

Identification and Functional Characterization of the 2-Hydroxy Fatty *N*-Acyl- $\Delta^3(E)$ -desaturase from *Fusarium graminearum**^[5]

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$\Delta^3(E)$ -Unsaturated fatty acids are characteristic components of glycosylceramides from some fungi, including also human- and plant-pathogenic species. The function and genetic basis for this unsaturation is unknown. For *Fusarium graminearum*, which is pathogenic to grasses and cereals, we could show that the level of Δ^3 -unsaturation of glucosylceramide (GlcCer) was highest at low temperatures and decreased when the fungus was grown above 28 °C. With a bioinformatics approach, we identified a new family of polypeptides carrying the histidine box motifs characteristic for membrane-bound desaturases. One of the corresponding genes was functionally characterized as a sphingolipid- $\Delta^3(E)$ -desaturase. Deletion of the candidate gene in *F. graminearum* resulted in loss of the $\Delta^3(E)$ -double bond in the fatty acyl moiety of GlcCer. Heterologous expression of the corresponding cDNA from *F. graminearum* in the yeast *Pichia pastoris* led to the formation of $\Delta^3(E)$ -unsaturated GlcCer.

Glycosphingolipids represent a structurally and functionally diverse group of membrane lipids ubiquitously distributed among all eukaryotes. They are characteristic constituents of the endomembrane system and highly enriched in the plasma membrane (1–3).

Fungi and some marine invertebrates synthesize glucosylceramides (GlcCers)² with distinctive structural modifications of the ceramide backbone not found in mammalian and plant glycosphingolipids (4, 5). Fungal GlcCer mainly consists of a diunsaturated, C9-methyl-branched sphingoid base, (4*E*,8*E*)-9-

methyl-sphinga-4,8-dienine, which is *N*-acylated with an α -hydroxylated fatty acid. The fatty acyl moiety can be further modified by the introduction of a $\Delta^3(E)$ -double bond (Fig. 1), whose function is still unclear.

The occurrence of the $\Delta^3(E)$ -double bond is restricted to eukaryotic fungi, including human- and plant-pathogenic species (reviewed in Ref. 5). Depending on the organism, the proportion of $\Delta^3(E)$ -unsaturated sphingolipids varies. In the two dimorphic fungi *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, the GlcCer of the mycelial form shows a higher proportion of unsaturation than the GlcCer of the yeast form. In galactosylceramides (GalCer) of *Aspergillus fumigatus* (a fungus without a yeast form), the proportion of $\Delta^3(E)$ -unsaturated fatty acids is higher than in GlcCer, and in *Sporothrix schenckii*, the double bond is restricted to GalCer of the yeast form (6–8). These data suggest that the $\Delta^3(E)$ -desaturation might be important for the yeast-mycelium phase transition of dimorphic fungi, although it depends on the fungus whether the proportion of $\Delta^3(E)$ -unsaturated fatty acids is higher in the yeast form or in the mycelium.

$\Delta^3(E)$ -Double bonds are also found in other lipid molecules. Phosphatidylglycerol in photosynthetically active plastids carries mostly $\Delta^3(E)$ -hexadecenoic acid in the *sn*-2 position (Fig. 1). The gene locus for the putative desaturase was named *fad4*, but the actual sequence is still not known (reviewed in Ref. 9). The $\Delta^3(E)$ -unsaturation can also be found in common polyunsaturated C₁₈-fatty acids of seed triacylglycerols in some higher plants. One example is caleic acid, (3*E*,9*Z*,12*Z*)-octadeca-3,9,12-trienoic acid (Fig. 1), representing a major fatty acid in triacylglycerols from seeds of *Stenachaenum macrocephalum* and *Calea urticaefolia* (10–12).

Many enzymes necessary for modifications of the ceramide backbone (glycosyltransferases, desaturases, hydroxylases, C9-methyltransferase) have already been identified and functionally characterized in plants and fungi (13–21). However, a gene corresponding to a desaturase with $\Delta^3(E)$ -regioselectivity has not yet been cloned.

All sphingolipid modifying desaturases and hydroxylases belong to a large superfamily of membrane-bound enzymes. Therefore, we expected that the Δ^3 -desaturase would also belong to this group. Members of this superfamily contain three characteristic sequence motifs, the histidine boxes HX_{3–4}H, HX_{2–3}HH, and (H/Q)X_{2–3}HH (22). Some of these sequences are fused to a cytochrome *b5* domain. We expected that the fatty acid α -hydroxylase Scs7p from *Saccharomyces cerevisiae*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) FJ176922 and FJ176923.

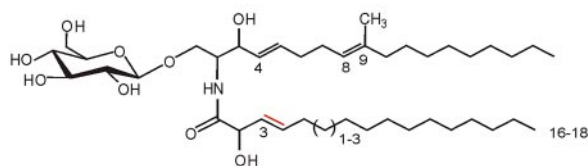
^[5] The on-line version of this article (available at <http://www.jbc.org>) contains three supplemental tables and a supplemental figure.

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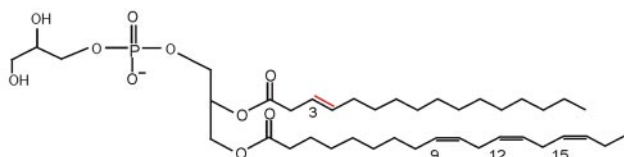
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² The abbreviations used are: GlcCer, glucosylceramide; GalCer, galactosylceramide; ESI FT-ICR MS, electrospray ionization-mass spectrometry; GLC, gas liquid chromatography; GLC-MS, gas chromatography-mass spectrometry; FAME, fatty acid methyl ester; TMS-FAMES, trimethylsilylated FAMES; PG, phosphatidylglycerol; Δ^3 Fg, $\Delta^3(E)$ -desaturase candidate from *F. graminearum*; CM, complete minimal medium; AFP, antifungal peptide; 18:0, stearic acid; 18:1, oleic acid; 18:3, α -linolenic acid; 18:0(2-OH), 2-hydroxyoctadecanoic acid; 18:1(2-OH), 2-hydroxyoctadecenoic acid.

Fungal glucosylceramide



Plant plastidial PG



Caleic acid from TAG of plant seeds

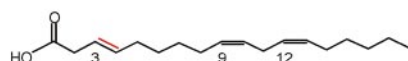


FIGURE 1. **Lipids containing acyl groups with a $\Delta 3(E)$ -double bond.** *Top*, glucosylceramides from eukaryotic fungi show a characteristic consensus structure. The long-chain base is linked to an α -hydroxylated C_{16} or C_{18} fatty acid, which is $\Delta 3(E)$ -unsaturated. The sugar head group can be either glucose (as shown) or galactose. *Middle*, PG in chloroplasts of higher plants usually carries (3*E*)-hexadec-3-enoic acid in the *sn*2-position. *Bottom*, caleic acid, (3*E*,9*Z*,12*Z*)-octadeca-3,9,12-trienoic acid, can be found, for example, in the plants *C. urticaefolia* and *Stenachaenum macrocephalum*, representing a major component of seed triacylglycerols (TAG).

would show the highest sequence similarity to the $\Delta 3(E)$ -desaturase because it represents the enzyme with the closest similarity regarding regioselectivity.

