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Isolation and characterization of *Neurospora crassa* mutants resistant to antifungal plant defensins

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Abstract

Twenty-five *Neurospora crassa* mutants obtained by chemical mutagenesis were screened for increased resistance to various antifungal plant defensins. Plant defensin-resistant *N. crassa* mutants were further tested for their cross-resistance towards other families of structurally different antimicrobial peptides. Two *N. crassa* mutants, termed MUT16 and MUT24, displaying resistance towards all plant defensins tested but not to structurally different antimicrobial peptides were selected for further characterization. MUT16 and MUT24 were more resistant towards plant defensin-induced membrane permeabilization as compared to the *N. crassa* wild-type. Based on the previously demonstrated key role of fungal sphingolipids in the mechanism of growth inhibition by plant defensins, membrane sphingolipids of MUT16 and MUT24 were analysed. Membranes of these mutants contained structurally different glucosylceramides, novel glycosylinositolphosphorylceramides, and an altered level of steryl glucosides. Evidence is provided to link these clear differences in sphingolipid profiles of *N. crassa* mutants with their resistance towards different plant defensins.

Keywords: Mode of action; Membrane permeabilization; Sphingolipid; Glucosylceramide; Glycosylinositolphosphorylceramide; Steryl glucoside; Heliomicin

1. Introduction

Multicellular organisms produce a battery of antimicrobial peptides and proteins to defend themselves against microbial attack or injury. One particular family of antimicrobial peptides, occurring in different plant species, are plant defensins (reviewed in Broekaert et al., 1997; Thomma et al., 2002). Plant defensins are small (45–54 amino acids) highly basic cysteine-rich peptides that are apparently ubiquitous throughout the plant kingdom. The global fold of plant defensins comprises a cysteine-stabilized αβ motif (CSαβ motif) consisting of an α-helix and a triple-stranded β-sheet, organized in a βαββ architecture and stabilized by four disulfide bridges (Thomma et al., 2002). Regarding amino acid composition, the plant defensin family is quite diverse; sequence conservation is restricted to eight structurally important cysteines (Thomma et al., 2002). Some plant defensins are not found to display any antimicrobial activity, while others are demonstrated to have antifungal or antibacterial activity in vitro. Plant defensins appear not to be toxic to either mammalian or plant cells. Among plant defensins displaying antimicrobial activity, some peptides exhibit antifungal activity with an additional hyperbranching effect on specific target fungi, while others inhibit the growth of fungi without causing morphological changes (Broekaert et al., 1997, 1995; Osborn et al., 1995).

Further analysis of the antifungal activity demonstrated that the plant defensins DmAMP1 and RsAFP2, isolated from dahlia (*Dahlia merckii*) and radish (*Raphanus sativus*), respectively, induce an array of

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relatively rapid responses in fungal membranes, including increased K^+ efflux, increased Ca^{2+} uptake, membrane potential changes, and membrane-permeabilization (Thevissen et al., 1996, 1999). Using radiolabeled plant defensins, the existence of high-affinity binding sites for these defensins on fungal cells and plasma membrane fractions was demonstrated (Thevissen et al., 1997, 2000b). For most plant defensins, molecular components involved in signaling and possible intracellular targets remain unknown. Only in case of DmAMP1 and RsAFP2, part of the molecular basis of their inhibitory effect was recently elucidated. Through a genetic complementation approach, *IPT1* was identified as a gene determining sensitivity towards DmAMP1 in the yeast *Saccharomyces cerevisiae* (Thevissen et al., 2000a). *IPT1* encodes an enzyme involved in the last step of the biosynthesis of the inositolphosphoryl-containing sphingolipid mannosyldiinositolphosphorylceramide ($M(IP)_2C$[1]) (Dickson et al., 1997). *S. cerevisiae* strains carrying a non-functional *IPT1* allele lack $M(IP)_2C$ in their plasma membranes, bind less DmAMP1 compared to wild-type strains, and are highly resistant to DmAMP1-mediated membrane permeabilization and growth inhibition. Apparently, DmAMP1-susceptibility of different yeast strains is linked to the presence of $M(IP)_2C$ in their membranes (Im et al., 2003; Thevissen et al., 2000a). Similarly, the *GCS* gene was demonstrated to be an RsAFP2-sensitivity gene in the yeast *Pichia pastoris* (Thevissen, K., unpublished data; Thomma et al., 2003). This gene encodes a glucosyltransferase that catalyses the final step in the biosynthesis of glucosylceramides (GlcCer), membrane compounds that are structurally related to the inositolphosphoryl-containing sphingolipids (Leipelt et al., 2001). Sphingolipids like $M(IP)_2C$ and GlcCer are, along with sterols and phospholipids, one of the three major types of lipid compounds found in eukaryotic membranes. Possibly, membrane patches containing inositolphosphoryl-containing sphingolipids and/or glucosylceramides act as binding sites for the plant defensins (Thevissen et al., 2000a; Thomma et al., 2003). Subsequently, this interaction leads to insertion of the plant defensins into the membrane resulting in membrane destabilization. Whether such destabilization directly causes fungal cell growth inhibition or whether DmAMP1 and RsAFP2 act through intracellular targets is currently unknown.

