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# Functional characterization of sphingolipid C4-hydroxylase genes from *Arabidopsis thaliana*[1]

Petra Sperling[a,2], Philipp Ternes[a,2], Hermann Moll[b], Stephan Franke[c], Ulrich Zähringer[b], Ernst Heinz[a,*]

[a] *Institut für Allgemeine Botanik, Universität Hamburg, Ohnhorststr. 18, 22609 Hamburg, Germany*
[b] *Laborgruppe Immunchemie, Forschungszentrum Borstel, Parkallee 22, 23845 Borstel, Germany*
[c] *Organische Mikroanalytik, Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany*

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**Abstract In the genome of *Arabidopsis thaliana*, two genes were identified encoding isoenzymes for C4-hydroxylation of long chain bases (LCB) in plant sphingolipids. Both predicted proteins consist of 258 amino acid residues (77% identity) which show sequence similarity to di-iron-binding enzymes, such as *Sur*2p and *Erg*3p from yeast, involved in oxygen-dependent lipid modifications. Heterologous expression of these genes in a yeast *sur2*Δ-null mutant lacking C4-LCB hydroxylation resulted in the formation of D-*ribo*-$C_{18}$- and -$C_{20}$-phytosphinganine. The identity and stereochemical configuration of the isolated trihydroxybases was confirmed by electrospray ionization-mass spectroscopy, gas–liquid chromatography–mass spectrometry and $^1$H-nuclear magnetic resonance spectroscopy. These results represent the first functional identification of *SUR2* genes from plants as well as from any organism other than yeast. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Desaturase; Hydroxylase; Phytosphinganine; Sphingolipid; *SUR2*; *SYR2*

## 1. Introduction

Sphingolipids are ubiquitous membrane components in eukaryotic cells and in a few bacteria [1]. Recent interest is focussing on the role of sphingolipids in signal transduction pathways and other cellular processes in mammals and yeast [2,3], whereas in plants, hardly anything is known about their function. Sphingolipid synthesis starts with the condensation of acyl-CoA (mainly palmitoyl-CoA) and serine to yield 3-ketosphinganine, which is reduced to yield sphinganine (D-*erythro*-2-amino-1,3-dihydroxyalkane). This long chain base (LCB) is *N*-acylated with a fatty acid of 14–26 carbon atoms to form a ceramide. Depending on the source, this basic structure can be further modified by differences in chain length, degree of unsaturation, methyl branching, and insertion of additional hydroxy groups. Complex sphingolipids such as cerebrosides, phytoglycolipids, and membrane anchors of plasma membrane proteins are formed by the addition of various sugar residues or other polar phosphate-containing head groups to the ceramide. In mammals, the sphingoid moiety is mostly 4-*trans*-sphingenine (sphingosine) [4,5], whereas in yeasts, sphinganine is hydroxylated at the C4-position to give $C_{18}$- and $C_{20}$-phytosphinganine [6–8], although the preferred substrate specificity (free LCB, ceramide or phytoglycolipid) of the hydroxylating enzyme is not yet clear. The *SUR2*/*SYR2* gene encoding C4-LCB hydroxylation has been identified from *Saccharomyces cerevisiae* by gene disruption [9,10]. It has been shown that the *SUR2* gene is not essential for growth as no defect in vegetative growth or stress resistance was observed in a yeast null mutant strain [9,10]. On the other hand, inactivation of *SUR2* relieves the inhibitory effect of high $Ca^{2+}$-concentrations on strains which accumulate inositol phosphoryl ceramides as a consequence of a block in their derivatization by mannosylation [3]. This may point to a participation of the hydroxylated LCB in the control of ER-localized $Ca^{2+}$-stores. Another, not fully understood effect of *SUR2* deletion is the resulting resistance to growth inhibition by the fungicide syringomycin E [3,10]. In *Candida lipolytica* as well as in several other organisms including plants even tetrahydroxybases may occur [11–13], suggesting the presence of a C5-LCB hydroxylase. In plants, sphinganine can be either desaturated to sphingosine or C4-hydroxylated to yield phytosphinganine, most of which is further desaturated to yield *cis*/*trans*-stereoisomers of $\Delta^8$-unsaturated LCB [14,15]. Recently, the gene for a $\Delta^8$-LCB desaturase was identified from higher plants [16,17], but the genes responsible for $\Delta^4$-desaturation and for C4-hydroxylation of LCB in plants were still unknown. In this study, we describe the functional identification of two *Arabidopsis thaliana* *SUR2*-like genes essential for phytosphinganine synthesis in plants by complementation of a yeast null mutant lacking C4-LCB hydroxylation.