Here we describe the identification, cloning, and functional characterization of a sphingolipid- $\Delta 3(E)$ -desaturase from the plant-pathogenic eukaryotic fungus *Fusarium graminearum*. This monomorphic fungus grows only in the mycelial form and contains GlcCer but no GalCer (23). We used phylogenetic profiling to identify a candidate gene, which was cloned and subjected to deletion and expression studies.

EXPERIMENTAL PROCEDURES

Bioinformatics—The NCBI non-redundant protein sequence data base (www.ncbi.nlm.nih.gov) was searched using PSI-BLAST (24) with the α -hydroxylase Scs7p from *S. cerevisiae* and the fatty acid $\Delta 11$ -desaturase from *Thalassiosira pseudonana* (25) as queries. The resulting sequences were checked for the presence of histidine box motifs and analyzed as described in Refs. 20 and 4 using ClustalX (26). With this strategy, 10 desaturase-like sequences were found in the completely sequenced genome of *F. graminearum*. A putative function could clearly be assigned to 9 of the 10 desaturase sequences by phylogenetic classification within the superfamily of membrane-bound desaturases. The remaining desaturase-like sequence not clustering with desaturases of known regioselectivity was chosen as a query gene for the subsequent BLAST searches of other eukaryotic genomes. Further phylogenetic profiling resulted in promising orthologous candidates forming a single desaturase family restricted to eukaryotes such as *F. graminearum* (Fg05382, Acc-Nr. XP_383758), *Magnaporthe grisea* (XP_368852), *A. fumigatus* (XP_748510), *Aspergillus*

nidulans (XP_662689 and XP_658567), and *Neurospora crassa* (XP_958382).

Growth of *Pichia pastoris*—A preculture was grown in YPD medium (2% bacto tryptone, 1% yeast extract, 1% D-glucose) for 24 h at 30 °C with shaking (180 rpm). 200 ml of fresh YPD medium were inoculated 1:100 from the preculture and incubated again for 24 h at 30 °C. Gene expression was induced by replacing YPD with minimal methanol medium (1.3% yeast nitrogen base without amino acids, 0.5% methanol) and further incubation for 24 h. Cells were harvested by centrifugation.

Growth of *F. graminearum*—For induction of conidiation, mycelium of *F. graminearum* strain 8/1 (27) was grown on agar plates at 18 °C under near UV light and white light with a 12-h photoperiod for ~10 days (28). For transformation and/or lipid analysis, *F. graminearum* was grown in complete minimal medium (CM (29)) for 2–3 days with shaking (150 rpm) and a standard temperature of 28 °C. To determine the temperature-dependent occurrence of the $\Delta 3(E)$ -double bond in GlcCer, equal amounts of conidia were grown in 100 ml of CM at 18, 28, and 30 °C, respectively, for 3 and 7 days.

Construction of a Disruption Vector for *F. graminearum*—5′- and 3′-flanking regions of Fg05382 were amplified by a standard PCR program using *Pwo*-polymerase (peqlab) and the specific primers 5′-agagctcgagCCTAAAGCGACCTACACATATGA-3′ and 5′-atctagatctTGTTAATTTGATGTGCGCATGAGA-3′ (5′-flanking region, 722-bp fragment) and 5′-agagatctCACGATATCGATTACTTCATGATC-3′ and 5′-agaggcgccgcTCACCATGGCAGTTCAAGCTTAT-3′ (3′-flanking region, 964-bp fragment). All primers provided recognition sites for the restriction enzymes BglII, NotI, or XhoI (underlined). The amplified sequences were cloned into the vector pGEM[®]-T (Promega). As a selection marker, a hygromycin-resistance cassette (*hph*, previously cloned into pGEM[®]-T, with BglII restriction sites at either end) was cloned into the BglII site between the 5′- and 3′-flanking regions, resulting in the construction of p $\Delta 3$ FgKO. For transformation of *F. graminearum*, the construct was linearized with XhoI and NotI (all restriction enzymes were obtained from New England Biolabs).

Cloning of the Putative $\Delta 3(E)$ -Desaturases—cDNA from *F. graminearum* strain 8/1 was used as template for amplification of the complete open reading frame using a proofreading *Pwo*-polymerase (peqlab) and a standard PCR program. The specific primer sequences with restriction sites for EcoRI (forward) or NotI (reverse) were: 5′-atctgaattcattATGGCCGAA-CACCTCGTCT-3′ and 5′-atctgcccgcCTACTGCCTTAACTTCTT-3′. The resulting PCR product was ligated into the vector pGEM[®]-T, and its identity was confirmed by sequencing. The isolated fragment was cloned into the EcoRI and NotI restriction sites of the *Pichia* expression vector pPIC3.5 (Invitrogen), resulting in the plasmid p $\Delta 3$ Fg-PIC, which was linearized prior to transformation of *P. pastoris* with BglII.

Transformation of *F. graminearum*—Protoplasting and chemical transformation of *F. graminearum* were carried out as described in Ref. 30 with modifications. 50 ml of CM were inoculated with 30,000 conidia and incubated for 24 h at 28 °C with shaking (150 rpm). Mycelium was homogenized with an Ultra-Turrax blender, and the volume of CM was increased to 200 ml

$\Delta 3$ -Desaturase from *Fusarium*

and incubated overnight at 28 °C. The collected mycelium was washed twice with sterile water and dried on sterile Whatman paper. 1 g of mycelium was suspended in 20 ml of an enzyme mix (3% GlucanexTM, 5% driselase, both from Sigma-Aldrich, dissolved in 700 mM NaCl, pH 5.6) and digested for 3 h at 28 °C with shaking at 75 rpm. Protoplasts were separated from undigested hyphal material by filtration through gauze and nylon membrane (first filtration, 100- μ m pore size; second filtration, 40- μ m pore size). The protoplasts were sedimented (5 min, 850 \times g), washed twice with ice-cold 700 mM NaCl-solution, and subsequently resuspended in 1 ml of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.0, 50 mM CaCl₂) and stored on ice until transformation. For transformation, 1×10^7 protoplasts were diluted in STC to a final volume of 1 ml. 30 μ g of the linearized knock-out-cassette were added, and the protoplasts were incubated on ice for 10 min. 1 ml of PTC (60% polyethylene glycol 3350, 10 mM Tris-HCl, pH 7, 50 mM CaCl₂) was added followed by incubation for 20 min on ice. The protoplast suspension was mixed gently with 8 ml of STC and plated in aliquots of 750 μ l on regeneration medium (0.1% yeast extract, 0.1% bacto tryptone, 1 M sucrose, 1.6% granulated agar; 10 ml poured in 94-mm Petri dishes). After incubation for 12–24 h at 28 °C, the plates were overlaid with 10 ml of selective agar (1.2% granulated agar in water, 200 μ g ml⁻¹ hygromycin B) and further incubated. Transformants were obtained about 7 days after transformation. They were transferred to fresh CM plates containing 100 μ g ml⁻¹ hygromycin B and incubated at 28 °C. Single transformants were isolated and checked for the correct integration of the knock-out cassette by PCR and southern-blotting.

