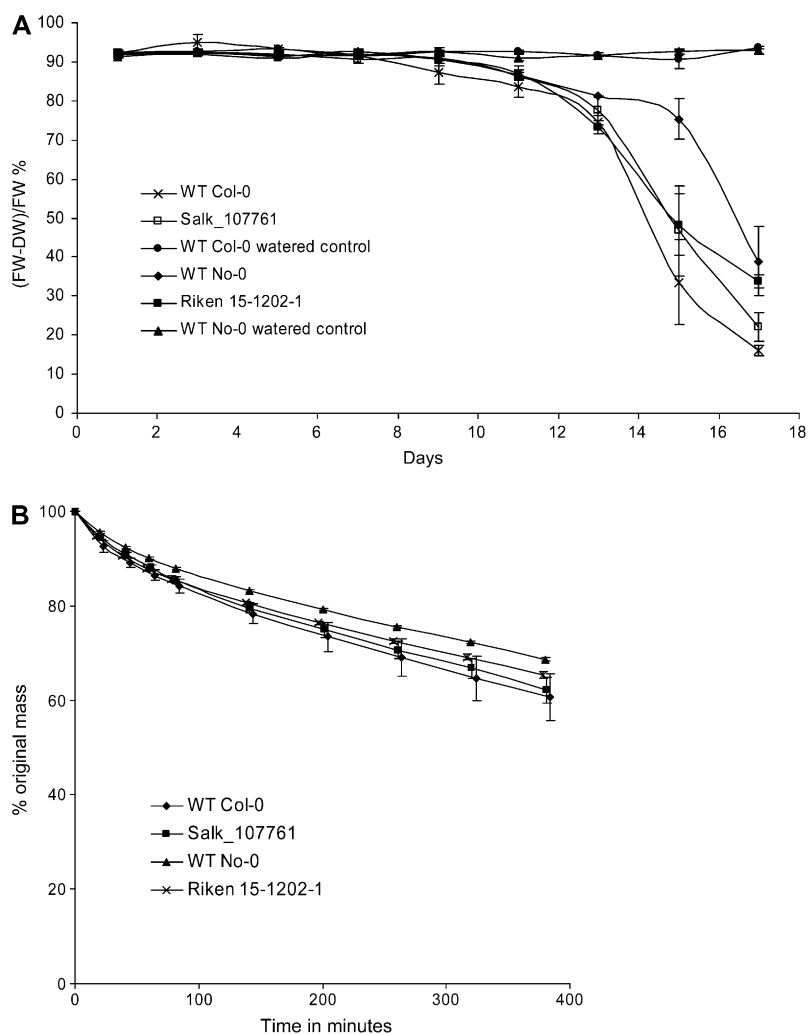


Figure 5. Phenotypic characterization of mutants for altered drought response. **A**, The wild type (WT) and the respective insertion mutant were grown to first bolt stage and then water was withheld. As a control, wild-type material was also fully watered. Leaves were harvested from drought-treated and control plants every 2 d for 17 d, and the (fresh weight – dry weight)/fresh weight [(FW-DW)/FW] percentage was calculated. As can be seen, there is no significant difference between the wild-type (Col-0 and No-0) and the mutant (SALK-107761 and RIKEN 15-1202-1) lines in terms of their decrease in fresh weight in response to drought. Each point represents an average ($n = 3$), and error bars show SE. **B**, The wild type and the respective insertion mutants were assessed for their ability to regulate water loss through their transpiration stream via excision of roots and measurement of total mass as an indicator of water loss. Measurements were taken over a 7-h period, and data are expressed as percentages of the original starting mass (in grams) of the rootless plant. Plant material used was as for **A**. As can be seen, neither insertion mutant had any significant difference in mass loss. Each point represents an average ($n = 3$), and error bars show SE.