## 2. Materials and methods

### 2.1. Isolation of SUR2 genes from A. thaliana

A BLAST search with the *SUR2* gene from *S. cerevisiae* revealed good sequence similarity with the hypothetical proteins of the pre-

*Corresponding author. Fax: (49)-40-42816 254.
E-mail: eheinz@botanik.uni-hamburg.de

[1] The nucleotide sequences reported in this paper can be accessed through the GenBank™/EBI Data Bank with accession numbers AC012188 and AC013289.
[2] Both authors contributed equally to this work.

*Abbreviations:* ESI-MS/MS, electrospray ionization-mass spectroscopy/mass spectroscopy; GLC-MS, gas–liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography; LCB, long chain base; ORF, open reading frame; PCR, polymerase chain reaction; TLC, thin-layer chromatography

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dicted genes F14L17.5 and T6C23.16 in the *A. thaliana* DNA database (accession numbers AC012188 and AC013289). These genomic sequences were used to construct specific primers (extended by a *Kpn*I and *Xba*I restriction site, respectively) for polymerase chain reaction (PCR) amplification of the two 777-bp coding regions from *A. thaliana* cDNA. For first strand cDNA synthesis, mRNA was isolated from developing *A. thaliana* leaves using dynabeads (Dynal) and transcribed using the SuperScript Preamplification System (Gibco BRL). The PCR products were ligated into the *Kpn*I and *Xba*I restriction sites behind the *GAL1* promoter of the yeast expression vector pYES2 (Invitrogen) yielding pF14L17.5 and pT6C23.16, respectively. Correct cloning was confirmed by sequencing.

### 2.2. Expression in a S. cerevisiae sur2Δ-null mutant

The plasmids pYES2 (empty vector), pF14L17.5 and pT6C23.16 were transformed into the *sur2*Δ-null mutant strain of *S. cerevisiae* [9] as described before [16]. Transformed cells were grown in complete minimal-dropout-uracil medium (CMdum) supplemented with 2% raffinose as the only carbon source [18]. For expression experiments, 50-ml test cultures in CMdum were aerobically grown for 24 h at 30°C, then induced by the addition of 1.8% galactose ((w/v); final concentration) and finally grown to saturation for another 24 h at 30°C.

### 2.3. LCB analysis

Transgenic yeast cells were harvested by centrifugation for 15 min at 2800×*g* and washed with water once. Cell pellets (≈650 mg) were subjected to barium hydroxide hydrolysis to liberate the LCBs, which were converted into their dinitrophenyl derivatives and analyzed by reversed-phase HPLC, as described before [16], and in addition, by electrospray ionization-mass spectroscopy/mass spectroscopy (ESI-MS/MS) (MAT 95XL-Trap instrument, with direct infusion as well as coupled to HPLC).

### 2.4. Gas–liquid chromatography–mass spectrometry (GLC–MS) analysis

LCBs obtained from transgenic yeast cells (9.6 g fresh weight) were separated into the dihydroxybase ($R_F = 0.38$) and trihydroxybase fraction ($R_F = 0.17$) by preparative thin-layer chromatography (TLC) in chloroform/methanol/ammonia (40:40:1; v/v/v). Acetylation and final TLC purification [16] yielded peracetylated LCB fractions (∼100 μg each) of sphinganines (d18:0 and d20:0) and phytosphinganines (t18:0 and t20:0), respectively.

GLC–MS analysis was performed with a HP-5MS column (30 m, Hewlett Packard) using a temperature gradient from 200°C (3 min)→330°C at 5°C $min^{-1}$. EI- and CI-mass spectra were recorded as described [16,19]. For determination of the absolute configuration (D- or L-*ribo*), free phytosphinganines were converted into their diastereomeric amides using (*S*)-(+)-hydroxybutyric acid and (*R*)-(−)-hydroxybutyric acid *N*-hydroxysuccinimide esters [20] and separated as per-*O*-acetylated derivatives by GLC–MS by the same gradient as described above.