In the present study we screened several *Neurospora crassa* mutants, obtained by chemical mutagenesis, for increased resistance to various plant defensins. In addition, these *N. crassa* mutants were tested for their cross-resistance towards other families of structurally different antimicrobial plant proteins. Based on the previously demonstrated key role of inositolphosphoryl-containing sphingolipids and GlcCer in the mechanism of fungal growth inhibition by certain defensins (Thevissen et al., 2000a; Thomma et al., 2003), two plant defensin-resistant *N. crassa* mutants were characterized with respect to plant defensin-induced membrane permeabilization and complex lipid composition.

[1] *Abbreviations used:* AMP, antimicrobial peptide; AFP, antifungal peptide; GlcCer, glucosylceramide; GlcSte, steryl glucoside; GIPC, glycosylinositolphosphorylceramide; $M(IP)_2C$, mannosyldiinositolphosphorylceramide; WT, wild-type.

2. Materials and methods

2.1. Materials and microorganisms

The antifungal proteins DmAMP1 and Hs-AFP1 (Osborn et al., 1995), RsAFP2 and RsAFP2 (Terras et al., 1992), AcAMP1 (Broekaert et al., 1995), Ace-AMP1 (Cammue et al., 1995), and IbAMP4 (Tailor et al., 1997) were isolated as previously described. The insect defensin-like peptide heliomicin was kindly provided by Dr. P. Bulet (Atheris Laboratories, Switzerland) and Dr. J.L. Dimarcq (Entomed, Strasbourg, France). Ethyl methanesulfonate (EMS) was obtained from Sigma. SYTOX Green was purchased from Molecular Probes (Eugene, Oreg.). *N. crassa* strains (FGSC collection number 987, MAT A, termed wild-type (WT); and FGSC collection number 988, MAT a) and *N. crassa* mutants (derived from FGSC 987) were grown on half-strength potato dextrose broth agar (PDA, 12 g/l PDB, Difco, Detroit, Mich.; 15 g/l agar, Difco).

2.2. Chemical mutagenesis of N. crassa and isolation of RsAFP2-resistant N. crassa mutants

Conidiospores of the *N. crassa* WT were mutagenized by treatment with ethylmethanesulfonate (EMS). To this end, 100 μl aliquots of conidiospores ($\sim 10^7$ spores/ml in 20% (v/v) glycerol) were resuspended in 100 μl 100 mM sodium phosphate (pH 7.5). After adding 1 μl EMS solution, the spore suspension was incubated at 25 °C with continuous shaking (300 rpm). After 45 min, EMS was inactivated by addition of 200 μl 5% thiosulfate. To calculate the rate of spontaneous mutagenesis, an additional aliquot of conidiospores was treated similarly, except that EMS was omitted. Spore suspensions were diluted in 70 ml half-strength PDB containing 4 μM RsAFP2. The suspensions were then divided in 100 μl subcultures in 96-well flat-bottom microtiter plates (Greiner) which were subsequently incubated at room temperature for 5 days and scored for growth on a regular basis. Putative mutants were transferred to PDA plates.

