A Higher Plant Δ8 Sphingolipid Desaturase with a Preference for (*Z*)-Isomer Formation Confers Aluminum Tolerance to Yeast and Plants[C][OA]

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Three plant cDNA libraries were expressed in yeast (*Saccharomyces cerevisiae*) and screened on agar plates containing toxic concentrations of aluminum. Nine cDNAs were isolated that enhanced the aluminum tolerance of yeast. These cDNAs were constitutively expressed in Arabidopsis (*Arabidopsis thaliana*) and one cDNA from the roots of *Stylosanthes hamata*, designated *S851*, conferred greater aluminum tolerance to the transgenic seedlings. The protein predicted to be encoded by *S851* showed an equally high similarity to Δ6 fatty acyl lipid desaturases and Δ8 sphingolipid desaturases. We expressed other known Δ6 desaturase and Δ8 desaturase genes in yeast and showed that a Δ6 fatty acyl desaturase from *Echium plantagineum* did not confer aluminum tolerance, whereas a Δ8 sphingobase desaturase from Arabidopsis did confer aluminum tolerance. Analysis of the fatty acids and sphingobases of the transgenic yeast and plant cells demonstrated that *S851* encodes a Δ8 sphingobase desaturase, which leads to the accumulation of 8(*Z*/*E*)-C_{18}-phytosphingenine and 8(*Z*/*E*)-C_{20}-phytopshingenine in yeast and to the accumulation of 8(*Z*/*E*)-C_{18}-phytosphingenine in the leaves and roots of Arabidopsis plants. The newly formed 8(*Z*/*E*)-C_{18}-phytosphingenine in transgenic yeast accounted for 3 mol% of the total sphingobases with a 8(*Z*):8(*E*)-isomer ratio of approximately 4:1. The accumulation of 8(*Z*)-C_{18}-phytosphingenine in transgenic Arabidopsis shifted the ratio of the 8(*Z*):8(*E*) isomers from 1:4 in wild-type plants to 1:1 in transgenic plants. These results indicate that *S851* encodes the first Δ8 sphingolipid desaturase to be identified in higher plants with a preference for the 8(*Z*)-isomer. They further demonstrate that changes in the sphingolipid composition of cell membranes can protect plants from aluminum stress.

Trivalent cations are toxic to most plant cells. The increased prevalence of soluble aluminum (Al^{3+}) cations in acid soils is a major limitation to plant production around the world. Aluminum disrupts a range of cellular processes, including nutrient acquisition, cell wall loosening, nuclear division, cytoskeleton stability, cytoplasmic calcium homeostasis, hormone transport, and signal transduction (Taylor, 1988; Kochian, 1995; Matsumoto, 2000). Many of these symptoms occur rapidly and some workers have concluded that aluminum toxicity is initiated by interactions occurring in the extracellular compartment (Horst, 1995) and cell membranes. Aluminum accumulates rapidly in the highly charged cell wall and near the fixed charges and polar groups on the plasma membrane surface, which can displace calcium from critical sites in the apoplasm, alter physical properties of the plasma membrane, change membrane lipid composition, block ion channels, and disrupt signal transduction processes by interfering with phospholipase C metabolism (Haug and Caldwell, 1985; Rengel, 1992; Shi and Haug, 1992; Kinraide et al., 1994; Ryan et al., 1994; Jones and Kochian, 1995; Piña-Chable and Hernández-Sotomayor, 2001; Mantinez-Estévez et al., 2003; Stival da Silva et al., 2006). Whereas it remains unclear which, if any, of these reactions are primary causes for aluminum toxicity in plants, it is plausible that aluminum-dependent changes in cell membrane structure and function contribute to the overall stress encountered in acid soils. Consistent with this idea are reports demonstrating that aluminum can reduce membrane fluidity of the Archaebacterium *Thermoplasma acidophilum* by binding to the polar head groups of phospholipids (Deleers et al., 1986) and alter the lipid composition of plant roots (Lindberg and Griffiths, 1993; Zhang et al., 1997; Peixoto et al., 2001; Stival da Silva et al., 2006). Previous reports have also shown that genetically engineered changes to the lipid composition of plant and yeast (*Saccharomyces cerevisiae*) membranes can affect their susceptibility to chilling, photoinhibition, drought, fungal toxins, and ion toxicity (Avery et al., 1996; Nishida and Murata, 1996; Delhaize et al., 1999; Thevissen et al., 2000; Los and Murata, 2004; Zhang et al., 2005; Stival da Silva et al., 2006).

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In contrast to the complexity of aluminum toxicity, the genetics of aluminum resistance can be relatively simple. For instance, the mechanism for aluminum resistance in some cereal species, such as wheat (*Triticum aestivum*; Raman et al., 2005) and barley (*Hordeum vulgare*; Minella and Sorrells, 1997) is controlled by single major genes. The aluminum resistance gene from wheat, *TaALMT1*, encodes a membrane protein that facilitates the aluminum-dependent release of malate anions from the root apices. These organic anions protect the root cells by chelating the aluminum cations in the apoplasm (Delhaize et al., 1993; Sasaki et al., 2004).

The aim of this study was to identify novel plant genes that confer aluminum tolerance using a strategy that does not make any assumptions about function. Using a yeast expression system, we isolated nine plant cDNAs that conferred increased tolerance to aluminum stress. One of these cDNAs from *Styolsanthes hamata* also enhanced the aluminum tolerance of Arabidopsis (*Arabidopsis thaliana*). We established that this cDNA encodes a Δ8 sphingolipid desaturase that preferentially produces the 8(*Z*)-isomer of phytosphingenine.

RESULTS

Plant cDNAs Conferring Aluminum Tolerance to Yeast

Bakers' yeast was transformed with cDNA libraries prepared from the nodules of soybean (*Glycine max*), roots of *S. hamata*, and cluster roots of white lupin (*Lupinus albus*). These libraries were chosen because they were either prepared from a species well adapted to acid soils (*S. hamata*) or from tissues predicted to contain proteins that facilitate organic anion efflux from root cells (soybean nodules; cluster roots of white lupin). Transformed yeast cells were screened on agar plates with aluminum concentrations sufficient to inhibit the growth of cells containing an empty vector. Plasmids isolated from aluminum-tolerant colonies were amplified in *Escherichia coli* and retransformed into wild-type yeast to confirm that the aluminum-tolerance phenotype was caused by expression of the cDNA inserts and was not due to spontaneous mutations. Nine different cDNAs conferred increased aluminum tolerance to yeast cells. Figure 1, A to C, illustrates the increase in aluminum tolerance provided by one of nine cDNAs. The cDNAs were sequenced and their likely function determined by comparing their putative translation products with the nonredundant protein database using the BLASTx algorithm (http://www.ncbi.nlm.nih.gov/BLAST; Table I).