### 2.5. $^1$H-nuclear magnetic resonance (NMR) spectroscopy

1D and 2D homonuclear ($^1$H,$^1$H-COSY) correlated $^1$H-NMR experiments were performed at 600 MHz (Bruker AvanceDRX-600). NMR spectra of the peracetylated trihydroxybase fraction (75 μg) and D-*ribo*-phytosphinganine (Sigma) dissolved in 500 μl $CDCl_3$-$d_1$ were recorded at 300 K with reference to internal $CDCl_3$ ($\delta_H = 7.190$, $\delta_C = 77.0$). All NMR experiments were performed using standard Bruker software (XWINNMR, version 2.5).

## 3. Results and discussion

### 3.1. Comparison of A. thaliana SUR2-like genes with other desaturase-like enzymes

Cloning and sequencing of fatty acyl desaturases from different organisms has shown that membrane-bound desaturases contain three characteristic histidine motifs, which are believed to coordinate a di-iron cluster in the active site [21]. Furthermore, sequence comparisons have revealed that regioselectivity spanning C5–C15 of the acyl chain appears to place greater constraints on the sequence of a fatty acid desaturase or a related enzyme than the actual reaction chemistry (i.e. desaturation, hydroxylation, acetylenation or epoxidation) or the phylogenetic position of the eukaryotic organism [22]. Therefore, we predicted that a plant C4-LCB hydroxylase should be more closely related to the yeast *Sur2*p protein sequence than to other desaturase-like enzymes. A BLAST search analysis of the GenBank database using the *S. cerevisiae SUR2* gene revealed significant similarity to two *A. thaliana* genes F14L17.5 and T6C23.16, which were deduced from the genomic sequence of chromosome 1. In contrast to our BLAST search, both predicted genes were annotated to show similarity to sterol desaturases (*ERG3*). Specific primers derived from the genomic sequences were used to amplify the open reading frame (ORF) coded by each gene via PCR using cDNA transcribed from leaf mRNA. Sequencing of the PCR products revealed that the corresponding ORFs encode two putative proteins of 258 amino acids. The protein sequences of F14L17.5 and T6C23.16 show 77% identity and 90% similarity to each other, 37 and 40% identity to a hypothetical membrane protein from *Schizosaccharomyces pombe*, as well as 28–31% identity to an ORF from *Candida albicans* and to the yeast Sur2p protein (Fig. 1). Only 15% identity is observed to C4-sterol methyloxidases (*ERG25*), 10–13% identity to C5-sterol desaturases (*ERG3*) and C25-cholesterol hydroxylases (*CH25H*) from different organisms. All of these proteins, including all five Sur2p sequences, are charac-

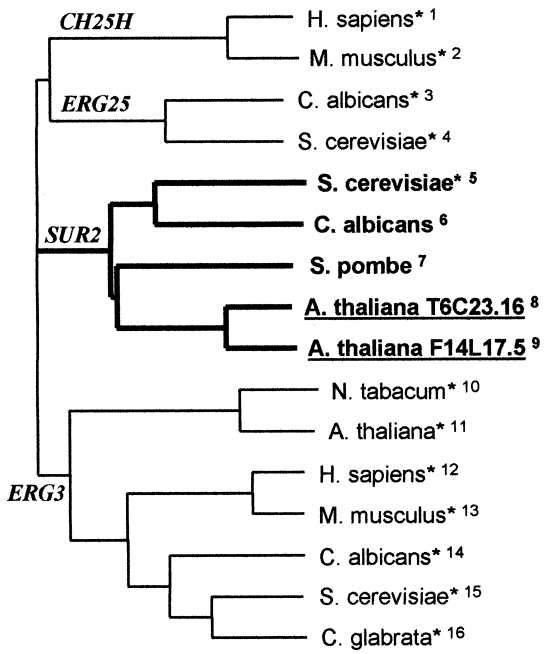


Fig. 1. Dendrogram of C4-LCB hydroxylases and sterol desaturase-like enzymes. Full length amino acid sequences were aligned and used to generate a phylogenetic tree using the programs CLUSTAL X and TreeView. Sequences with experimental evidence for function are designated with a asterisk (*). GenBank database accession numbers are given, if not stated otherwise: C25-cholesterol hydroxylases (*CH25H*): [1]AAC97481, [2]AAC97480, C4-sterol methyloxidases (*ERG25*): [3]AAC06014, [4]P53045, C4-LCB hydroxylases (*SUR2*): [5]AAA16608, [6]ORF 6.4041 on contig 6-2307 (*C. albicans* sequence data available from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida), [7]CAA21900, [8]AC013289, [9]AC012188, C5-sterol desaturases (*ERG3*): [10]AAD04034, [11]AAD38120, [12]BAA33729, [13]BAA33730, [14]AAC99343, [15]P32353, [16]P50860.