Transformation of *P. pastoris*—The expression construct pd3Fg-PIC was linearized with BglIII. Electrocompetent cells of *P. pastoris* strain GS115 were generated, and transformation by electroporation was carried out according to the manufacturer's instructions (Invitrogen). Single colonies were checked by PCR for the correct integration of the construct and used for lipid analyses.

Isolation of Glucosylceramide—Approximately 2–3 g of fresh weight of *P. pastoris* cells or mycelium of *F. graminearum* were suspended in 5 ml of H₂O 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. To increase the yield of *F. graminearum* lipids, the mycelium was homogenized with an Ultra-Turrax blender during the first extraction step.

The lipid extract was washed by phase partitioning with chloroform/methanol/0.45% NaCl (8:4:3), the organic phase was collected, and the solvents were subsequently evaporated. The lipids in the residue were redissolved in chloroform, and glucosylceramide was purified by preparative TLC on silica gel 60 plates (Merck), developed in chloroform/methanol 85:15. For NMR spectroscopy and mass spectrometry, the purified GlcCer was peracetylated (with acetic anhydride in pyridine 1:1) overnight at room temperature and repurified by TLC in diethyl ether.

Analysis of Fatty Acids—Fatty acid methyl esters (FAMES) were obtained from GlcCer by incubation with sulfuric acid (2 N in methanol with 2% 2,2-dimethoxy propane) overnight at

80 °C and extracted with petrol ether. Because fungal GlcCers mostly contain α -hydroxylated fatty acids, the FAMES were converted to their trimethylsilyl derivatives. For this purpose, purified FAMES were dissolved in 100 μ l of petrolether. After the addition of 100 μ l of *N,O*-bis(trimethylsilyl)trifluoro-acetamide (BSTFA, with 1% trimethyl-chlorosilane, Sigma-Aldrich), the samples were incubated at 70 °C for 60 min. Trimethylsilylated fatty acid methyl esters (TMS-FAMES) were analyzed by GLC and GLC-MS.

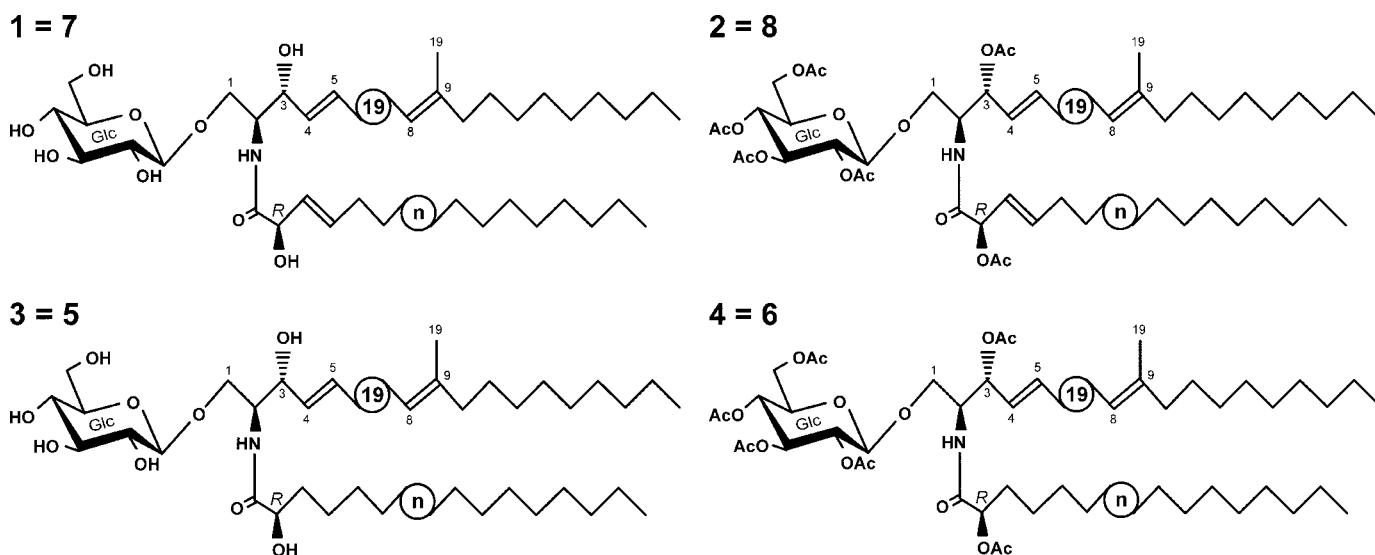
Stereochemical Analysis of Hydroxy Fatty Acids—Identification of the (*R*)- and (*S*)-stereoisomers of 2-hydroxy- $\Delta 3(E)$ fatty acids was performed as described previously (31, 32) with the following modifications. FAMES (50–100 μ g), isolated as described above, were first reduced with H₂/PtO₂ in chloroform/methanol (9:1, v/v) at room temperature (2–5 min).

GLC Analysis—GLC analysis was carried out in a CP3900 (Varian, Palo Alto, CA) with a flame ionization detector using a Select FAME[®] column (Varian; 50 m, 0.25 mm inner diameter, 0.25- μ m film thickness) with a temperature gradient starting from 100 °C (hold for 1 min) to 200 °C at 3 °C/min and continuing with 10 °C/min to 275 °C (8-min hold). Helium was used as carrier gas. Chromatograms were recorded and processed using the Galaxie chromatography data system (Varian).

GLC-MS—GLC-MS analysis was done on an HP 5975 inert XL mass selective detector (Agilent Technologies, Waldbronn, Germany) using an HP-5 MS fused silica column (30 m, 0.25-mm inner diameter, 0.25- μ m film thickness) with a temperature gradient starting from 150 °C (3 min) to 320 °C at 5 °C/min. Electron impact mass spectra were carried out at 70 eV.

Electrospray Ionization Fourier Transform Ion Cyclotron Mass Spectrometry (ESI FT-ICR MS)—ESI FT-ICR MS was performed in the positive ion mode using a high resolution APEX-QE instrument (Bruker Daltonics, Billerica, MA) equipped with a 7-tesla actively shielded magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Samples were dissolved at a concentration of ~ 10 ng/ μ l in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and 30 mM ammonium acetate adjusted with acetic acid to pH 5 and sprayed at a flow rate of 2 μ l/min. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature was set to 175 °C. The obtained mass spectra were charge deconvoluted, and the mass numbers given refer to the monoisotopic masses of the neutral molecules.