Thus, it appears that there is natural variation in the accumulation of different forms of sphingolipids between ecotypes, and as such, the absence of $\Delta 4$ -unsaturated LCBs such as sphingosine and sphinga-4,8-dienine in No-0 would argue that such LCBs are not essential for any particular biological process (in agreement with observations of the SALK insertion line). Interestingly, both Col-0 and No-0 ecotypes showed similar (low) levels of $\Delta 4$ -desaturase At4g04930 transcript accumulation (Supplemental Fig. S1). In the case of the No-0 ecotype, it is possible (based on the higher levels of phytosphingosine present) that a much more active C4-hydroxylase out-competes the sphingolipid $\Delta 4$ -desaturase for the same substrates (see Fig. 1A for representation; Sperling et al., 2001). It is also noteworthy that No-0 not only lacks $\Delta 4$ -unsaturated sphingolipids but also contains reduced levels of GlcCers in floral tissue, compared with Col-0. In addition to using the MS-based method described above, we also used highly optimized HPLC analysis of total LCBs (described in Tonon et al., 2005) isolated from the same floral material used for the sphingolipidomic analysis. As shown in Supplemental Figure S3, the LCB profiles

of total floral sphingolipids in Col-0 and the SALK insertion mutant are very similar apart from the absence of sphinga-4,8-diene in the mutant, confirming the results presented above. Equally, it is again clear that the No-0 ecotype does not accumulate this particular LCB, irrespective of whether or not the sphingolipid $\Delta 4$ -desaturase gene is insertionally inactivated (Supplemental Fig. S3). The observation that sphingolipid LCB composition is subject to natural variation may also facilitate quantitative genetic approaches to defining regulatory (homeostatic) processes.

Collectively, these data confirm the absence of $\Delta 4$ -unsaturated sphingolipids from the leaves of wild-type Arabidopsis plants and that a Col-0 background insertion mutant line lacks sphinga-4,8-dienine in sphingolipids of floral tissue. It is also important to highlight that our comprehensive analysis of over 160 different sphingolipids and their metabolites failed to identify significant amounts of sphingosine in either leaf or floral tissue (Supplemental Fig. S2; Supplemental Table S2). Such analyses do have limits to their ability to detect very low abundance compounds; however, our observations are in agreement with the restricted ex-

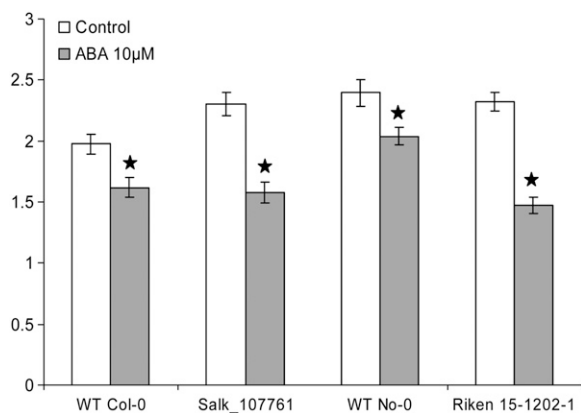


Figure 6. Stomatal aperture measurements of wild-type (WT) and mutant lines after ABA treatment. Stomatal apertures were measured from wild-type and mutant leaves treated with or without ABA for 2 h. Data are from three independent experiments, and measurements were carried out double blind. Each bar represents an average ($n = 3$), and error bars show SE. Significant differences ($P < 0.01$; stars) between the treated and untreated samples were determined by Student's t test.

pression pattern of the sphingolipid $\Delta 4$ -desaturase gene and also with previous sphingolipid analyses in several different studies (Sullards et al., 2000; Markham et al., 2006, Markham and Jaworski, 2007).