A cDNA Encoding a Lipid Desaturase Confers Aluminum Tolerance to Arabidopsis

Nine cDNAs were expressed in Arabidopsis under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Aluminum tolerance of several T_1 lines from each construct was compared with wild-type plants by estimating relative root growth on agar plates (data not shown). One cDNA from the *S. hamata* library, designated *S851*, enhanced the aluminum tolerance of all five independently transformed T_1 lines tested (data not shown) and this cDNA was investigated further. It is possible that some of the other cDNAs are able to increase the aluminum tolerance of T_2 material, but this was not tested in this study. We generated T_2 populations from the T_1 plants expressing *S851* and selected two independent lines, *At-S851-H4* and *At-S851-H6*, which were homozygous for antibiotic resistance. Expression of the *S851* transgene in these two lines was confirmed by northern-blot analysis (data not shown). The aluminum tolerance of the *At-S851-H4* and *At-S851-H6* lines was estimated on agar in plates and pots. Relative root growth was consistently 20% to 100% greater in the homozygous transgenic lines compared to wild-type plants over a range of aluminum concentrations (Fig. 2). The stimulation of root growth observed in some treatments is likely to be caused by the often-reported amelioration of proton stress by low concentrations of aluminum (Kinraide, 1993).

In addition to aluminum, we tested whether yeast expressing *S851* cDNA (designated here as *Sc_pYES3-S851*) was more tolerant of other toxic cations than yeast cells transformed with the empty vector (designated here as *Sc_pYES3*). *Sc_pYES3-S851* conferred enhanced tolerance to gadolinium (Gd^{3+}), but no

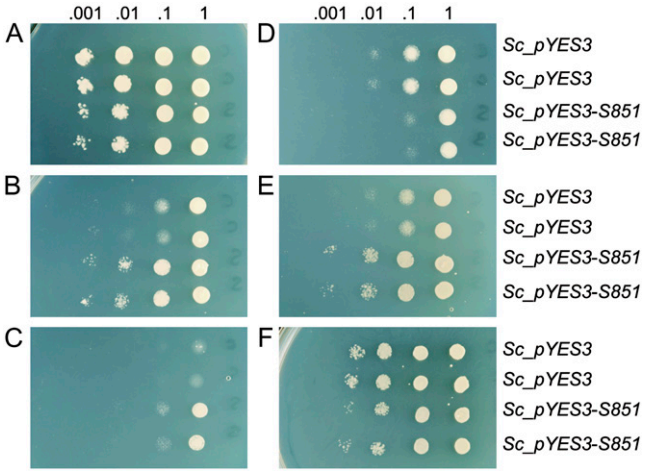

Figure 1. The *S851* cDNA from *S. hamata* confers aluminum and gadolinium tolerance to transgenic yeast cells. Yeast strains transformed with an empty vector (*Sc_pYES3*) or transformed with a vector containing the *S851* cDNA (*Sc_pYES3-S851*) were grown in SMM − ura medium. Cultures were diluted to an OD_{600} = 1.0 in sterile water before a series of 10-fold dilutions was prepared for each of two independent cultures of each strain. Aliquots (10 μL) of each dilution were added to agar plates containing SMM − ura with a range of toxic cations. A, Control agar. B, 200 μM $AlCl_3$. C, 400 μM $AlCl_3$. D, 3.6 mM $MnCl_2$. E, 700 μM $GdCl_3$. F, 600 μM $LaCl_3$. Results are typical of those obtained from at least two independent experiments. [See online article for color version of this figure.]

Table I. *Putative function of the nine plant cDNAs isolated from their ability to confer aluminum tolerance to yeast cells*
cDNAs were compared to the nonredundant database using the BLASTx algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

Source of the cDNA Libraries	Clone Name	Length	Putative Function	% Identity (e Value)	GenBank Accession No.
		bp			
S. hamata (roots)	S851	1,764	Lipid desaturase	70% (0)	EF640314
	S1157	1,616	Similar to Leu-rich repeat and extensin-like proteins	49% ($2e^{-79}$)	EF640315
	S853	1,203	Heat-shock protein	95% (e^{-147})	EF640316
	S453	493	No similarity		EF640317
White lupin (cluster roots)	La18	610	Similar to dynein light chain	67% ($6e^{-39}$)	EF640318
	La97	1,642	GDP dissociation inhibitor	92% (0)	EF640319
	La31	1,473	Chalcone synthase	97% (0)	EF640320
	La93	789	Similar to unknown protein	68% ($4e^{-39}$)	EF640321
Soybean (nodule)	N552	1,661	Acyl-CoA oxidase	79% (0)	EF640322

consistent changes were observed for lanthanum (La^{3+}) or manganese (Mn^{2+}; Fig. 1, D–F). The homozygous Arabidopsis lines, *At-S851-H4* and *At-S851-H6*, were also tested on agar plates containing a range of gadolinium concentrations, but neither showed greater tolerance than wild-type plants (data not shown).

Function of the Protein Encoded by *S851*

The predicted translation product of *S851* is equally similar (approximately 70% amino acid identity) to two distinct lipid-modifying enzymes: a Δ6 fatty acyl lipid desaturase and a Δ8 sphingolipid desaturase. Δ6 desaturase enzymes add a double bond to linoleic acid ($18:2^{\Delta 9,12}$) and α-linolenic acid ($18:3^{\Delta 9,12,15}$) to produce γ-linolenic acid ($18:3^{\Delta 6,9,12}$) and stearidonic acid ($18:4^{\Delta 6,9,12,15}$), respectively. Δ8 sphingolipid desaturases create double bonds in long-chain bases (also called sphingobases), such as sphinganine (d18:0), phytosphinganine (t18:0), or 4-sphingenine ($18:1^{\Delta 4}$) to produce *E*- and *Z*-isomers of 8-sphingenine ($d18:1^{\Delta 8}$), 8-phytosphingenine ($t18:1^{\Delta 8}$), or 4,8-sphingadienine ($d18:2^{\Delta 4,8}$), respectively.

A phylogenetic comparison between the predicted S851 protein and other Δ6 fatty acyl lipid desaturase and Δ8 sphingobase desaturase proteins of known function revealed that S851 grouped with most of the Δ8 sphingolipid desaturase proteins (Fig. 3). However, Δ6 fatty acyl lipid desaturases from *Echium plantagineum* and *Borago officinalis* also clustered with the Δ8 desaturase enzymes, making it difficult to predict S851 function from this cladogram. To establish the function of the S851 protein, we tested the ability of other Δ6 and Δ8 desaturase enzymes to confer aluminum tolerance to yeast. In addition, the fatty acid and long-chain base compositions were measured in transgenic yeast and Arabidopsis expressing S851.