NMR Spectroscopy—One-dimensional ¹H and two-dimensional homonuclear and H-detected heteronuclear and ¹H,¹³C-correlation spectra were recorded on a 600-MHz spectrometer (Bruker Avance DRX-600) at 300 K. Spectra were taken from 0.1–0.5 mg of glucosylceramide, isolated from *F. graminearum* wild type and the $\Delta 3(E)$ -desaturase expression mutant of *P. pastoris*, in 3-mm tubes (Ultra Precision, Norell, Landisville, NJ). The peracetylated samples were dissolved in 0.25 ml of deuterated chloroform (99.96%, Cambridge Isotope Laboratories, Andover, MA) using tetramethylsilane ($\delta_H = 0.000$) and chloroform ($\delta_C = 77.0$) as internal reference. The native glycolipids were dissolved in 0.25 ml of deuterated methanol (99.96%, Deutero, Kastellaun, Germany) using methanol ($\delta_H = 3.34$, $\delta_C = 48.86$) as internal reference. One-dimensional (¹H-) and two-dimensional homonuclear (¹H,¹H-COSY (correlation



SCHEME 1. GlcCer structures as determined by NMR and ESI/MS. The circled *n* refers to the heterogeneity of the fatty acid (C_{16} or C_{18}).

spectroscopy), TOCSY (total correlation spectroscopy), ROESY (rotational frame Overhauser spectroscopy)) and H-detected heteronuclear (^1H , ^{13}C -HMQC (heteronuclear multiple-quantum coherence experiment)) experiments were recorded and processed using standard Bruker software (XWINNMR, version 3.5).

RESULTS

Glucosylceramide from F. graminearum Contains $\Delta^3(E)$ -Unsaturated Fatty Acids—GlcCer of *F. graminearum* strain PH-1 is known to contain $\Delta^3(E)$ -unsaturated fatty acids (23). To check whether this is also true for the strain 8/1, which was used in this study, GlcCer was isolated, purified, and subjected to ESI FT-ICR MS analysis (data not shown). Scheme 1 shows the GlcCer structures as determined by NMR spectroscopy and ESI/MS. The native GlcCer, **1**, had main peaks originating from $[M+H]^+$ and $[M+Na]^+$, yielding a molecular mass of 753.5785 Da, which is almost identical to the calculated mass for $C_{43}H_{79}O_9N$ (753.5755 Da). This mass is in agreement with fungal GlcCer containing the sphingoid base (4*E*,8*E*)-9-methylsphingina-4,8-dienine linked to a hydroxylated monounsaturated C_{18} -fatty acid, **1**. The same observations could be made with the peracetylated derivative of GlcCer, **2** (data not shown). The position and configuration of the double bond in the fatty acyl moiety was analyzed by NMR spectroscopy (for coupling constants, see supplemental Tables 1 and 2) and assigned as $\Delta^3(E)$. Therefore, strain 8/1 was suitable for this study.

The Extent of $\Delta^3(E)$ -Unsaturation in Fusarium Varies with the Growth Temperature—Many fungi respond to decreasing temperatures with increasing unsaturation of membrane lipids (33). We thought it might be worthwhile to check whether the $\Delta^3(E)$ -content of GlcCer in *F. graminearum* is also affected by variations in temperature. Therefore, *F. graminearum* strain 8/1 was grown in CM for 3 and 7 days at different temperatures, and the lipids were extracted. The composition of total fatty acids (supplemental Fig. 1) was analyzed by GLC and quantified. Linolenic acid (18:3) represented $\sim 30\%$ of total fatty acids isolated from cells grown at 18 °C. With increasing growth tem-

perature, the proportion of 18:3 decreased to 12% (28 °C) and 10% (30 °C), respectively. This is in line with the observations made by Suutari (33).

Because GlcCer represents only a small proportion of total lipids, the typical 2-hydroxy-unsaturated fatty acids could not be detected in the samples of total cellular fatty acids. Therefore, GlcCer was purified, and its fatty acids were analyzed using GLC (Fig. 2A), showing that the degree of unsaturation varied. GlcCer of cultures grown at 18 °C contained almost exclusively Δ^3 -unsaturated hydroxy fatty acids. At 28 °C, $\sim 75\%$ of the hydroxy fatty acids were unsaturated, whereas the level of unsaturation decreased to 25% at a growth temperature of 30 °C. No differences in the extent of Δ^3 -unsaturated fatty acids in GlcCer were measured between cultures grown at the same temperature but for different incubation periods (3 or 7 days). The proportion of (3*E*)-double bonds in GlcCer of *F. graminearum* greatly changed within a very narrow temperature range (28–30 °C), which is in contrast to the continuously changing proportion of linolenic acid in phosphoglycerolipids (Fig. 2B).

Identification of a Putative Sphingolipid $\Delta^3(E)$ -Desaturase Family with Members from Euscomycete Fungi—A bioinformatics strategy developed by Ternes *et al.* (20) was adapted to identify candidate sequences for $\Delta^3(E)$ -desaturases. Using PSI-BLAST with the *S. cerevisiae* sphingolipid α -hydroxylase Scs7p (14) and the Δ^{11} -acyl group desaturase from *T. pseudonana* (25) as queries, a collection of sequences with the histidine box motifs was assembled. The final collection of desaturase-like sequences was grouped into families, and a putative biochemical function was assessed to each family based on published experimental results (Fig. 3). This led to the identification of one additional family matching the following criteria. 1) The family comprised only sequences with so far unknown function, 2) it was present in all completely sequenced genomes of euscomycetes, 3) it was missing in yeasts and in all other fungi, and 4) it was also missing in animals. Initially, the candidate family contained six sequences from *A. fumigatus*, *A. nidulans*, *F. graminearum*,