Phenotypic Characterization of Arabidopsis Insertion Mutants

Having demonstrated that mutants disrupted in the sphingolipid $\Delta 4$ -desaturase gene lacked $\Delta 4$ -unsaturated sphingolipids, we wished to determine if this absence had any functional consequences. Gross phenotypic appearances of SALK_107761.42.15.x and RIKEN 15-1202-1 showed no obvious differences compared with their respective wild types (Supplemental Fig. S4); equally, a more detailed examination of a range of growth and developmental parameters showed no differences (data not shown). In the case of the RIKEN insertion mutant, these observations are in agreement with a large-scale "phenome" study that included this allele but reported no alterations to growth and development (Kuromori et al., 2006). In view of the previous reports of a role for S-1-P in Ca^{2+} -mediated guard cell closure in Arabidopsis (Coursol et al., 2003) and *Commelina* (Ng et al., 2001) and that drought stress resulted in an increase of S-1-P in this latter species, we sought to establish if the insertion mutants had altered responses to drought. As discussed above, although no $\Delta 4$ -unsaturated sphingolipids were detected in analysis of total rosette leaves, it was hypothetically possible that sphingosine and S-1-P might be present in specific cell types via the discrete expression of sphingolipid $\Delta 4$ -desaturase At4g04930, leading to underrepresentation in microarray and Q-PCR studies of whole tissues. Thus, the availability of bona fide knockout alleles of this gene allowed this scenario to be evaluated.

Two different protocols were used to evaluate the performance of mutant plants compared with wild-type plants under drought-induced conditions. First, in an experiment similar to that used by Ng et al. (2001), in which a 2-fold increase in S-1-P was reported in unwatered *Commelina* plants after 11 d, Arabidopsis plants were withheld water for 17 d, with leaf samples being harvested every 2 d. Fresh and dry weights of well-watered controls and drought-treated plants were obtained for wild-type controls and mutant lines. As can be seen in Figure 5A, there is no statistically significant difference in response to drought between the controls and mutants in terms of the plant's ability to regulate water loss. A second experimental approach was adopted to determine if maintenance of water status through guard cell closure was altered in these mutants, using well-established methods previously used to identify Arabidopsis mutants altered in their response to ABA and drought resistance (Xiong et al., 2001; Chini et al., 2004). This method determines a plant's ability to restrict water loss through (ABA-mediated) guard cell closure and has been used to characterize mutants such as *aba2*, *aba3*, *abi1*, and *sad1* (Leon-Kloosterziel et al., 1996; Xiong et al., 2001; Chini et al., 2004). Plants were grown to just prior to bolt initiation stage under well-watered conditions, and then all aerial parts of the plants were severed from the roots with a scalpel. The fresh weights of these intact rootless plants were then determined over 7 h, indicating the rate of mass loss (water) via the transpiration stream. As can be seen in Figure 5B, no significant differences between wild-type controls and insertion mutants were observed.

In addition to these indirect measurements of water relations in Arabidopsis, we also measured the stomatal aperture of mutants and wild-type plants in the presence or absence of ABA ($10 \mu M$; using a slightly modified method based on that described in Vahisalu et al., 2008). As can be seen in Figure 6, stomata of the

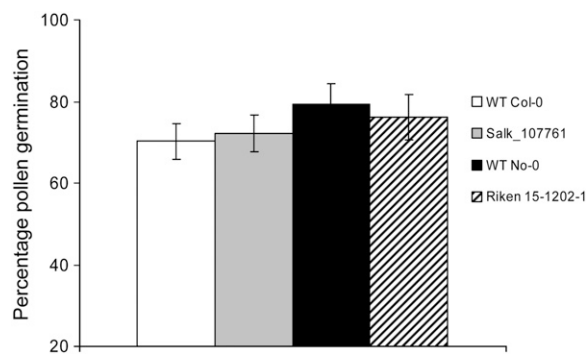


Figure 7. Pollen viability of mutants. Pollen from wild-type (WT) and insertion mutant flowers was assayed for germination, with percentage germination assessed by microscopic examination. Plant material used was as for Figure 5. No significant difference between the wild type and mutants was observed. Each bar represents an average ($n = 3$), and error bars show SE.

mutant lines responded well to ABA compared with wild-type stomata. All four lines (Col-0, SALK_107761, No-0, and RIKEN 15-1202-1) showed a highly significant ($P = 0.01$) stomatal closure in response to ABA treatment, indicating that there was no difference between wild-type and mutant plants in the response to this hormone.