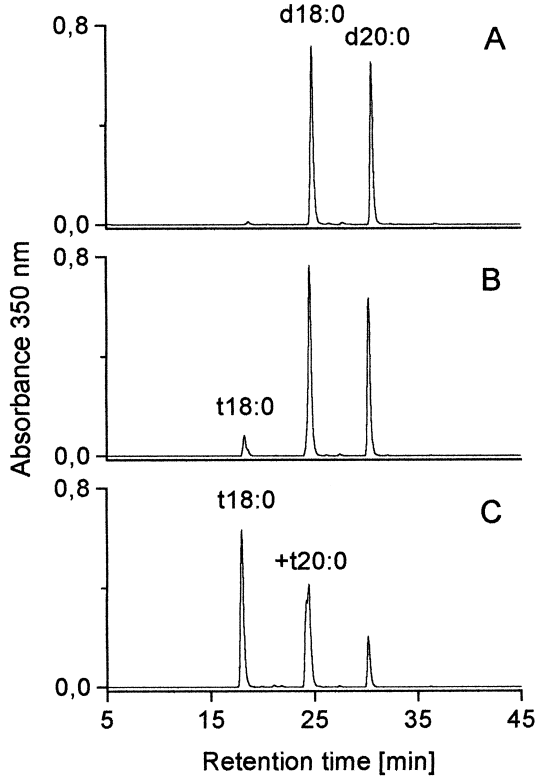


Fig. 2. Formation of D-*ribo*-phytosphinganine in a *S. cerevisiae* mutant lacking C4-LCB hydroxylation by heterologous expression of homologous plant isoenzymes. A: $C_{18}$- and $C_{20}$-sphinganine (d18:0 and d20:0) produced by yeast *sur2*Δ-null mutant cells harboring the empty vector pYES2. Formation of $C_{18}$- and $C_{20}$-phytosphinganine (t18:0 and t20:0) in *sur2*Δ-cells expressing either pT6C23.16 (B) or pF14L17.5 (C) encoding isoenzymes of the C4-sphinganine hydroxylase from *A. thaliana*. LCB from whole yeast cells were converted into their dinitrophenyl derivatives and analyzed by reversed-phase HPLC [16].

terized by three histidine boxes. Therefore, the assumption mentioned above also seems to include desaturase-like enzymes working further towards the carboxyl end, i.e. with a C4-regioselectivity. In contrast to all eukaryotic $\Delta^5$-, $\Delta^6$- and $\Delta^8$-fatty acid desaturases or to $\Delta^8$-LCB desaturases [23,24], no fusion to cytochrome $b_5$, the electron donor required in extraplastidial desaturase-like reactions, is observed in the C4-LCB hydroxylase sequences from yeast, *C. albicans*, *S. pombe* and *A. thaliana*.

### 3.2. Reconstitution of phytosphinganine synthesis in a yeast *sur2*Δ-null mutant by expression of *A. thaliana* isoenzymes