Initial analyses of the major fatty acid compositions in transgenic yeast and Arabidopsis showed no changes associated with the expression of the *S851* cDNA (Table II). A cDNA encoding a Δ6 fatty acyl desaturase isolated from *E. plantagineum* (Zhou et al., 2006) was expressed in yeast (*Sc_pYES3-Δ6*) and tested on agar plates containing toxic levels of aluminum. The *Sc_pYES3-Δ6* and *Sc_pYES3* cells were equally sensitive to aluminum and both grew significantly slower than the *Sc_pYES3-S851* cells (Fig. 4A). To confirm that the Δ6 fatty acyl desaturase was functional in yeast, we analyzed the lipid content of these cells grown in the presence and absence of linoleic acid, a substrate for Δ6 desaturase enzymes. When linoleic acid was excluded from the medium (control), the major fatty acid composition of all strains was similar (Fig. 5). When linoleic acid was included in the medium, it was activated to acyl-CoA and incorporated into membrane lipids where it accounted for over 40% of the total fatty acid content in all strains. The palmitoleic and oleic acid fractions showed a concomitant decrease of approximately 60%. Inclusion of linoleic acid was also associated with γ-linolenic acid accumulation in the *Sc_pYES3-Δ6* strain only where it accounted for 1% of the total fatty acid content (Fig. 5). No γ-linolenic acid was detected in the *Sc_pYES3* or *Sc_pYES3-S851* strains. This result confirms that the Δ6 desaturase enzyme from *E. plantagineum* was functional when substrate for the enzyme was available and indicates that *S851* cDNA does not encode a Δ6 fatty acyl desaturase.

Yeast cells were then transformed with a known Δ8 sphingolipid desaturase from Arabidopsis (GenBank accession no. AF001394; strain *Sc_pYES3-Δ8*) and its aluminum tolerance compared with control cells. The *Sc_pYES3-Δ8* strain was more tolerant to aluminum stress than the *Sc_pYES3* control (Fig. 4B). The finding that the aluminum tolerance of yeast is increased by expression of a known Δ8 desaturase is consistent with the hypothesis that *S851* encodes a Δ8 sphingolipid desaturase, but not a Δ6 fatty acyl desaturase.

Sphingobase Analyses of Yeast and Plant Cells Expressing *S851*

Analysis of the sphingobases released from the *Sc_pYES3-S851* yeast strain identified Δ8-unsaturated long-chain bases C_{18}- and C_{20}-phytosphingenine, which were not present in the control strain (Fig. 6, A and B).

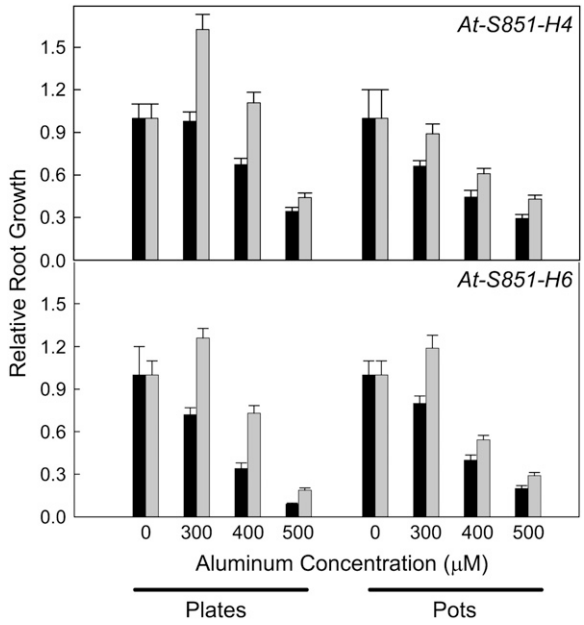

Figure 2. The *S851* cDNA from *S. hamata* confers aluminum tolerance in transgenic Arabidopsis plants. *S851* was constitutively expressed in Arabidopsis under the control of the CaMV 35S promoter. Two homozygous lines, *At-S851-H4* and *At-S851-H6* (gray bars), and wild-type plants (black bars) were grown on sterile nutrient agar supplemented with 0, 300, 400, or 500 μM aluminum chloride (pH 4.8–4.9). Relative root growth (RRG), defined as (root length with aluminum)/(root length without aluminum), was estimated after 14 d by (1) growing seedlings on plates supported in an almost vertical orientation (plates) or (2) growing seedlings in sterile pots that forced the roots to grow directly into the agar (pots). Data show the mean and SES of the RRG (n = 20–30). To account for the accumulation of errors associated with deriving RRG, the SES were calculated as follows: $SE_{RRG} = RRG\,[(SE_x/x)^{2+}(SE_y/y)^2]^{1/2}$, where x and y represent the mean root length in the control treatment and the mean root length in the aluminum treatment. The root growth in the zero aluminum (control) treatments for the wild-type and *At-S851-H4* lines was (in mm): 16.0 ± 0.1 and 19.8 ± 0.1, respectively, for the plates and 16.6 ± 0.2 and 14.7 ± 0.2 for the pots. The root growth in the control treatments for the wild-type and *At-S851-H6* lines was (in mm): 15.9 ± 0.2 and 12.8 ± 0.1, respectively, for the plates and 15.5 ± 0.1 and 14.5 ± 0.1 for the pots.

These novel 8(*Z*)- and 8(*E*)-C_{18}-phytosphingenines accounted for 3 mol% of the total sphingobases in *Sc_pYES3-S851* with an 8(*Z*):8(*E*) ratio of 4:1. Although the identities of most of the sphingobases in Figure 6 were confirmed by HPLC/mass spectrometry (MS) with electrospray ionization, the 8(*Z*):8(*E*) ratio of C_{20}-phytopshingenines could not be estimated accurately because 8(*E*)-C_{20}-phytosphingenine coeluted with the C_{19}-phytosphinganine present in yeast.

Changes in the sphingobase composition of Arabidopsis plants expressing *S851* were also consistent with Δ8 sphingolipid desaturase activity. The main sphingobases in wild-type Arabidopsis plants, 8(*Z*)-C_{18}-phytosphingenine, 8(*E*)-C_{18}-phytosphingenine, and C_{18}-phytosphinganine, comprised 15, 58, and 27 mol%, respectively, of the total with an 8(*Z*):8(*E*) ratio of approximately 0.3 (Fig. 6C; Table III). This profile is generally consistent with previous analyses of the Arabidopsis ecotypes Columbia C24 (Sperling et al., 2005) and Wassilewskija (Bonaventure et al., 2003). In the *At-S851-H4* and *At-S851-H6* lines, these same sphingobases represented 43, 42, and 15 mol%, respectively (Fig. 6, D and E; Table III). Separate analyses of the sphingobases in leaves and roots resemble the data obtained with whole plants (Table III). The increase in the 8(*Z*)-isomer of C_{18}-phytosphingenine shifted the 8(*Z*):8(*E*) ratio closer to 1.0. These data confirm that *S851* cDNA from *S. hamata* encodes a sphingolipid desaturase, which introduces a Δ8 double bond into phytosphinganine in both heterologous systems tested. The enzyme is stereo-unselective, but exhibits a strong preference for *Z*-isomer formation.