Collectively, these data indicate that $\Delta 4$ -unsaturated sphingolipids and/or their metabolites do not play a primary role in modulating water relations or response to drought stress in Arabidopsis. This is perhaps surprising given the study of Ng et al. (2001), which indicted a role for S-1-P in *Commelina* guard cell closure (based on exogenous application of 4–6 μM S-1-P) and also reported an increase in the endogenous levels of this phosphorylated LCB in drought-treated leaves. Our analysis of sphingolipid LCBs from *Commelina* leaf tissue indicates that sphingosine is a very minor component (Supplemental Fig. S2), although this does not preclude the presence of S-1-P in *Commelina*. Based on our current studies in Arabidopsis using insertion mutants lacking any capacity to synthesize sphingosine, it is clear that this particular LCB(-1-P) either does not play a dominant role in these processes or is functionally redundant. In that respect, it is interesting that Coursol et al. (2005) reported a different LCB-1-P, phytosphingosine-1-P (t18:0-1-P), as being involved in Arabidopsis guard cell closure. Very recently, Chen et al. (2008) demonstrated a crucial role for these C4-hydroxylated LCBs in Arabidopsis growth and development.

Having not observed a direct role for sphingosine and other $\Delta 4$ -unsaturated sphingolipids in drought stress, control of transpiration rate, or stomatal closure, we considered any other potential roles for these LCBs. As noted above, the sphingolipid $\Delta 4$ -desaturase At4g04930 is only expressed at significant levels in flowers, predominantly in pollen. Interestingly, mutations in the *Drosophila* DES-1 ortholog result in flies with defective spermatogenesis (Endo et al., 1996). Therefore, we investigated the formation and viability of pollen in our Arabidopsis mutants, although the initial isolation and characterization of these insertion alleles indicated that both mutant alleles (SALK and RIKEN) were fertile, implying no gross defect in pollen development or viability. Pollen from control and mutant lines were visualized by double staining with fluorescein diacetate and propidium iodide, allowing the discrimination between live and dead pollen (McConn and Browse, 1996). However, no difference was observed in the percentage of dead pollen cells between control and mutant lines (data not shown). Second, pollen from wild-type control and mutant lines was germinated in vivo in the presence of 2% Suc, and the percentage germination rate was determined according to the methods of Footitt et al. (2007). As can be seen in Figure 7, no significant differences in rates between wild-type controls and mutants were observed, indicating no role for sphingosine or $\Delta 4$ -unsaturated sphingolipids in pollen viability or germination.

CONCLUSION

The aim of this study was to determine the function of the Arabidopsis gene At4g04930, assumed to encode a sphingolipid $\Delta 4$ -desaturase, and also to investigate the role of $\Delta 4$ -unsaturated LCBs in Arabidopsis growth and development. We have provided, to our knowledge, the first functional characterization of a higher plant sphingolipid $\Delta 4$ -desaturase via complementation of a *P. pastoris* mutant lacking this activity. Arabidopsis insertion mutants disrupted in At4g04930 lacked any detectable transcripts for this gene and also did not contain any $\Delta 4$ -unsaturated sphingolipids such as sphingosine or S-1-P. Our data, however, indicated a role for $\Delta 4$ -unsaturated LCBs in the channeling of ceramides for the synthesis of GlcCers in certain tissues, although these insertion mutants displayed normal growth and development and did not indicate any perturbation to drought tolerance, transpiration rate, or pollen viability. Based on all of these data, and bearing in mind that At4g04930 transcripts are undetectable in most Arabidopsis tissues, we consider it unlikely that sphingosine and S-1-P play a significant role in the life cycle of Arabidopsis. We believe that our approach, in which the in vivo capacity to synthesize sphingosine (and hence S-1-P) is abolished by insertional gene inactivation and biochemically confirmed by a high-resolution HPLC-electrospray ionization-MS/MS analysis of Arabidopsis sphingolipids, provides definitive evidence that $\Delta 4$ -unsaturated sphingolipids are nonessential in this plant species. In addition, in view of the absence of sphingosine and S-1-P in virtually all Arabidopsis tissues, we would suggest that activities recently described as “sphingosine kinase” (Worrall et al., 2008) should be renamed “LCB kinase.”