2.3. Antifungal activity assay

Fragments from the edges of the mycelium lawns were transferred to 50 ml half-strength PDB and incubated

for 24–48 h at 25 °C with continuous shaking. Aliquots (500 μl) of these cultures were transferred to 2 ml polypropylene microcentrifuge tubes with o-ringed screw caps each containing five glass beads (1-mm diameter). The mycelium was fragmented by high speed reciprocal shaking using a Phastprep apparatus (Bio 101/Savant, Farmingdale). The obtained mycelium fragment suspensions were 100-fold diluted for use in antifungal activity assays. Antifungal activities of protein samples were assayed by microspectrophotometry as described previously (Broekaert et al., 1990; Terras et al., 1992). Incubation medium was either half-strength PDB or Synthetic Medium for Fungi 1 (SMF1, (Thevissen et al., 1999)). Antifungal activity assays were repeated twice.

2.4. SYTOX Green uptake assay

The SYTOX Green uptake assay was performed as described previously (Thevissen et al., 1999) except for the preparation of the suspension of mycelium fragments. *N. crassa* WT (10^5 conidiospores/ml) and mutants (fragments from the mycelium on the agar plates) were incubated in 50 ml half-strength PDB cultures for 20 and 36 h, respectively. Fragmentation of the mycelium in SMF1 was achieved by vortexing vigorously. Aliquots of this mycelium suspension were used in the SYTOX Green uptake assay.

2.5. Fungal isolates, growth conditions, and crossing procedures

For the purpose of lipid extraction, cultures of *N. crassa* WT and mutants, grown on solid YPD agar plates (10 g/L yeast extract; 20 g/L peptone; and 20 g/L glucose) at room temperature, were transferred to 1.2-L liquid PDB in 2.5-L Fernbach flasks and shaken for 3 days at 25 °C, 250 rpm. Mycelia were harvested by filtration through cheesecloth, washing off excess medium with water. Excess water was removed by gentle pressure, and mycelia either stored at −80 °C until extraction or processed immediately as described below. Crossing of *N. crassa* isolates of opposite mating type was performed as described previously (Davis and De Serres, 1970).

2.6. Solvents for extraction, anion exchange chromatography, and high performance thin layer chromatography

Solvent A, chloroform/methanol (1:1, v/v); solvent B, isopropanol/hexane/water (55:25:20, v/v/v, upper phase discarded); solvent C, chloroform/methanol/water (30:60:8, v/v/v); solvent D, isopropanol/hexane/water (55:40:5 v/v/v); solvent E, chloroform/methanol/2 N ammonium hydroxide (40:10:1 v/v/v); and solvent F, chloroform/methanol/water (50:47:14 v/v/v, containing 0.035% w/v $CaCl_2$).

2.7. Extraction and purification of sphingolipids and steryl glucosides

Extraction and purification of sphingolipids were carried out as described previously (Levery et al., 2002; Toledo et al., 1995, 1999, 2000, 2001), with minor modifications. Briefly, sphingolipids were extracted by homogenizing mycelia (40–80 g wet weight) in a glass-walled blender, once with 200 ml of solvent A, two times with 200 ml of solvent B, and once more with 200 ml of solvent A. The four extracts were pooled and dried on a rotary evaporator. The dried residue was partitioned between water and 1-butanol pre-saturated with water (200 ml each) with vigorous shaking in a separatory funnel. The lower (water) layer was removed, and similarly extracted four more times with equal volumes of water-saturated 1-butanol. The five 1-butanol extracts were combined in a round-bottom flask and evaporated to dryness. The dried residue from this step was then treated with 20 ml methanol–water–1-butanol (4:3:1 v/v/v) containing 25–30% methylamine at 55 °C for 4 h (flask tightly stoppered), with occasional agitation. After removal of the reagent solution by rotary evaporation, the residue was resuspended in a minimal volume of solvent C and applied to a column of DEAE-Sephadex A-25 (Ac^- form). Neutral lipids, including putative glucosylceramides (GlcCer) and steryl glucosides (GlcSte), were eluted with five volumes of solvent C. The neutral fraction was then dried and taken up in solvent D prior to analytical or preparative HPTLC as described below. Acidic lipids, including putative glycosylinositolphosphorylceramides (GIPC), were eluted with five volumes of 0.5 M sodium acetate in MeOH. The acidic fraction was dried, dialysed exhaustively against deionized water, redried, and taken up in solvent B prior to analytical HPTLC, as described below.