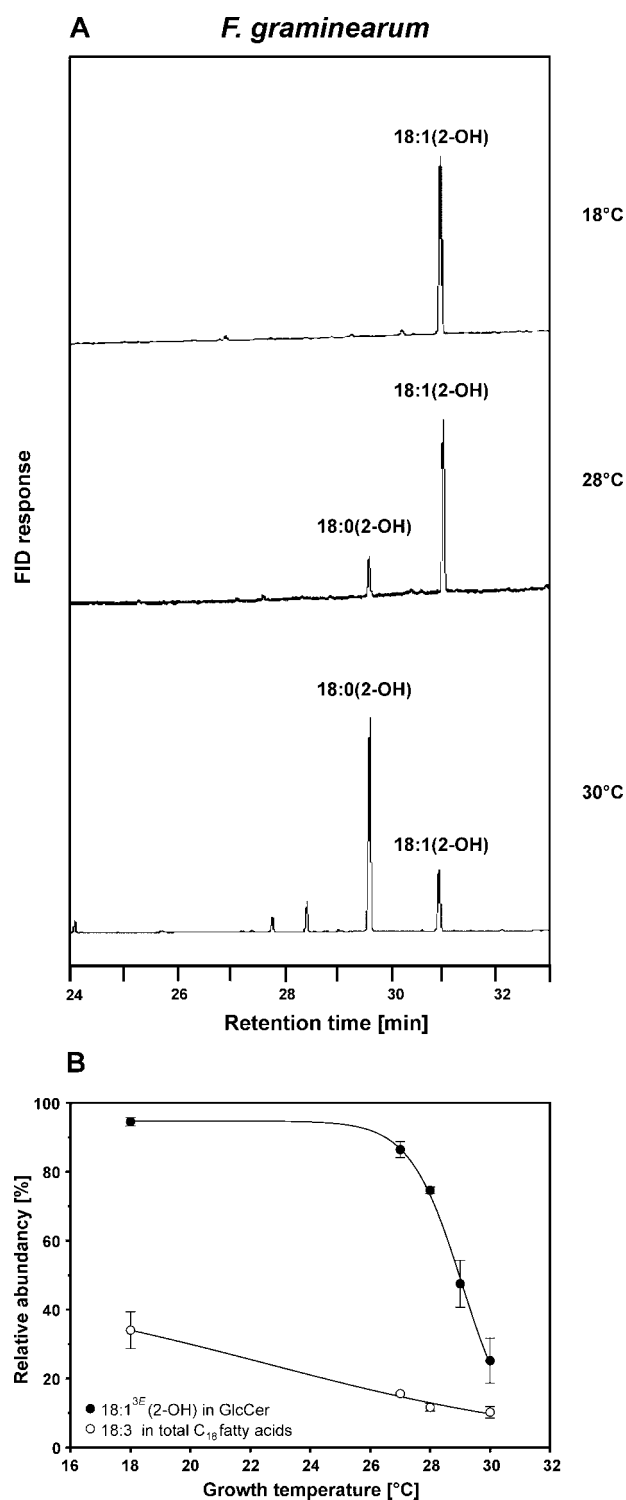


FIGURE 2. The extent of $\Delta 3(E)$ -unsaturation in GlcCer of *F. graminearum* varies depending on the growth temperature. *A*, TMS-FAMEs were derived from GlcCer of *F. graminearum* grown at 18, 28, and 30 °C and analyzed by GLC. *FID*, flame ionization detector. *B*, relative amounts of 18:1^{3E} in fatty acids of GlcCer and of 18:3 in total C₁₈ fatty acids. The level of $\Delta 3(E)$ -unsaturation in GlcCer decreased significantly when *F. graminearum* was grown at temperatures above 28 °C. Data were obtained from at least two independent experiments.

M. grisea, and *N. crassa*, but with more sequenced genomes of eucaryotes being published, this number could be raised (supplemental Table 3).

The polypeptide sequences of the candidate family vary in length between 400 and 500 amino acids. They neither possess a C-terminal nor an N-terminal cytochrome *b₅* domain, but they contain the three histidine boxes characteristic for membrane-bound desaturases. For all putative proteins, four transmembrane helices were predicted, using the program TMHMM (34). This is in line with topology predictions for other membrane-bound desaturases (Ref. 13, and reviewed in Ref. 35). Positions of the histidine boxes and putative transmembrane domains are shown in Fig. 4. Desaturases can generally be divided in two groups based on a different spacing between the first and the second histidine box. The $\Delta 3(E)$ -desaturase candidate family belongs to the group with a long spacing like the sphingolipid- $\Delta 4(E)$ - and the sphingolipid- $\Delta 8$ -desaturases (Fig. 3). The α -hydroxylases, which were expected to be more closely related to $\Delta 3$ -desaturases than other desaturases, however, belong to the group with short spacing. From the identified putative $\Delta 3(E)$ -desaturase family, we selected the candidate protein from *F. graminearum* to elucidate its apparent enzymatic function.

Cloning of the Candidate Sphingolipid- $\Delta 3(E)$ -Desaturase Gene from *F. graminearum*—Genomic DNA isolated from *F. graminearum* strain 8/1 was used as a template for PCR. The complete open reading frame (including two predicted introns) coding for the candidate sequence was amplified and cloned into the vector pGEM[®]-T. Sequencing revealed seven alterations when compared with the published sequence Fg05382 (XP_383758) from the sequenced strain PH-1. These changes led to three amino acid differences in the predicted polypeptide, indicating variations between the isolates PH-1 and 8/1. Also, cDNA obtained from *F. graminearum* was amplified by PCR, cloned into pGEM[®]-T, and sequenced. Except for the introns, genomic and cDNA sequences were identical, both displaying the same differences to XP_383758 from strain PH-1. The genomic sequence and the cDNA sequence are deposited in GenBank[™] under accession numbers FJ176922 (genomic DNA) and FJ176923 (cDNA).

Deletion of the $\Delta 3(E)$ -Desaturase Candidate Gene in *F. graminearum* Led to a Loss of $\Delta 3$ -Unsaturated Fatty Acids in GlcCer—For a functional assignment of the newly cloned sequence of *F. graminearum*, a knock-out cassette was constructed. 5'- and 3'-flanking regions of the candidate gene were amplified by PCR and cloned into pGEM[®]-T. A selection marker cassette, providing resistance to hygromycin, was ligated into the BglII-restriction sites between the flanking regions. Protoplasts of *F. graminearum* were transformed with the linearized knock-out construct, and single colonies were checked for the correct integration of the knock-out cassette with PCR and Southern blotting. GlcCer of several deletion mutants were isolated, and their fatty acid compositions were analyzed by GLC (Fig. 5). The resulting fatty acid profiles were dominated by 2-hydroxyoctadecanoic acid, whereas $\Delta 3(E)$ -unsaturated components were not detected. ESI FT-ICR MS analysis (data not shown) of the native GlcCer of the knock-out mutant, **3**, resulted in main peaks originating from [M+H]⁺ and [M+Na]⁺, yielding a molecular mass of 755.5917 Da almost identical to the value calculated for C₄₃H₈₁O₉N (755.5911 Da) and indicating GlcCer with a saturated fatty acyl