However, it should also be emphasized that we believe that it is very likely that other LCBs and LCB-1-Ps play important roles in plant-specific processes. Such data are starting to emerge regarding the role of phytosphingosine (Coursol et al., 2005; Shi et al., 2007; Chen et al., 2008) and also $\Delta 8$ -unsaturated LCBs (Ryan et al., 2007). The significance of sphingolipids and their phosphorylated metabolites is still an evolving story in many eukaryotic systems, and it is hoped that studies such as this can contribute to the development of more precise models for their form and function in different organisms. In addition, our observation of the unexpected role of $\Delta 4$ -unsaturated *N*-acylated LCBs in GlcCer synthesis highlights our still only partial understanding of sphingolipid metabolism in plants and yeast.

MATERIALS AND METHODS

Construction of a Phylogenetic Tree

The amino acid sequences of sphingolipid $\Delta 4$ -desaturase genes from Arabidopsis (*Arabidopsis thaliana*) were aligned using the EMBOSS multiple alignment program EMMA. The phylogenetic tree was created using the PHYLIP software package. To test the reliability of the final tree, 100 bootstrap samples were made of the alignment and distance matrices created for each bootstrap sample using PROTDIST. The distance matrices were converted into

trees using the neighbor-joining method, and a consensus tree was created with numbers indicating how many times each branch was observed in the bootstrap sets.

Generation of a Sphingolipid $\Delta 4$ -Desaturase Deletion Mutant of *Pichia pastoris*

Generation of the knockout cassette for the deletion of the $\Delta 4$ -desaturase was performed by restriction enzyme-based cloning. The ORF including 500 bp of flanking upstream and downstream sequences was amplified from genomic DNA with *PfuTurbo* DNA polymerase (Stratagene). The primers used were Delta4-F-*EcoRI* and Delta4-R-*HindIII* (Supplemental Table S3). The primers included adapter sequences (underlined in Supplemental Table S3) containing the indicated restriction sites. The PCR products were purified by gel electrophoresis and ligated into the multiple cloning site of the Litmus38i vector (New England Biolabs) using the restriction sites *EcoRI* and *HindIII*. A part of the ORF was subsequently deleted by digestion with *XbaI* and *XmnI*. The zeocin resistance cassette from the GAPZ-B vector (Invitrogen) was amplified using the primers Zeo-F-*XbaI* and Zeo-R-*XmnI* and ligated into the interrupted ORF using the indicated restriction sites (underlined in Supplemental Table S3).

Transformation of *P. pastoris* Cells by Electroporation

The vector containing the knockout cassette was linearized and used for the transformation of electrocompetent GS115 (his4) cells (Invitrogen). Transformants were selected on yeast potato dextrose media plates containing 100 mg L⁻¹ zeocin. Correct integration of the knockout cassette was tested by PCR with the primer pairs Delta4-Test-F/Zeo-int-R and Zeo-int-F/Delta4-Test-R (data not shown).

Cloning of the Arabidopsis $\Delta 4$ -Desaturase

The complete coding sequence of the putative $\Delta 4$ -desaturase from Arabidopsis was amplified with *Pwo* polymerase (Peqlab) using a standard PCR program and the specific primers d4At-F and d4At-R on cDNA (generated from RNA isolated from floral tissue). PCR products were ligated in the *P. pastoris* expression vector pPIC3.5 (Invitrogen) in the *EcoRI* and *NotI* restriction sites of the multiple cloning site. The cloned complete coding sequence was checked by sequencing.

Transformation of *P. pastoris*

Chemical competent cells from *P. pastoris* strain GS115- $\Delta 4$ -desaturase-KO were transformed with the *ScaI* and *NdeI* linearized construct described above. The strains GS115 (= wild-type) and GS115- $\Delta 4$ -desaturase-KO were also transformed with linearized empty vector pPIC3.5. Single colonies were isolated and used for lipid analysis.

Liquid Culture

A preculture was grown in liquid minimal glycerol medium (1.34% yeast nitrogen base without amino acids, 1% glycerol) for 24 h at 30°C with shaking (180 rpm). A total of 200 mL of minimal glycerol medium was inoculated 1:100 from the preculture and incubated again for 24 h at 30°C. Gene expression was induced by the addition of methanol to a final concentration of 0.5% and further incubation for 24 h. Cells were harvested by centrifugation.