2.8. High performance thin layer chromatography and preliminary characterizations

Both, analytical and preparative HPTLC, were performed on silica gel 60 plates (Merck, Darmstadt, Germany) using solvents E or F for neutral or acidic lipids, respectively. Samples were dissolved in solvents D or B for neutral or acidic lipids, respectively, and applied by streaking from 10 μl Micro-caps (Drummond, Broomall, PA). For analytical HPTLC, detection was made by Bial's orcinol reagent (orcinol 0.55% [w/v] and H_2SO_4 5.5% [v/v] in ethanol/water 9:1 [v/v]; the plate is sprayed and heated briefly to ~200–250 °C). For preparative HPTLC of neutral glycolipids (putative GlcCer and GlcSte), samples were streaked lengthwise on 10×20-cm plates. Separated glycolipid bands were

visualized under UV after spraying with primulin (Aldrich; 0.01% in 80% aqueous acetone). Bands were marked by pencil and individually scraped from the plate. Glycolipids were then isolated from the silica gel by repeated sonication in solvent A followed by centrifugation. Following concentration of the extract, primulin was removed by passage through a short column of DEAE-Sephadex A-25 in Solvent C. Preliminary characterizations of putative GlcCer and GlcSte fractions were carried out by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and electrospray-ionization mass spectrometry (ESI-MS) as described previously (Levery et al., 2000, 2002; Toledo et al., 1999, 2000, 2001).

2.9. Statistical analysis

To determine a possible correlation between SYTOX Green uptake and antifungal activity, *P* values of the corresponding data were calculated with Microsoft Excel software, by calculating the analysis of variance. If the *P* value of the data was lower than 0.05, it was concluded that the data were significantly correlated.

3. Results

3.1. Chemical mutagenesis of N. crassa and isolation of RsAFP2-resistant N. crassa mutants

Neurospora crassa mutants were generated by chemical mutagenesis of *N. crassa* WT using ethylmethanesulphonate (EMS) and selected for resistance towards 4 μM of the radish plant defensin RsAFP2. The frequency of appearance of RsAFP2-resistant mutants was eight times higher in chemically mutagenized populations compared to non-mutagenized populations ($1/4 \times 10^5$ fungal spores versus $1/3.4 \times 10^6$ fungal spores, respectively). Twenty-five *N. crassa* mutants were isolated (MUT1–MUT25) and characterized further.

3.2. Antifungal activity assay and cross-resistance of N. crassa mutants

N. crassa WT grows superficially on solid medium whereas the RsAFP2-resistant *N. crassa* mutants tend to grow into the agar and have a very compact appearance (Fig. 1). These mutants grow about 10-fold slower than the *N. crassa* WT (1-cm radial growth/96 h versus 1-cm radial growth/10 h, respectively). In addition, the ability to form ascospores seems to be lost for most of these mutants: when mutants were crossed to wild-type isolates of the opposite mating type, neither fruiting bodies nor ascospores were observed (data not shown). Crossing of the corresponding wild-type isolates, on the contrary, resulted in normal ascospore formation.