DISCUSSION

Nine plant cDNAs from three different plant libraries were isolated for their ability to confer aluminum tolerance to yeast cells. One cDNA (*S851*) that originated from the acid soil-tolerant forage species, *S. hamata*, also increased the aluminum tolerance of Arabidopsis

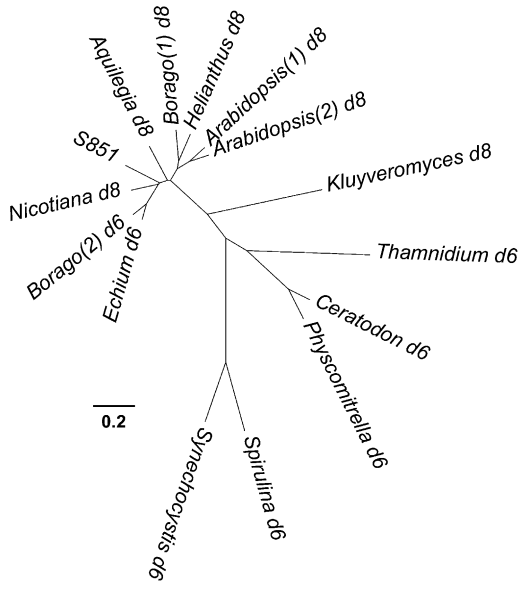

Figure 3. Phylogenetic tree of Δ6 fatty acyl lipid desaturase and Δ8 sphingobase desaturase proteins. Phylogenetic and molecular evolutionary analyses of the proteins were conducted using *MEGA*, version 3.1 (Kumar et al., 2004). Default settings for the protein alignment and neighbor-joining phylogenetic calculations were used. All proteins have had their function verified experimentally as being a Δ6 fatty acyl desaturase enzyme (d6) or a Δ8 sphingobase desaturase enzyme (d8). Full species names and GenBank accession numbers are as follows: *Aquilegia vulgaris* (AF406816) Arabidopsis (gene 1; AF001394); Arabidopsis (gene 2; BX820915); *B. officinalis* (gene 1; AF133728); *B. officinalis* (gene 2; U79010); *Ceratodon purpureus* (AJ250735); *E. plantagineum* (AY952780); *Helianthus annuus* (X87143); *Kluyveromyces lactis* (AB085690); *N. tabacum* (ABO31111); *Physcomitrella patens* (AJ222980); S851 (EF640314); *Spirulina platensis* (X87094); *Synechocystis* sp. (L11421); and *Thamnidium elegans* (AY941161). Scale bar represents the number of substitutions per site.

Table II. *Analysis of the major fatty acids in the total lipids extracted from yeast and Arabidopsis expressing S851 cDNA from S. hamata*

Fatty acids were analyzed as their methyl ester derivatives by gas chromatography. Results show the contribution of each major fatty acid as mol% of the total fatty acid extract. Data represent the mean and SE where $n = 3$ for yeast, and $n = 7$ to 9 for Arabidopsis. Dashes indicate that no peaks were detected.

Yeast and Plant Lines	Major Fatty Acids							
	Myristic	Palmitic	Palmitoleic	16:3	Stearic	Oleic	Linoleic	α-Linolenic
					mol%			
Yeast								
pYES3	1.9 ± 0.4	22.4 ± 0.5	36.9 ± 1.7	–	8.1 ± 0.5	27.0 ± 0.9	–	–
pYES3_S851	1.8 ± 0.2	25.6 ± 4.7	34.8 ± 3.5	–	7.7 ± 0.4	26.5 ± 2.5	–	–
Arabidopsis								
Wild type	0.5 ± 0.1	20.5 ± 4.5	0.4 ± 0.1	15.8 ± 0.2	1.8 ± 0.5	4.4 ± 2.0	16.0 ± 3.7	40.8 ± 7.3
At-S851-H4	0.8 ± 0.2	20.3 ± 4.0	0.4 ± 0.1	10.1 ± 6.6	2.6 ± 0.9	8.3 ± 4.5	13.3 ± 1.2	40.3 ± 6.9
At-S851-H6	0.6 ± 0.2	18.2 ± 2.5	1.3 ± 0.7	10.3 ± 5.8	2.9 ± 0.7	8.3 ± 3.9	13.4 ± 1.3	41.6 ± 5.4

seedlings. A previous attempt to isolate aluminum tolerance genes by screening a cDNA expression library in yeast identified several genes that were effective in yeast (Delhaize et al., 1999), but these did not prove to be effective when overexpressed in tobacco (*Nicotiana tabacum*) plants (E. Delhaize, unpublished data). Yeast is a powerful technique for isolating plant genes that confer aluminum tolerance regardless of their role in the plant from which they originate. However, it is clear that the transgenes will not always confer the same phenotypes in the single-celled system as they do in intact plants. For instance, many genes identified to be important for aluminum tolerance in yeast encode components of signal transduction pathways and cell wall metabolism (Kakimoto et al., 2005). Therefore, the phenotype conferred by a transgene may depend on a signal pathway or cell wall structure being conserved between the species. Despite this potential problem, there are examples of plant cDNAs conferring aluminum tolerance to both yeast and plants. For instance, Ezaki et al. (2000) showed that genes encoding a GDP dissociation inhibitor from Arabidopsis and a blue copper protein from tobacco enhanced the aluminum tolerance of transgenic Arabidopsis and yeast. Interestingly, one of the other cDNAs isolated here from white lupin (La97), which increased the aluminum tolerance of yeast, but not Arabidopsis, also encodes a GDP dissociation inhibitor. The reason La97 did not increase the aluminum tolerance of Arabidopsis might be due to subtle differences in functions of the two proteins or because the increase in tolerance was insufficient to be clearly identified in the segregating T_1 lines.

The putative protein encoded by *S851* showed an equally strong similarity to a Δ6 fatty acyl lipid desaturase and a Δ8 sphingolipid desaturase. We demonstrated that the S851 protein did not have Δ6 fatty acyl lipid desaturase activity because the *Sc_pYES3-S851* yeast strain failed to accumulate γ-linolenic acid under any conditions tested. Analyses of the sphingobases released from transgenic yeast and Arabidopsis indicated that *S851* encodes a Δ8 (*Z*/*E*)-sphingolipid desaturase. We showed that another Δ8 sphingolipid desaturase enzyme from Arabidopsis also confers aluminum tolerance to yeast, which is consistent with the finding of Stival da Silva et al. (2006). By contrast, expression of a known Δ6 fatty acyl lipid desaturase in yeast provided no protection from aluminum stress. Our results indicate that the sphingolipid composition can protect yeast and Arabidopsis from aluminum toxicity, but whether this occurs by altering membrane structure or by specific biochemical interactions is not clear. The *Sc_pYES3-S851* yeast strain also showed greater tolerance to toxic concentrations of another trivalent cation, gadolinium, which indicates that Δ8 unsaturated sphingolipids have the potential to provide tolerance to other ionic stresses.

Sphingolipids do not possess the ester-glycerol linkages common in most membrane lipids, but are composed of a long-chain amino alcohol base that forms an amide linkage to a fatty acid. This basic ceramide structure can be further modified by glycosylation, hydroxylation, and desaturation. Δ8 unsaturated sphingobases can exist as the *E* (trans)- or *Z* (cis)-isomer and all Δ8 sphingolipid desaturases isolated so far from higher plants preferentially form the *E* isomer (see Sperling and Heinz, 2003). The lipid desaturase characterized here is notable for being the first bifunctional Δ8 sphingolipid desaturase enzyme from higher plants to preferentially synthesize the *8*(*Z*)-isomer of phytosphingenine.