For a functional identification, the ORFs of the predicted genes F14L17.5 and T6C23.16 amplified by PCR were cloned into a yeast expression vector to give pF14L17.5 and pT6C23.16., respectively. These, and the empty vector pYES2 were transformed into a *sur2*Δ-null mutant strain of *S. cerevisiae* lacking the C4-sphinganine hydroxylase. Transformed yeast cells were grown to saturation after induction with galactose, and LCBs were liberated by barium hydroxide hydrolysis from their complex sphingolipids [25]. The extracted LCBs were converted into their dinitrophenyl derivatives [1] and analyzed by reversed-phase HPLC (Fig. 2) as described before [16,17]. In agreement with previous reports [9,10], *sur2*Δ-mutant cells harboring the empty vector pYES2 contain only $C_{18}$- and $C_{20}$-sphinganine (D-*erythro*-2-amino-1,3-dihydroxyoctadecane and -eicosane, d18:0 and d20:0, respectively), but no $C_{18}$- and $C_{20}$-phytosphinganine (*D-ribo*-2-amino-1,3,4-trihydroxyoctadecane and -eicosane; t18:0 and t20:0), which predominate in wild type yeast cells (Fig. 2A). A significant increase in $C_{20}$-sphinganine was observed with increasing culturing time (data not shown) suggesting that a lack of the trihydroxy phytosphinganine in *sur2*Δ-mutant cells is compensated by this dihydroxy $C_{20}$-LCB. In wild type yeast cells, it is the adaptation to heat stress which results in a similar accumulation of $C_{20}$-LCBs [26,27]. Transformants expressing pT6C23.16 and pF14L17.5 accumulated in addition to these dihydroxy sphinganines a new LCB in yields of 7 and 43 mol percent, respectively, of the total LCBs detected in the HPLC profiles (Fig. 2B,C). Its shorter retention time in reversed-phase HPLC was identical with that of the reference substance $C_{18}$-phytosphinganine from yeast (data not shown) [16]. Further evidence for the identity of the newly formed LCB was obtained by on-line ESI-MS analysis of the individual peaks during HPLC separation of the dinitrophenyl derivatives and ESI-MS/MS analysis of selected pseudomolecular ions. The new LCB expressed in both transgenic yeast strains displayed identical mass spectra regarding both the pseudomolecular ion $[M-H]^-$ at $m/z = 482$ and its fragmentation pattern (data not shown). In addition, these spectra were identical with those measured for the reference compound D-*ribo*-phytosphinganine. *Sur2*Δ-cells with pF14L17.5 also produced some $C_{20}$-phytosphinganine (t20:0) coeluting with d18:0 (double peak in Fig. 2C). These results suggest that the genes T6C23.16 and F14L17.5 encode isoenzymes of the *A. thaliana* C4-LCB hydroxylase. Moreover, our data show that the substrate specificity of the plant enzyme is not limited to $C_{18}$-species, but that also $C_{20}$-sphinganine is accepted.

### 3.3. GLC–MS analysis of LCB

The structural assignment of the newly formed trihydroxy LCB was based on the assumption that it was derived by hydroxylation form D-*erythro*-sphinganine [1,28]. Therefore, the newly introduced hydroxyl group had to be characterized with regard to relative (*ribo* or *lyxo*) and absolute (D- or L-) configuration. For this purpose, suitable derivatives were prepared and analyzed by NMR spectroscopy and mass spectrometry, respectively.

Unequivocal proof for the identity of the new LCB as phytosphinganine was obtained by GLC–MS [19]. The LCBs liberated from *sur2*Δ-cells expressing pF14L17.5 were separated into their di- and trihydroxybase fractions by TLC followed by peracetylation. Peracetylated d18:0 and d20:0 were identified by GLC–EI-MS in relative proportions of ~15:1 (19.05 min and 21.56 min; $m/z = 144$, 102, 84). The peracetylated t18:0 and t20:0 were detected in a ~7:1 ratio, with t18:0 having the same retention time (20.14 min) and fragmentation pattern ($m/z = 352$, 292, 146, 144, 102, 84) as the reference compound D-*ribo*-phytosphinganine. The second component (22.62 min, $m/z = 380$, 320, 144, 102, 84) was identified as peracetylated t20:0.

### 3.4. $^1$H-NMR analysis of ribo-phytosphinganine derivatives

The 1D and 2D $^1$H NMR spectra of the peracetylated t18:0 and t20:0 and of the reference compound t18:0 (D-*ribo*-phytosphinganine) were found to be identical (data not shown), expressing a diagnostic H-4-signal ($\delta \approx 4.94$ ppm). Therefore,