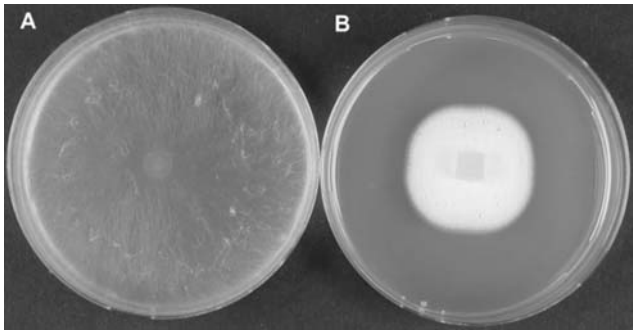

Fig. 1. Growth of *N. crassa* (A) WT and (B) MUT16 on solid PDA plates.

Therefore, antifungal activity assays were performed on outgrowing mycelium fragments of these mutants. Resistance and cross-resistance of these mutants towards different types of antifungal peptides was evaluated. These included the plant defensins RsAFP2, Hs-AFP1, and DmAMP1, isolated from radish (*R. sativus*), *Heuchera sanguinea*, and dahlia (*D. merckii*) respectively, and three structurally unrelated antimicrobial plant proteins, namely AceAMP1, extracted from onion seeds and sharing structural analogies to plant non-specific lipid transfer proteins (Cammue et al., 1995), AcAMP1, isolated from *Amaranthus caudatus* seeds and sharing sequence similarity to the cysteine/glycine-rich domain of chitin-binding proteins (Terras et al., 1992) and IbAMP1, a highly basic cysteine-rich peptide from seeds of *Impatiens balsamina* (Tailor et al., 1997).

As summarized in Table 1, mutants can be divided in three groups based on sensitivity to different antimicrobial proteins. The largest group of mutants showed resistance to all antifungal peptides tested (group I). A second group (II) of mutants was found to be resistant to all plant defensins, but remained sensitive towards at least one of the structurally unrelated antifungal peptides. A last group (group III) consisted of mutants with various resistance patterns towards the different antimicrobial compounds tested. The proposed classification of these *N. crassa* mutants should not be considered as mutually exclusive since several mutants, e.g., MUT2 and MUT14, displayed intermediate resistance characteristics (between groups I and III). Because of their specific resistance to plant defensins, mutants of the second group (MUT 16 and MUT24) were chosen for further study. Both mutants appeared to be at least 16-fold more resistant to the plant defensins tested as compared to *N. crassa* WT (Table 1). In addition, heliomicin, a plant defensin-like antifungal insect peptide (Lamberty et al., 2001; Thomma et al., 2002) displayed activity against *N. crassa* WT (IC_{50} value = 0.04 μM), whereas heliomicin was not active against the *N. crassa* mutants MUT16 and MUT24 (IC_{50}-value >20 μM).

Table 1
Activity of antifungal peptides against *N. crassa* strains

Strain	IC_{50} (μM)[a]					
	RsAFP2	Hs-AFP1	DmAMP1	AceAMP1	IbAMP2	AcAMP2
Wild-type	1.25	0.4	0.4	2	2.5	0.3
Group I						
MUT1	>20	>20	>20	17	>20	>20
MUT3	>20	>20	>20	>20	20	20
MUT4	>20	>20	>20	>20	>20	>20
MUT5	>20	>20	>20	>20	>20	>20
MUT6	>20	>20	>20	>20	>20	20
MUT7	>20	>20	>20	>20	>20	20
MUT8	>20	>20	>20	>20	>20	>20
MUT9	>20	>20	>20	>20	>20	20
MUT10	>20	>20	>20	>20	>20	>20
MUT15	>20	>20	>20	>20	>20	20
MUT17	>20	>20	>20	>20	>20	>20
MUT20	>20	>20	>20	>20	>20	15
MUT21	>20	>20	>20	>20	>20	>20
Group II						
MUT12	>20	20	>20	4.2	10	5.0
MUT16	**>20**	**20**	**20**	**<0.1**	**2.5**	**0.3**
MUT19	>20	20	>20	4.2	10	5.0
MUT24	**>20**	**20**	**>20**	**1.7**	**12**	**5.0**
Group III						
MUT2	>20	>20	>20	17	20	10
MUT11	>20	>20	>20	>20	10	5.0
MUT13	>20	>20	>20	8.3	>20	10
MUT14	>20	>20	>20	>20	20	10
MUT18	>20	1.2	20	8.3	5.0	2.5
MUT22	4	>20	>20	4.2	10	5.0
MUT23	>20	>20	>20	8.3	>20	15
MUT25	>20	>20	>20	2.0	>20	10