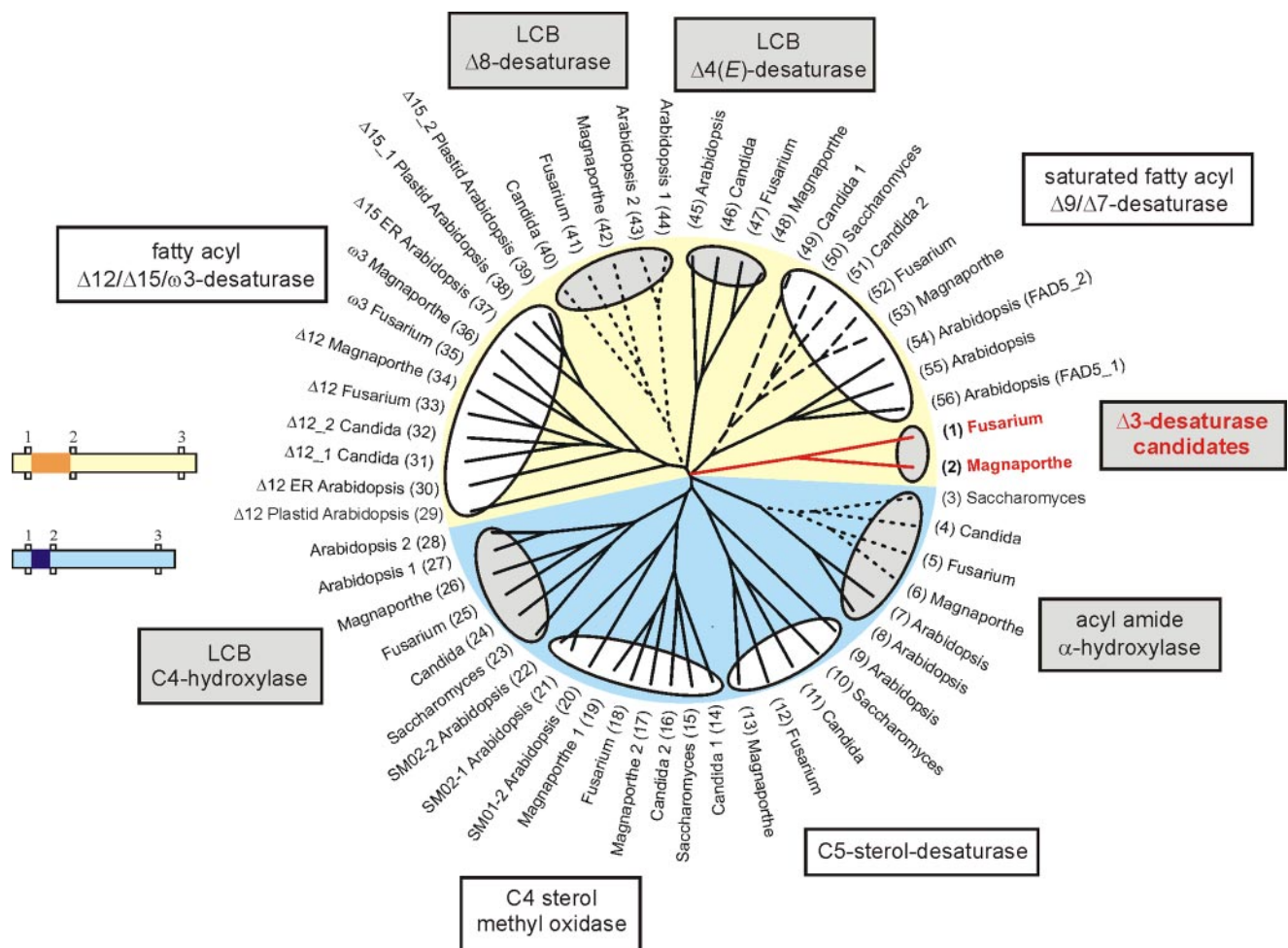


FIGURE 3. Dendrogram showing desaturase-like sequences from *F. graminearum*, *M. grisea*, *Candida albicans*, *S. cerevisiae*, and *A. thaliana*. Sphingolipid-modifying enzymes are shaded in gray, and the $\Delta 3(E)$ -desaturase candidates are marked in red. Cytochrome b_5 -fusion proteins are indicated by dashed lines (C-terminal fusion) and dotted lines (N-terminal fusion), respectively. The yellow and light blue background indicates groups of desaturase and hydroxylase sequences distinguished by a different spacing between the first and the second histidine box. The small pictures illustrate the distance between these histidine boxes, which is 25–34 amino acids in the sequences with yellow background and 7–18 amino acids in the sequences with light blue background. The spacing is highlighted in dark yellow and dark blue, respectively. GenBank protein accession numbers are: 1) XP_383758, 2) XP_368852, 3) NP_013999, 4) XP_717303, 5) XP_385340, 6) XP_361446, 7) NM_129030, 8) NM_11820, 9) NP_192948, 10) NP_013157, 11) XP_713612, 12) XP_382678, 13) XP_363987, 14) XP_722703, 15) NP_011574, 16) XP_713456, 17) XP_360827, 18) XP_390006, 19) XP_369331, 20) NP_567670, 21) NP_850133, 22) NP_563789, 23) NP_010583, 24) XP_715359, 25) XP_382698, 26) XP_369735, 27) AAF43928, 28) AAG52550, 29) NP_194824, 30) NP_850139, 31) XP_722258, 32) XP_714324, 33) XP_385960, 34) XP_365283, 35) XP_388066, 36) XP_362963, 37) NP_180559, 38) NP_187727, 39) NP_196177, 40) XP_719958, 41) XP_390021, 42) XP_361026, 43) NP_182144, 44) AAD00895, 45) NP_192402, 46) XP_722116, 47) XP_390550, 48) XP_001404401, 49) XP_714854, 50) NP_011460, 51) XP_717653, 52) XP_386360, 53) XP_363864, 54) NP_565721, 55) BAC43716, and 56) NP_172098. ER, endoplasmic reticulum; LCB, long-chain base.

moiety, 18:0(2-OH). The molecular mass corresponding to $\Delta 3$ -unsaturated GlcCer was not detected. The obtained molecular mass for the peracetylated derivative, **4**, was also almost identical to that calculated for GlcCer with a saturated C_{18} fatty acid (data not shown). These data show that the identified gene is essential for $\Delta 3$ -unsaturation of GlcCer. To verify that it encodes the actual desaturase, the corresponding cDNA from *F. graminearum* was expressed in the yeast *P. pastoris*.

The Heterologous Expression of the Candidate Gene from *F. graminearum* Resulted in the Formation of $\Delta 3$ -Unsaturated Fatty Acids—Ceramide or GlcCer of *P. pastoris* was considered as a suitable substrate for the putative $\Delta 3(E)$ -desaturase because the only difference for the corresponding sphingolipids from *F. graminearum* is the missing $\Delta 3$ -double bond. The complete open reading frame of the candidate cDNA of *F. graminearum* was cloned into the *P. pastoris* expression vector pPIC3.5 under control of the inducible *AOX1* promoter. The

plasmid was linearized and used for transformation of *P. pastoris* strain GS115. GlcCer was isolated from several *P. pastoris* transformants and used for fatty acid analysis. As shown in Fig. 6, the expression of the heterologous desaturase from *F. graminearum* ($\Delta 3Fg$) led to the formation of unsaturated fatty acids. The amide-linked fatty acyl moiety of GlcCer consisted of 50–100% 2-hydroxyoctadecenoic acid, 18:1(2-OH). This level of unsaturation in *P. pastoris* cells expressing $\Delta 3Fg$ is remarkably high when compared with the activity of other desaturases in heterologous expression systems (20).