Table 1
Comparison of $^1$H-NMR-data of peracetylated D-*ribo*- (**1**) and L-*lyxo*-phytosphinganine (**2**) from the literature [29], D-*ribo*-phytosphinganine (Sigma P2795) from yeast (**3**) and phytosphinganine produced by *sur2*Δ-yeast mutant cells expressing the *A. thaliana* gene F14L17.5 (**4**)

| Proton | Chemical shifts $\delta$ (ppm) | | | | Coupling constants $J$ (Hz) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | |
| H-1a | 4.00 | 3.96 | 4.002 | 4.003 | 11.7 | 11.7 | 11.7 | 11.7 | $J_{1a,1b}$ |
| H-1b | 4.29 | 4.22 | 4.290 | 4.290 | 4.8 | 4.6 | 4.8 | 4.9 | $J_{1b,2}$ |
| H-2 | 4.47 | 4.52 | 4.472 | 4.472 | 3.1 | m | 2.7 | 3.1 | $J_{2,1a}$ |
| NH-2 | 5.98 | 5.77 | 5.907 | 5.938 | 9.4 | 9.7 | 9.4 | 9.4 | $J_{2,NH-2}$ |
| H-3 | 5.10 | 5.08 | 5.095 | 5.099 | 8.3 | m | 8.3 | 8.3 | $J_{2,3}$ |
| H-4 | 4.93 | 5.11 | 4.938 | 4.937 | 3.2 | m | 3.1 | 3.0 | $J_{3,4}$ |
| NAc | 2.03 | 1.96 | 2.026 | 2.027 | | | | | |
| OAc | 2.05 | 2.06 | 2.048 | 2.049 | | | | | |
| OAc | 2.05 | 2.08 | 2.048 | 2.080 | | | | | |
| OAc | 2.08 | 2.11 | 2.079 | 2.101 | | | | | |

$^1$H-NMR spectra of **3** and **4** were recorded at 600 MHz, 300 K in $CDCl_3$-$d_1$ and refer to the internal chloroform signal ($\delta_H$ 7.260 ppm), those of **1** and **2** at 400 MHz [29]. m, multiplet.

these and the GLC-MS data prove that the third hydroxyl group in the LCBs is located at C4.

C4-hydroxylation of D-*erythro*-sphinganine can lead either to the D-*ribo*- or L-*lyxo*-phytosphinganine and, thus, the relative stereoconfiguration (*ribo*- or *lyxo*-) was investigated in detail by $^1$H-NMR-spectroscopy using peracetylated phytosphinganines [29]. Table 1 summarizes the $^1$H-NMR data of peracetylated trihydroxybases from *sur2*Δ-cells expressing pF14L17.5 and the reference compound D-*ribo*-phytosphinganine together with literature data (D-*ribo*- and L-*lyxo*-phytosphinganine) [29]. With the exception of the NH-signal, all diagnostic protons (H-1–H-4) allowed the unequivocal assignment of the newly formed trihydroxybase to its *ribo*-phytosphinganine derivative (identical to peracetylated D-*ribo*-phytosphinganine) which also correlated well with literature data [29]. Since the L-*lyxo*-phytosphinganine differs from the D-*ribo*-phytosphinganine in the diagnostically unresolved multiplet ($\delta$ 5.08–5.11 ppm) the newly formed phytosphinganine could be unequivocally assigned to the *ribo*-configuration.

### 3.5. Determination of the absolute configuration of D-ribo-phytosphinganine by GLC–MS

For the determination of the absolute configuration of phytosphinganine (D-*ribo* or L-*ribo*), the trihydroxybase fraction as well as D-*ribo*-phytosphinganine (2*S*,3*S*,4*R*) (reference substance) were derivatized with (*S*)-(+)- and (*R*)-(−)-3-hydroxybutyric acid, respectively, followed by per-*O*-acetylation and analyzed by GLC-MS. The resulting per-*O*-acetylated diastereomeric amide pair of the reference D-*ribo*-phytosphinganine was nearly baseline-resolved into the (*S*)-(+)- (retention time 24.50 min) and the (*R*)-(−)-3-hydroxybutyric acid derivative (24.61 min). The per-*O*-acetylated acid amides of the trihydroxybase fraction showed the same retention times (24.49 and 24.61 min) and EI-MS fragmentation ($m/z$ = 331, 318, 230, 170, 110, 69) as the reference compound, thus allowing the phytosphinganine formed by *sur2*Δ-cells expressing pF14L17.5 to be assigned to the D-configuration. In conclusion, HPLC, EI-MS and NMR analysis clearly show that the gene F14L17.5 from *A. thaliana* encodes a C4-sphinganine hydroxylase, which converts D-*erythro*-sphinganine into D-*ribo*-phytosphinganine in a stereospecific manner.

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