Isolation of GlcCer

Approximately 5 g fresh weight of *P. pastoris* cells was suspended in 5 mL of water and boiled in a water bath for 15 min. The cells were sedimented by centrifugation, and the lipids were extracted by shaking in 10 mL of chloroform:methanol (1:1) overnight at 8°C, followed by 9 mL of chloroform:methanol (2:1) for at least 4 h at 8°C. The lipid extract was washed by phase partitioning with chloroform:methanol:0.45% (w/v) NaCl (8:4:3), and the solvents subsequently evaporated. The lipids were redissolved in chloroform, and GlcCer was purified by preparative thin-layer chromatography on silica gel 60 plates (Merck) developed in chloroform:methanol (85:15, v/v).

Analysis of the Sphingolipid LCB Composition

The LCB composition of *P. pastoris* glucosylceramides was analyzed as described by Ternes et al. (2006). Similar methods were used for the analyses of Arabidopsis and *Commelina communis* sphingolipid LCBs, according to the methods of Borner et al. (2005) and Tonon et al. (2005). Briefly, samples (yeast pellet or plant tissues) were subjected to strong alkaline hydrolysis in 10% barium hydroxide in dioxane. The resulting free LCBs were converted to dinitrophenyl derivatives and analyzed by reverse-phase HPLC with detection by absorbance at 350 nm. Identification was via comigration with known standards (Avanti Polar Lipids) and atmospheric pressure chemical ionization-MS (Tonon et al., 2005).

Sphingolipidomic Analysis

Sphingolipid analysis was carried out exactly as described by Markham and Jaworski (2007). Analyses were carried out on pooled material (leaves and flowers) from a number of individual plants grown in the same growth cabinet. A minimum of three technical replicates were run from each pooled sample.

Growth of Arabidopsis and Identification of Arabidopsis $\Delta 4$ -Desaturase Insertion Mutants

Arabidopsis plants were grown and maintained as described previously (Footitt et al., 2007) in soil, with plants grown to maturity in controlled-environment rooms (16 h of light at 23°C and 70% relative humidity/8 h of dark at 18°C and 80% relative humidity). During the light phase, the incident photosynthetically active radiation was 150 to 175 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the soil level. The position of plant trays was rotated to minimize light effects. Arabidopsis seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (Salk_107761.42.15.x, Col-0 ecotype) and the RIKEN BioResource Centre (insertion mutant RIKEN 15-1202-1, No-0 ecotype). PCR was used to identify and isolate homozygous mutants from segregating populations using a gene-specific primer in conjunction with a primer to the insertion module. Genomic DNA was extracted from leaf material, and PCR was performed at 94°C for 15 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and then 72°C for 7 min using HotStart Taq Pol master mix (Qiagen). The following primers were used for the identification of RIKEN 15-1202-1 homozygous individuals: 930F (5'-TCTCTCTCGTTTGACTTCC-3'), 930R (5'-TGCTAAGAAGAGATTGTGGTT-3'), and Ds3-2a (5'-CCGGATCGTATC-GGTTTTCCG-3'). The following primer combination was used for the identification of Salk_107761.42.15.x homozygous individuals: SalkLP (5'-AGGTTGTCGAAGAAACAACG-3'), SalkRP (5'-TGATGAACCTCC-CAGTAGCCAG-3'), and LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3').