[a] Concentration required for 50% inhibition of fungal growth as defined under 'Materials and methods'. IC_{50}-values were calculated after 48 h of incubation and are means of duplicate measurements. Experiments were at least repeated twice. Standard errors were less than 8.5%. Mutants in bold were further characterized.

3.3. SYTOX Green uptake assay

Possible membrane permeabilization induced by RsAFP2-, Hs-AFP1-, and DmAMP1 in these mutants was tested using a SYTOX Green uptake assay. SYTOX Green is a high affinity nucleic acid stain that fluoresces upon nucleic acid binding. It can only penetrate cells with compromised plasma membranes (Matsuzaki et al., 1997; Roth et al., 1997; Schmitt and Compain, 1995). Previously, we have shown that DmAMP1, RsAFP2, and Hs-AFP1 induce membrane permeabilization on intact hyphae of *N. crassa* WT (Thevissen et al., 1999). Similarly, these plant defensins also induced membrane permeabilization on mycelium fragments of *N. crassa* WT (Fig. 2, upper panel). A significant correlation between the induced hyphal permeabilization and the antifungal activity of the plant defensins (Fig. 2, lower panel) could be observed. In case of *N. crassa* mutants MUT16 and MUT24, no significant permeabilization was detected upon incubation of mycelium fragments in the presence of different concentrations of RsAFP2, Hs-AFP1, and DmAMP1 (Fig. 2, both).

3.4. HPTLC profile of neutral and acidic lipid fractions of N. crassa WT, MUT16, and MUT24

Previously, we have shown that DmAMP1-susceptibility of yeast strains is linked to the presence of $M(IP)_2C$ (Im et al., 2003; Thevissen et al., 2000a). We performed high performance thin layer chromatography (HPTLC)-analysis of the acidic fraction of lipids of *N. crassa* WT and mutants MUT16 and MUT24, representing mainly inositolphosphoryl-containing sphingolipids (termed glycosylinositolphosphorylceramides (GIPC)). Bands were visualized upon orcinol staining, i.e., violet staining indicating the presence of hexose. In the HPTLC of Fig. 3A, the corresponding orcinol$^+$ acidic lipid GIPC-profiles of *N. crassa* WT, MUT16 and MUT24 are compared. The WT profile exhibited four bands with Rf values higher than those in the mutant profiles. These may represent mono- or diglycosyl-IPCs, possibly segregated into two components each based on differences in hydroxylation of the ceramide fatty-*N*-acyl moiety. By contrast, the mutant GIPC profiles appeared to be distinct from the WT, but similar to each other.