A connection between Δ8 unsaturated sphingobases and aluminum tolerance in plants was previously investigated by Stival da Silva et al. (2006). They showed that the heterologous expression of a Δ8 (*E*/*Z*)-sphingolipid desaturase from Arabidopsis in hybrid maize (*Zea mays*) led to an 8-fold increase in the 8(*E*)-phytosphingenine content of a homozygous T_2 line, which changed the 8(*Z*):8(*E*) ratio from 5:1 (wild type) to approximately 1:3 (transgenic plants). However, those transgenic maize plants were scored as being more sensitive to aluminum stress. This contrasts with our findings, which show that expression of a similar desaturase from *S. hamata* increases the aluminum tolerance in Arabidopsis. There are several possible explanations for why one Δ8 sphingolipid

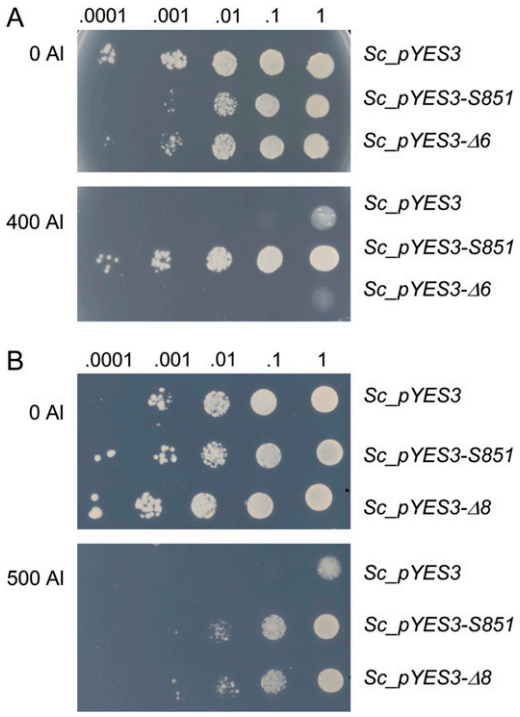

Figure 4. The ability of different plant desaturases to confer aluminum tolerance to transgenic yeast cells. A, Yeast transformed with an empty vector (*Sc_pYES3*), a vector containing the *S851* cDNA (*Sc_pYES3-S851*), or a vector containing a Δ6 fatty acyl lipid desaturase isolated from *E. plantaguneum* (*Sc_pYES3-Δ6*) were grown in SMM − ura medium with or without 400 μM $AlCl_3$. B, Yeast transformed with an empty vector (*Sc_pYES3*), a vector containing the *S851* cDNA (*Sc_pYES3-S851*), or a vector containing a known Δ8 sphingolipid desaturase isolated from Arabidopsis (*Sc_pYES3-Δ8*) were grown in SMM − ura medium with or without 500 μM $AlCl_3$. Serial dilutions of the cultures were prepared as described in Figure 1. Results are typical of those obtained from at least two independent experiments. [See online article for color version of this figure.]

desaturase enzyme confers aluminum tolerance, whereas another does not. For instance, maize has a naturally high content of 8(*Z*)-unsaturated bases and is generally more aluminum tolerant than Arabidopsis. These attributes could influence the magnitude of phenotype generated by expression of a Δ8 sphingolipid desaturase in maize. Furthermore, as noted above, the Δ8 sphingolipid desaturase from Arabidopsis preferentially forms the (*E*)-isomer of 8-phytosphingenine (Sperling et al., 1998; Sperling and Heinz, 2003), whereas the enzyme from *S. hamata* preferentially forms the (*Z*)-isomer. Introduction of a (*Z*)-double bond leads to a kink in the long-chain base, which is not generated by the formation of an (*E*)-double bond. This kink might induce some specific biochemical functions, generate changes to membrane structure, or possibly affect the functioning of the sphingolipid-rich lipid rafts (see below). The finding that both Δ8(*E*/*Z*) sphingolipid desaturase enzymes were able to increase the aluminum tolerance of yeast could be explained by the absence of any unsaturated sphingobases in wild-type yeast. Therefore, even a small accumulation of 8(*Z*)-phytosphingenine from either enzyme might be sufficient to improve its resistance to aluminum stress. Future studies will determine whether the stereochemistry of sphingobases influences their ability to confer aluminum tolerance by expressing the Δ8 sphingolipid desaturase genes from Arabidopsis and *S. hamata* in the same plant species.

Participation of membrane lipids, not just sphingolipids, in the perception and response to environmental signals is well known. For instance, unsaturated acyl lipids can ameliorate the damage caused by chilling stress and photoinhibition at low temperatures (Cossins, 1994; Nishida and Murata, 1996; Murata and Los, 1997) as well as drought stress (Zhang et al., 2005). Membranes become more rigid as temperature decreases and damage to cells can occur as membranes change from a liquid crystalline phase to a gel phase (see Los and Murata, 2004). Membranes with a higher percentage of unsaturated acyl lipids appear to incur less damage at low temperature, in part because fluidity is maintained.

In comparison to other membrane lipids, the functions of sphingolipids are poorly understood despite being a ubiquitous component of eukaryotic cells. Although in excess of 300 structurally different compounds have now been identified, sphingolipids typically constitute <5% of total lipids in yeast and plants. Interest in sphingolipid metabolism has increased as their roles in cell growth, membrane stability, stress response, and apoptosis have been elucidated (Thevissen et al., 2000; Sperling and Heinz, 2003; Worrall et al., 2003; Lynch and Dunn, 2004). More recently, microdomains or rafts with a high sphingolipid-to-protein ratio have been detected in the plasma membranes of plant cells (Mongrand et al., 2004; Borner et al., 2005).

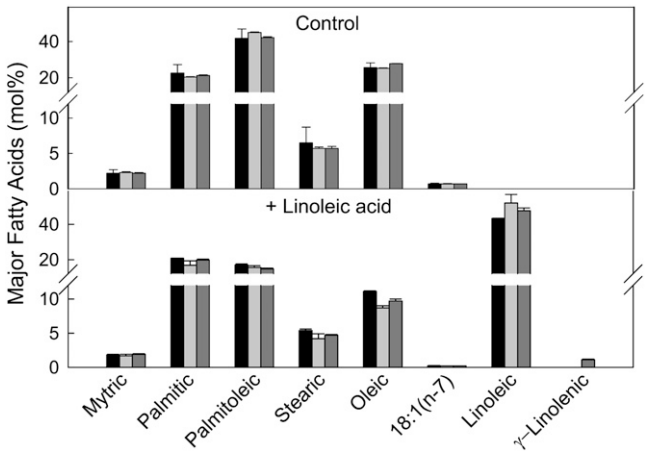

Figure 5. Effect of linoleic acid supplementation on the major fatty acid composition of lipids extracted from different yeast strains. Yeast cultures were grown in the presence or absence of 0.5 mM linoleic acid for 3 d. Bars represent *Sc_pYES3* (black), *Sc_pYES3-S851* (light gray), and *Sc_pYES3-Δ6* (dark gray). Note that γ-linolenic acid was only detected in the *Sc_pYES3-Δ6* strain grown with linoleic acid. Data show the mean and SD (n = 3) of each major fatty acid as mol% of the total fatty acid extract.