The identity of the fatty acids isolated from GlcCer of *P. pastoris* wild type and the expression mutant indicated in Fig. 6 was confirmed by ESI FT-ICR MS analysis, and the latter was also confirmed by NMR spectroscopy. Upon ESI FT-ICR MS analysis, *P. pastoris* wild type GlcCer, **5**, was found to have the same peaks as detected for the GlcCer of the *F. graminearum* knockout mutant, **3**, however with different intensities (data not

$\Delta 3$ -Desaturase from *Fusarium*

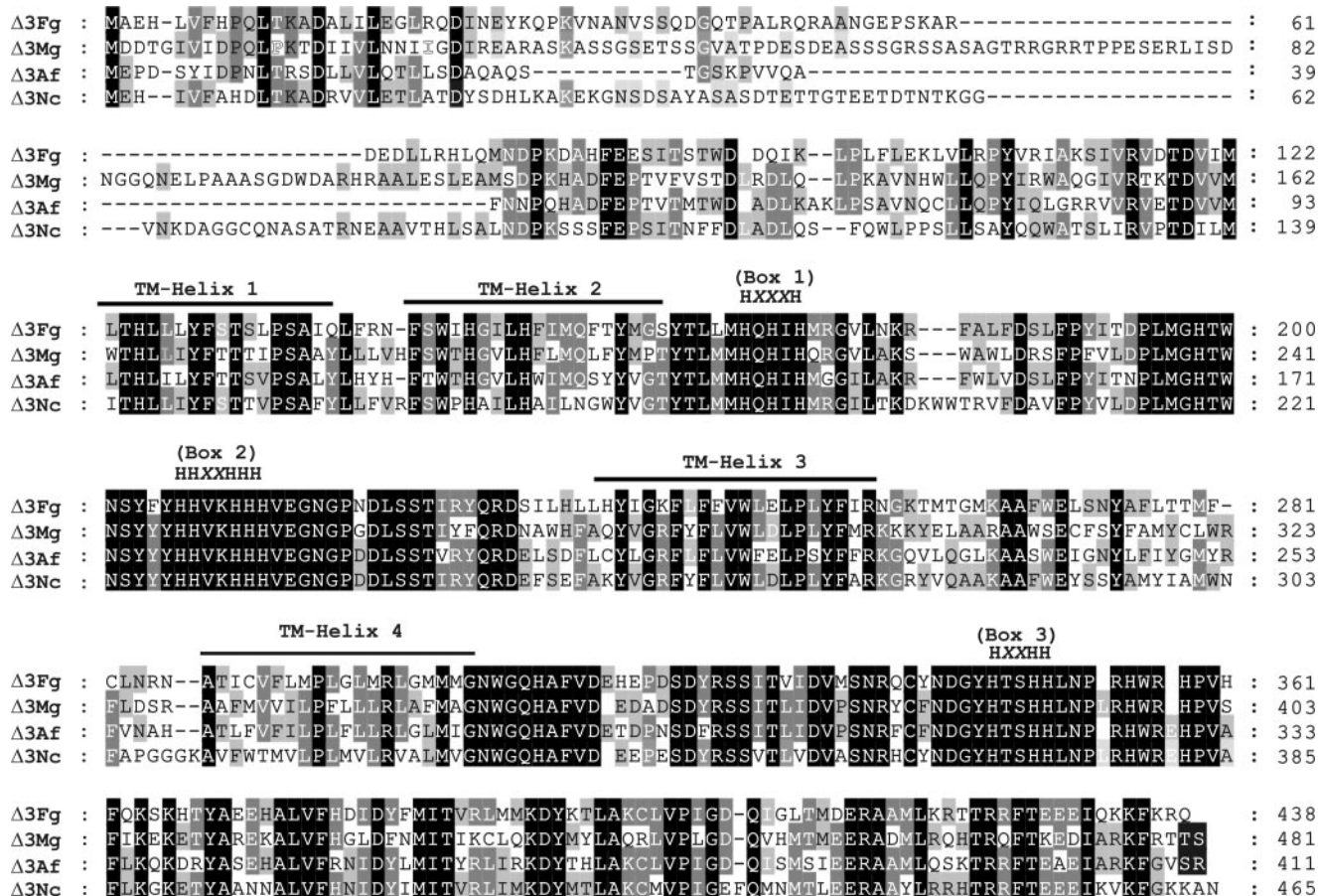


FIGURE 4. Sequence alignment of $\Delta 3(E)$ -desaturase candidate polypeptide sequences from $\Delta 3Fg$, *M. grisea* ($\Delta 3Mg$), *A. fumigatus* ($\Delta 3Af$), and *N. crassa* ($\Delta 3Nc$). Histidine boxes and predicted transmembrane helices (TM-Helix) are indicated.

shown). By contrast, in GlcCer of *P. pastoris* cells expressing $\Delta 3Fg$, 7, the same molecular mass as for GlcCer of *F. graminearum* wild type, 1, was identified, being in agreement with GlcCer containing 18:1(2-OH) (data not shown). These data were further corroborated by the ESI FT-ICR MS analysis of the peracetylated GlcCer derivatives of *P. pastoris* wild type and the mutant expressing $\Delta 3Fg$, 6 and 8, clearly revealing that the identified sequence encodes a desaturase.

To determine the position and configuration of the double bond introduced by the enzyme, NMR analysis was performed both with the native and with the peracetylated GlcCer isolated from *P. pastoris* expressing $\Delta 3Fg$, 7, 8 (supplemental Tables 1 and 2). In the 1H NMR spectrum, signals characteristic for the double bond between H-3' and H-4' assigned to the 2-hydroxy fatty acid showed diagnostic coupling constant for the (*E*)-configuration in the native ($J_{3,4} = 15.3$ Hz) and in the peracetylated ($J_{3,4} = 14.3$ Hz) GlcCer. These were further supported by downfield shifts of the protons (supplemental Table 1) as well as the corresponding ^{13}C resonances in the HMQC spectrum for C-3' and C-4', respectively (supplemental Table 2). All other signals were in good agreement with published data for the native GlcCer (6, 23) and the peracetylated derivative (36–38).

To complete the structural analysis, we further investigated the stereochemical position of the 2-hydroxy group in the fatty acid of GlcCer from *P. pastoris* and *F. graminearum*. GLC-MS analysis of the diastereomeric methoxy fatty acid-(*S/R*)-phenyl-

ethylamides clearly revealed that the 2-hydroxy fatty acids were all (*R*)-configured (not shown), in contrast to 2-hydroxy fatty acids found in bacterial GlcCer, where the configuration is usually (*S*), as described for example in Ref. 39. Based on these results, we could identify the unsaturated GlcCer as *N*-(*R*)-2'-hydroxy-(3*E*)-octadec-3-enoyl-1-*O*- β -D-glucopyranosyl-(4*E*, 8*E*)-9-methyl-sphinga-4,8-dienine.

DISCUSSION

$\Delta 3(E)$ -Double bonds are present in different classes of lipids (Fig. 1), such as fungal glycosylceramide, plant plastidial phosphatidyl-glycerol, and triacylglycerol from seeds of some higher plants. Therefore, we expected to find sequences both from fungi and from plants in the bioinformatics analysis. However, our strategy led to the identification of a desaturase subfamily, which contains exclusively sequences from eukaryotes, coding for acylamide- $\Delta 3(E)$ -desaturases. No plant homologue has been found, which would have been expected at least for fully sequenced and annotated genomes like *Arabidopsis thaliana*. This indicates that the putative PG- $\Delta 3(E)$ -desaturase (*fad4*) is of different origin.