Confirmation of the absence of $\Delta 4$ -desaturase transcript was obtained by real-time PCR. RNA was extracted from three independent biological replicates comprising 100 mg of inflorescence tissue (Supplemental Fig. S1) using an RNeasy plant RNA isolation kit with on-column DNase treatment (Qiagen) followed by RNA cleanup using the RNeasy plant RNA isolation kit. One microgram of total RNA was treated with the Turbo DNA-free kit (Ambion) and used as a template to synthesize cDNA using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen). PCR was performed on the ABI 7500 Real Time PCR System (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) according to the manufacturer's specification, with the cDNA equivalent of 17.5 ng of RNA in a 25- μL reaction volume. Reactions were performed in duplicate, and the absence of genomic DNA and primer dimers was confirmed by analysis of RT-minus and water control samples and by examination of dissociation curves. The primers for the Arabidopsis $\Delta 4$ -desaturase, T1J1F2.2 (5'-TGGAGATCTTTCGCGTATCTAATC-3') and T1J1R2.2 (5'-CATACCGCCTCAACAATG-3'), were designed using Primer Express version 2.0 (Applied Biosystems). Primers for the reference gene At4g34270 have been described (Czechowski et al., 2005). Calculation of normalized expression values was done after the method of Livak and Schmittgen (2001).

Drought Stress Experiments

Leaves were detached and weighed (one from a representative plant on each day; many plants were used in rotation so as not to defoliate them). Then the leaf was dried at 80°C and reweighed. Rosette plants were detached from the soil surface, placed under 30% relative humidity at 19°C, and weighed at

designated time points. Percentage of fresh weight was calculated based on the initial weight of the plants as described by Chini et al. (2004).

Pollen Germination in Vitro

Pollen germination assays were carried out as described by Footitt et al. (2007). For each line, pollen from two flowers was cultured in suspended drops in control medium [16% (w/v) polyethylene glycol-3550, 2% (w/v) Suc, 1 mM CaCl₂, 1 mM Ca(NO₃)₂, 1 mM MgSO₄, and 0.015% (w/v) boric acid, pH 6.5]. Pollen was incubated in a humid chamber for 16 h in hanging drops on microscope slides. Germination was scored by microscopic examination. Tubes of germinated pollen grains were visualized with a Carl Zeiss Axiovert 135 inverted microscope.

Stomatal Bioassays

Stomatal assays were performed by floating leaves for 2.5 h under continuous illumination (60–100 μE m⁻² s⁻¹) in MES/KCl buffer (5 mM KCl, 10 mM MES, and 50 μM CaCl₂, pH 6.15). Following the opening of stomata, leaves were treated with 10 μM ABA for another 2 h. The leaves were subsequently homogenized individually in a Waring blender for 30 s, and the epidermal fragments were collected on a 100-μm nylon mesh (SpectraMesh; Merck). Stomatal apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (QWin software; Leica). Experiments were repeated three times and by counting at least 20 stomata per leaf per treatment; this analysis was performed double blind, meaning that neither the experimenter measuring the apertures nor the researcher who provided the material was aware of the identity (i.e. genotype) of the four samples undergoing the ABA treatment.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Validation of insertion mutants by Q-PCR.

Supplemental Figure S2. LCB profile of plant sphingolipids showing resolution of Δ8 and Δ4 regioisomers and the absence of sphingosine in Arabidopsis.

Supplemental Figure S3. LCB profiles of the wild type and insertion mutants.

Supplemental Figure S4. Phenotypic appearance of wild-type and mutant Arabidopsis.

Supplemental Table S1. Microarray-derived tissue-specific expression profile for At4g04930.

Supplemental Table S2. LCB analysis of wild-type and mutant Arabidopsis.

Supplemental Table S3. List of PCR primers used in construction of the *P. pastoris* expression system.

Supplemental Data Set 1. Sphingolipidomic analysis of sphingolipids from the wild type and insertion mutants (leaves and floral tissue).

Supplemental Data Set 2. Sphingolipidomic analysis of free LCBs and LCP-1-Ps from the wild type and insertion mutants (leaves and floral tissue).

ACKNOWLEDGMENTS

This paper is dedicated to the memory of our colleague Petra Sperling. J.N. thanks Teresa Dunn for helpful discussions. We thank Allison van de Meene and Mitch Le Bloa of the Rothamsted Research Centre for Bioimaging for advice with microscopy. We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants and RIKEN Genomic Sciences Center for providing the *Ds* insertion mutant. We also thank Enid MacRobbie for the gift of the *Commelina* seeds.

Received September 6, 2008; accepted October 17, 2008; published October 31, 2008.

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