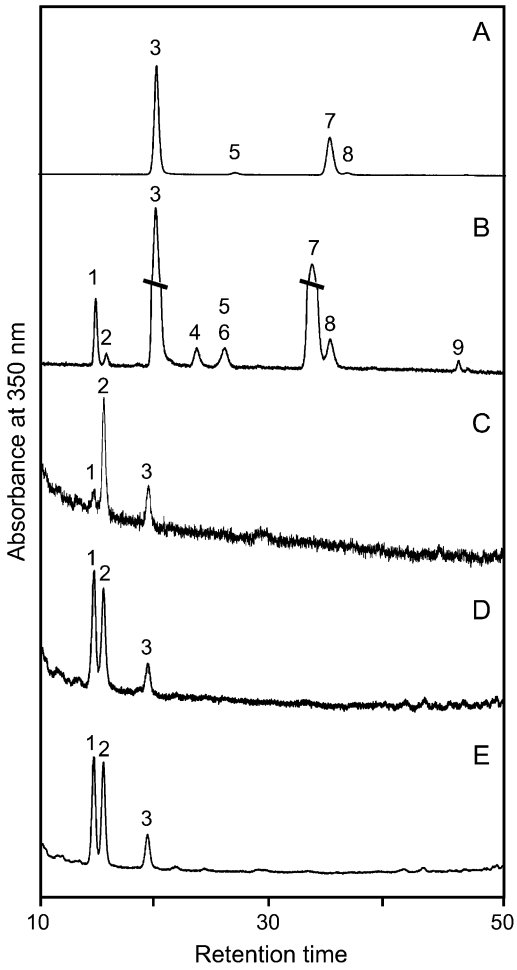

Figure 6. Changes in the long-chain base composition of yeast cells and Arabidopsis plants expressing the *S851* cDNA. Sphingobases were separated as dinitrophenyl derivatives by reversed-phase HPLC and detected at 350 nm. The different derivatized sphingobases are numbered according to increasing elution time with 1 = 8(*Z*)-C_{18}-phytosphingenine; 2 = 8(*E*)-C_{18}-phytosphingenine; 3 = C_{18}-phytosphinganine; 4 = 8(*Z*)-C_{20}-phytosphingenine; 5 = C_{19}-phytosphinganine coeluting with 6 = 8(*E*)-C_{20}-phytosphingenine; 7 = C_{20}-phytosphinganine; 8 = C_{18}-sphinganine; and 9 = C_{20}-sphinganine. INVSc2 cells expressing the *S851* gene from *S. hamata* (B) form 8(*Z*)- and 8(*E*)-phytosphingenines (1, 2, 4, 6), not present in INVSc2 cells expressing the empty vector pYES3 (A). The two Δ8 sphingolipid desaturases encoded in wild-type plants (C) preferentially form the Δ8 (*E*)-isomer (2), whereas expression of the *S851* cDNA in two independent homozygous lines, *At-S581-H4* and *At-S581-H6* (D and E), leads to a significant increase in the Δ8 (*Z*)-isomer (1). The identities of the *N*-(2,4-dinitrophenyl)-sphingobases (1–9) in samples B and E were confirmed by their negative ions $[M\text{-}H]^-$ of HPLC-electrospray ionization-MS and their relative elution times as previously described (Ternes et al., 2002).

These microdomains are characterized by their insolubility to nonionic detergents and a high proportion of the proteins associated with them are involved with stress responses, cellular trafficking, and cell wall metabolism (Morel et al., 2006). Interestingly, about 16% of them also exhibit putative fatty acid modification sites. These sphingolipid-enriched lipid rafts appear to provide a platform for protein binding and organization and may constitute signaling centers for specialized physiological functions (Morel et al., 2006). Unsaturated sphingolipids may have specific roles in cellular responses to stresses (Ohnishi et al., 1988; Imai et al., 2000; Kawaguchi et al., 2000) and a few studies have examined these responses in detail. For instance, sphingosine-1-P can modulate stomatal closure by linking the perception of abscisic acid to reduction in guard cell turgor, whereas dihydrosphingosine-1-P, a structurally similar base without the Δ4 double bond, has no effect (Ng and Hetherington, 2001; Ng et al., 2001).

Cerebrosides (glucosylceramides) and other more complex sphingolipids in Brassicaceae, such as glycosyl inositol phosphorylceramides, predominantly contain Δ8 unsaturated trihydroxy bases like 8-phytosphingenine ($t18{:}1^{\Delta 8}$) and only minor amounts of phytosphinganine and Δ8 unsaturated dihydroxy bases (Bonaventure et al., 2003). Furthermore, glycosyl inositol phosphorylceramides mostly contain the 8(*E*)-isomer of phytosphingenine, whereas cerebrosides typically have a larger proportion of the 8(*Z*)-unsaturated sphingobases (Sperling et al., 2005). Cerebrosides extracted from the roots and shoots of members of the Fabaceae (e.g. *Phaseolus* sp. and pea [*Pisum sativum*]) also contain appreciable proportions of the dihydroxy bases 8-sphingenine ($d18{:}1^{\Delta 8}$) and 4,8-sphingadienine ($d18{:}2^{\Delta 4,8}$; Imai et al., 1997; Sperling et al., 2005). A similar sphingobase profile can be expected in *S. hamata* because it also belongs to the Fabaceae. Future studies will attempt to confirm this prediction and investigate whether the known aluminum tolerance of *S. hamata* (ecotype Verano) can be attributed to the lipid profile of its membranes.

Desaturation of long-chain bases by sphingolipid desaturases probably occurs after ceramide formation, but the natural substrate of the desaturase encoded by *S851* is unclear. García-Maroto et al. (2007) speculated that distinct Δ8 desaturases might have preferences for the cerebroside or glycosyl inositol phosphorylceramide groups of sphingolipids. The sphingobase phytosphinganine (t18:0) is a candidate substrate for S851 (Sperling et al., 2000). However, the relatively low activity of S851 in yeast (with relatively abundant t18:0) compared to the plant tissues suggests that it may prefer one of the cerebrosides, such as monoglycosylceramides, which are absent from yeast, but present in higher plants. This idea is also consistent with the stereoselectivity of the S851 enzyme because cerebrosides generally contain a greater proportion of 8(*Z*)-unsaturated sphingobases.