In this study, we describe the identification and molecular characterization of the first desaturase with $\Delta 3(E)$ -regioselectivity. The enzyme introduces the double bond into an α -hydroxy fatty acyl moiety of fungal sphingolipids. The vicinal

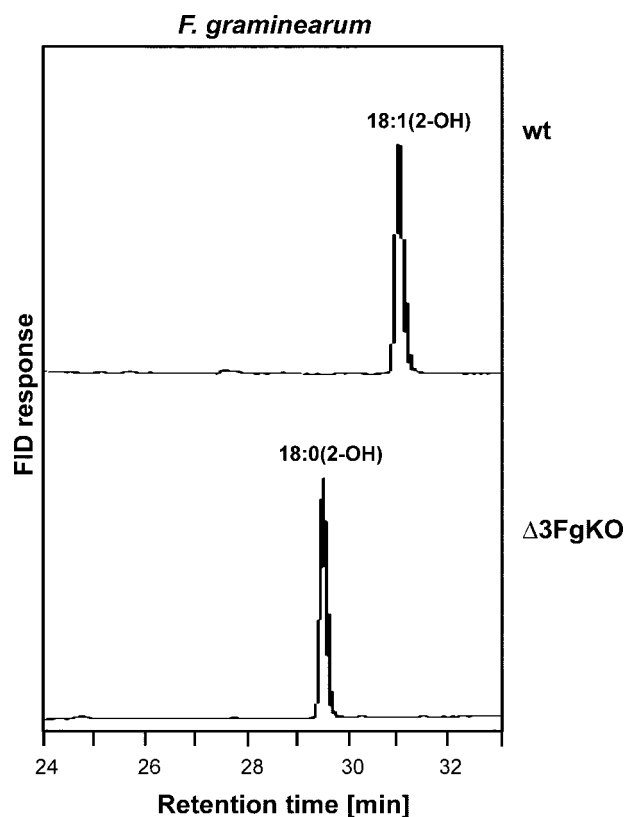


FIGURE 5. Deletion of the $\Delta 3(E)$ -desaturase candidate gene in *F. graminearum* led to a loss of unsaturated fatty acids in GlcCer. TMS-FAMES were derived from GlcCer of *F. graminearum* grown at 18 °C and analyzed by GLC. The knock-out mutant ($\Delta 3FgKO$) lacked the double bond in the hydroxy-fatty acid. FID, flame ionization detector; wt, wild type.

2-hydroxy group in the sphingolipid is most likely required for the $\Delta 3$ -desaturase activity.

The biological functions of $\Delta 3$ -double bonds in plastidial PG and fungal sphingolipids are not known. The fatty acid composition of plant plastidial PG is supposed to be important for chilling tolerance of plants (40, 41) and electron transport in photosystem II (reviewed in Ref. 42). However, the $\Delta 3(E)$ -unsaturation in position *sn2* is not crucial for these effects. A chemical mutant of *A. thaliana* lacking the $\Delta 3(E)$ -double bond in PG does not show a conspicuous phenotype regarding both chilling sensitivity and photosynthesis (43). The physical properties of the plastidial membrane are only slightly influenced by the introduction of a $\Delta 3(E)$ -double bond into PG (44).

We analyzed whether $\Delta 3(E)$ -unsaturation in *F. graminearum* is temperature-dependent and found that a shift in growth temperature from 28 to 30 °C resulted in a dramatic decrease of $\Delta 3(E)$ -unsaturated fatty acids in GlcCer, whereas the composition of total fatty acids remained almost unchanged. This substantial change in $\Delta 3(E)$ -unsaturation probably does not reflect an adaptation of membrane fluidity to varying temperatures, which has been described for *Z*-unsaturation in phosphoglycerolipids (33). These data rather suggest that the regulation of the $\Delta 3$ -desaturase differs from those of other fatty acid desaturases. The observed decrease in the level of unsaturation of GlcCer between 28 and 30 °C is not due to a temperature sensitivity of the enzyme because full activity could be achieved by

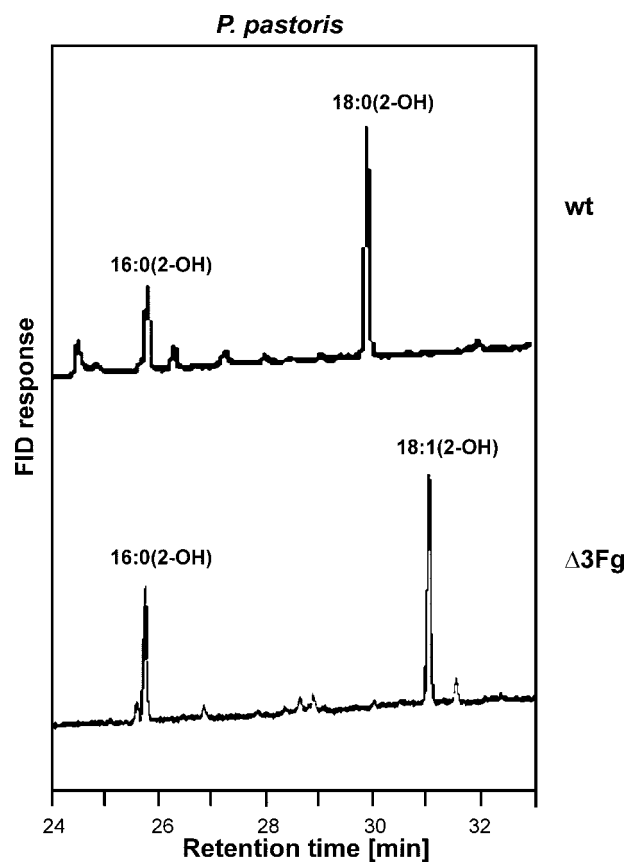


FIGURE 6. Heterologous expression of the candidate gene from *F. graminearum* in *P. pastoris* resulted in the formation of $\Delta 3(E)$ -unsaturated fatty acids. Fatty acids from GlcCer isolated from *P. pastoris* transformants were analyzed by GLC as TMS-FAMES. $\Delta 3Fg$ converted 2-hydroxyoctadecanoic acid 18:0(2-OH), efficiently (50–100%) into 2-hydroxyoctadecenoic acid, 18:1(2-OH). FID, flame ionization detector; wt, wild type.

heterologous expression in *P. pastoris* both at 28 °C and at 30 °C.

It seems likely that the $\Delta 3(E)$ -desaturase is specifically regulated by an unknown mechanism and may play a role in signaling processes. However, the $\Delta 3(E)$ -knock-out strain of *F. graminearum* does not show any obvious phenotype, and more detailed analyses of the mutant are required to ascertain the functions of the $\Delta 3(E)$ -desaturase for the life cycle of the fungus.

Because GlcCer from yeasts and filamentous fungi is critical for the activity of antifungal peptides (AFPs) from plants and insects (23, 45), we determined the growth inhibitory effect of two AFPs from the plant *Raphanus sativus* and the fungus *Aspergillus giganteus*. Although the *F. graminearum* wild type was sensitive to both AFPs, the $\Delta 3(E)$ -knock-out strain interestingly became resistant. In addition, the *P. pastoris* strain expressing the $\Delta 3$ -desaturase from *Fusarium* became hypersensitive to both AFPs (in comparison with the wild type). These data indicate that the $\Delta 3(E)$ -double bond in GlcCer is important for the interaction of the fungus with AFPs from *R. sativus* and *A. giganteus*. We will describe the experiments in a separate study.

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