S851 cDNA isolated from *S. hamata* encodes a Δ8 (*E*/*Z*)-sphingolipid desaturase with a preference for 8(*Z*)-isomer formation. Expression of this gene in yeast and Arabidopsis alters the sphingobase composition of the membranes and enhances the tolerance of these cells to aluminum stress. Future studies will investigate the role sphingolipid composition plays in the aluminum tolerance of *S. hamata*, whether the rise in

Table III. *Changes in the composition of the C_{18}-sphingobases released from Arabidopsis plants expressing S851 cDNA from S. hamata*

Sphingobases 8(*Z*)-t18:1 and 8(*E*)-t18:1 refer to the 8(*Z*)- and 8(*E*)-isomers of phytosphingenine, respectively, and t18:0 refers to phytosphinganine. Data represent the average of pooled plants sufficient for a sphingobase analysis.

Tissue Analyzed	Sphingobases				
	8(*Z*)-t18:1	8(*E*)-t18:1	t18:0	8(*Z*)-t18:1 as % of Total t18:1 Bases	Ratio 8(*Z*):8(*E*)
	mol%				
Whole plants					
Wild type	14.8	58.2	27.0	20.3	0.25
At-S851-H4	43.8	41.5	14.7	51.3	1.06
At-S851-H6	43.0	42.3	14.7	50.4	1.02
Leaves					
Wild type	17.1	62.9	20.0	21.4	0.27
At-S851-H4	40.0	38.9	21.1	50.7	1.03
At-S851-H6	39.1	40.8	20.1	48.9	0.96
Roots					
Wild type	16.8	40.1	43.1	28.5	0.42
At-S851-H4	34.5	25.7	39.8	57.3	1.34

the 8(*Z*)-isomer is paralleled by an increase in cerebrosides and how the two isomers 8(*Z*)-phytosphingenine and 8(*E*)-phytosphingenine affect membrane physiology and cellular metabolism.

MATERIALS AND METHODS

Plant cDNA Libraries

The three cDNA libraries screened in this study were chosen because they were either derived from acid-tolerant species or because the libraries were prepared from tissues predicted to contain proteins that facilitate organic anion efflux from root cells as described below. *Stylosanthes hamata* is a widely used tropical forage plant and ecotype Verano is suited to the infertile and acidic soils of Central and South America as well as northern Australia. White lupin (*Lupinus albus*) forms specialized structures, called cluster roots, on its lateral roots during the onset of phosphorus deficiency (see Ryan et al., 2001). These specialized roots release large amounts of citrate into the rhizosphere, which mobilizes poorly soluble reserves of phosphorus from the soil. In the nodule cells of legumes like soybean (*Glycine max*), the peribacteroid membrane surrounds the compartment containing the nitrogen-fixing bacteroids. Proteins on this membrane control the transport of malate from the plant to the bacteria, which is energetically similar to the efflux of malate from the plant cells.

The cDNA library from the cluster roots of white lupin was prepared by first germinating seeds on river sand for 2 weeks. The seedlings were then transferred to nutrient solution adjusted to pH 6.0 that contained 625 μM KNO_3, 250 μM $CaCl_2$, 250 μM $MgSO_4$, 12.5 μM $FeCl_3$, 6.26 μM Na_2EDTA, 11.5 μM H_3BO_4, 2.7 μM $MnCl_2$, 0.35 μM $ZnSO_4$, 0.3 μM $CuCl_2$, and 0.03 μM $(NH_4)_6Mo7O_{24}$ $4H_2O$. The formation of cluster roots was induced by maintaining the phosphate concentration at 2.5 μM for the first 2 weeks and being omitted thereafter. The nutrient solution was aerated and changed every week as well as on the day before sample collection. After 5 to 6 weeks of growth, cluster roots at different stages of development were collected in liquid nitrogen. Total RNA was extracted from the frozen cluster root tissue with TRIzol LS reagent (Invitrogen) and mRNA was isolated with the MessageMaker mRNA isolation system (Gibco-BRL). cDNA was prepared using the SuperScript plasmid system for cDNA synthesis kit (Gibco-BRL) and size fractioned into long (>1.0 kb) and short (<1.0 kb) pools. cDNAs were directionally ligated into the *Sal*I-*Not*I sites of a yeast (*Saccharomyces cerevisiae*) expression vector pYES3, which has the Gal-inducible promoter GAL1. pYES3 was modified from pYES2 as explained by Smith et al. (1995). The vectors were then cloned in *Escherichia coli* strain XL1-Blue (Stratagene). The plasmid library was recovered from *E. coli* and used to transform the yeast strain INVSc2 (*MATα his3-Δ1 ura3-52*; Invitrogen) as described by Gietz et al. (1995). Primary transformants were selected on supplemented minimal medium without uracil (SMM − ura; Rose et al., 1990), washed off plates with sterile water, and stored in 15% glycerol at −80°C.

cDNA libraries from soybean root nodules and from the roots of *S. hamata* (ecotype Verano) were ligated into the *Sal*I-*Not*I sites of pYES3 (Smith et al., 1995) and transformed into yeast (strain INVSc2) as described above.

Yeast Screen

Approximately 10^6 yeast transformants were screened as described previously (Delhaize et al., 1999) on SMM − ura with 2% Gal and buffered to pH 4.1 with 10 mM succinate. The selection medium also contained 500 or 600 μM $AlCl_3$, which was sufficient to prevent growth of cells transformed with the empty vector. Plasmids were isolated from aluminum-tolerant colonies, amplified in *E. coli*, and then retransformed into wild-type yeast.

Preparation of pYES3-Δ6 and pYES3-Δ8 Yeast Strains

A clone of the coding region of the Δ6 fatty acyl lipid desaturase from *Echium plantagineum* (GenBank accession no. AY952780) was provided by Xue-Rong Zhou (Commonwealth Scientific and Industrial Research Organization Plant Industry). A full-length coding region of the Δ8 sphingolipid desaturase from Arabidopsis (*Arabidopsis thaliana*; At3g61580; GenBank accession no. AF001394) was amplified from RNA isolated from middle maturity developing embryos with the following primers: 5′-GTTCGTCGTCAATGGCGGAA-3′ (forward) and 5′-CATTTAGCCATGAGTATTCAAAG-3′ (reverse). Reverse transcription-PCR was performed using the SuperScript one-step reverse transcription-PCR with platinum Taq (Invitrogen) kit following the manufacturer's instructions. Briefly, a 50-μL reaction contained 25 μL 2× reaction mix and 200 ng RNA, 1 μL each of two oligo primers, and 2 μL platinum Taq enzyme mix. Thermal cycling was 50°C for 30 min (one cycle), 94°C for 2 min (one cycle), 94°C for 15 s, 58°C for 30 s, 68°C for 1.5 min (40 cycles), and 68°C for 7 min (one cycle). PCR fragments obtained were cloned into pGEM T Easy (Promega) and sequenced. The insert was subsequently excised with *Not*I from pGEM T Easy vector and ligated into the *Not*I site of pYES3 in a sense orientation relative to the GAL1 promoter and transformed into yeast strain INVSc2.

Arabidopsis Transformation and Measurements of Aluminum Tolerance

Plant cDNAs were cloned into the pART7 plasmid (Gleave, 1992) to generate an expression cassette with the cDNA under the control of the CaMV 35S promoter. The plasmid was digested with *Not*I and the fragment that contained the expression cassette was ligated into the *Not*I site of the binary vector pPLEX502 (Schünmann et al., 2003). The binary vector was then transformed into *Agrobacterium tumefaciens* strain AGL1 by triparental mating.

Arabidopsis (ecotype Columbia) was transformed by the floral-dip technique as described by Clough and Bent (1998). The seeds were germinated and screened on Murashige and Skoog medium containing 50 μg/mL kanamycin to identify transgenic plants. Two independent homozygous T_2 lines with single inserts were identified from two T_1 populations that displayed approximately 75% resistance to kanamycin (indicating a single insert). To screen the T_1 seed and homozygous T_2 lines for aluminum tolerance, seeds were sterilized in a container filled with Cl_2 gas for 3 h followed by germination on agar medium in either sterile plates or pots that contained 1.67 mM KNO_3, 0.66 mM $CaCl_2$, 0.66 mM $MgSO_4$, 0.067 mM KH_2PO_4, 7.6 μM H_3BO_4, 1.8 μM $MnCl_2$, 0.23 μM $ZnSO_4$, 0.2 μM $CuCl_2$, 16.67 μM $FeCl_3$, and various concentrations of $AlCl_3$ with 5 mM succinic acid to buffer the medium at pH 4.8 to 4.9. Between 20 and 30 wild-type seeds and a similar number of seeds from one of the homozygous transgenic lines were spread along each half of a straight line across the middle of the agar plates. The plates were then held in a near-vertical position so that the line of seeds was horizontal. We noticed that the roots on these plates would sometimes lift off the agar and avoid the aluminum treatment. Therefore, aluminum tolerance was also measured in sterile pots, which forced the roots to penetrate the agar. In this arrangement, wild-type and transgenic seeds were spread around the periphery of the pots (6.5-cm diameter) to facilitate root length measurements at a later date. After positioning the seeds, the plates and pots were kept at 4°C for 2 d and then transferred to a temperature-controlled growth room (8-h darkness at 15°C and 16-h light at 20°C). Root lengths were measured after 14 d. These experiments were repeated at least three times for each homozygous line.

RNA Isolation and Northern-Blot Analysis

Total RNA isolated from Arabidopsis leaves was separated on a 1.5% denaturing formaldehyde gel, transferred onto a Hybond N^+ nylon membrane, and northern blots prepared according to Sambrook et al. (1989).

Fatty Acid Analyses

Total lipids of Arabidopsis leaves or yeast cells were extracted with methanol-chloroform according to the method described by Bligh and Dyer (1959). The fatty acid methyl ester (FAME) preparation and subsequent analysis of fatty acid composition by gas chromatography followed the method described by Liu et al. (2002). Briefly, after evaporating the solvent, lipid extracts were methylated in 2 mL of 0.02 M sodium methoxide for 1 h at 90°C. FAMEs were then extracted by adding 1.5 mL hexane and 2 mL water and vortexing. The upper phase, containing the FAMEs, was transferred to a microvial and separated in a SGE BPX70 column (0.25-mm diameter, 60-m length, and 2.5-μm film thickness) with gas chromatography (model 3400; Varian) using helium as carrier gas. Fatty acid composition was calculated as the percentage of each fatty acid represented in the total fatty acids.

Pretreatment of Yeast Cells in Linoleic Acid

Yeast cells were grown to OD_{600} 1.0 in 5 mL SMM − ura with 2% Glc at 30°C. Cells were collected by centrifugation, washed in sterile water, and resuspended in 5 mL SMM − ura containing 2% Gal, 0.5 mM linoleic acid (no. L1376; Sigma-Aldrich), and 1.0% NP-40, then incubated on a 20°C shaker for 3 d. Yeast cells were harvested by centrifuging and washed first with 1% NP-40, then with 0.5% NP-40, and finally with sterile water.

Preparation of Yeast and Plants for Sphingobase Analyses

Yeast cells transformed with the empty pYES3 vector (strain *Sc_pYES3*) or with pYES3 containing *S851* (strain *Sc_pYES3-S851*) were grown aerobically at 30°C for 2 d in complete minimal medium (minus uracil) supplemented with 2% (w/v) raffinose for 24 h. Expression of the transgene was induced by a further 24-h growth after addition of Gal (final concentration 1.8% [w/v]). Cells were harvested by centrifugation for 10 min at 1,500*g*, resuspended in water, boiled for 15 min to inactivate lipases, and centrifuged again.

Seeds (2.5 mg) of Arabidopsis (Columbia) wild-type and homozygous lines constitutively expressing S851, *At-S851-H4*, and *At-S851-H6*, were sterilized in 4% NaOCl and 0.02% (v/v) Triton X-100 for 10 min, washed three times in sterile water, and resuspended in 0.05% (w/v) agarose for plating. Plants were grown in a climate chamber for 3 weeks at 23°C, a 15-h light/9-h dark cycle, and 150 μE m^{-2} s^{-1} on sterile plates that contained 1× Murashige and Skoog salts, including B5 vitamins (M5519; Sigma), 1% (w/v) Suc, 2.3 mM MES-buffer adjusted to pH 5.8, and 0.8% plant agar. Homozygous lines were grown on plates supplemented with 50 μg kanamycin mL^{-1}. After 21 d, Arabidopsis plants were harvested, boiled in water for 15 min, and dabbed dry.

Sphingobase Analyses

Pellets of yeast cells (380 mg of fresh weight) and whole Arabidopsis plants, leaves, or roots (500 mg) were subjected to barium hydroxide hydrolysis as previously described (Ternes et al., 2002). After extraction by solvent partitioning, the sphingobases were converted to dinitrophenyl derivatives by reaction with 1-fluoro-2,4-dinitrobenzene and purified by thin-layer chromatography. HPLC separations were carried out by reverse-phase elution and detection at 350 nm. To identify compounds by spectrometric identification, reversed-phase HPLC-MS with electrospray ionization of dinitrophenyl-derivatized sphingobases was performed on a MAT 95 XL-Trap instrument (ThermoQuest) in negative ion mode, as previously described (Ternes et al., 2002). In the negative ion mode (*mass-to-charge ratio* $[m/z] = M_r - 1$), pseudomolecular ions corresponding to the dinitrophenyl derivatives of the sphingobases were detected at the expected retention times, with $m/z = 466$ for C_{18}-sphinganine, $m/z = 482$ for C_{18}-phytosphinganine, $m/z = 480$ for 8(*Z*)- and 8(*E*)-C_{18}-phytosphingenines, $m/z = 496$ for C_{19}-phytosphinganine, $m/z = 494$ for C_{20}-sphinganine, $m/z = 510$ for C_{20}-phytosphinganine, and $m/z = 508$ for 8(*Z*)- and 8(*E*)-C_{20}-phytosphingenines.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers provided in Table I.

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