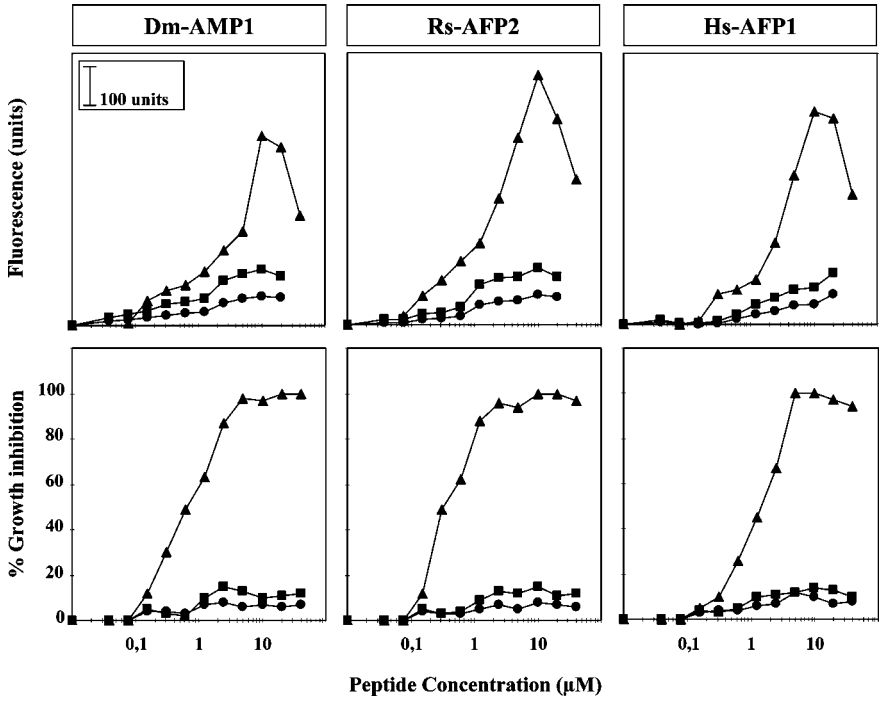

Fig. 2. Membrane permeabilization and growth inhibition induced by different plant defensins in *N. crassa* WT, MUT16, and MUT24. Dose–response curves of membrane permeabilization measured by SYTOX Green fluorescence (upper panel) and growth inhibition (lower panel) of *N. crassa* WT (triangles), *N. crassa* MUT16 (squares), and *N. crassa* MUT24 (circles) suspended in SMF1 are shown. Mycelium fragments were treated with DmAMP1, Hs-AFP1, and RsAFP2, and fluorescence was measured at different time points, only the values at 360 min are shown here. Values are means of duplicate measurements and correspond to one representative experiment of three. Standard errors were typically less than 7.5%.

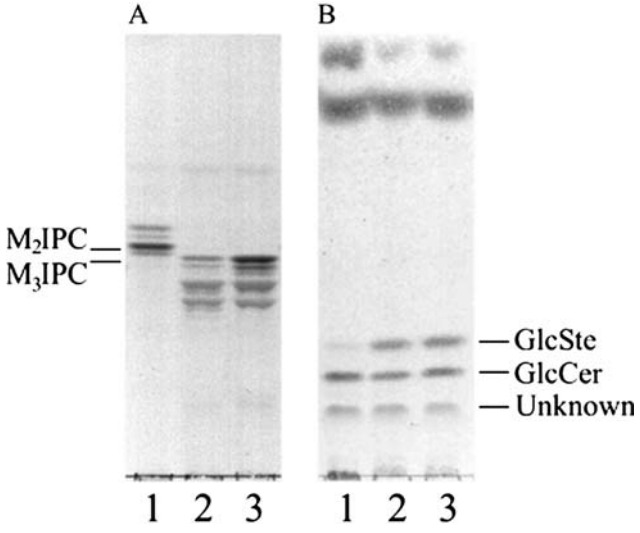

Fig. 3. HPTLC profiles of acidic and neutral glycolipids from *N. crassa* wild-type, MUT16, and MUT24 (lanes 1–3, respectively). (A) Acidic lipids, containing GIPC, were developed with solvent F and (B) neutral lipid fractions, containing steryl glucoside (GlcSte) and glucosylceramide (GlcCer), were developed with solvent E. Carbohydrate-containing bands were visualized with Bial's orcinol reagent. Labels on the right identify bands based on preliminary evidence from NMR spectroscopy and mass spectrometry of compounds isolated by preparative HPTLC. Labels on left show relative migration in solvent F of dimannosylinositol- and trimannosylinositolphosphorylceramides (M_2IPC and M_3IPC, respectively) isolated and characterized from *A. nidulans*. These are only indications of relative polarity of *N. crassa* GIPC, and no structural relationship is implied.

Several lower Rf (more polar) components were visible in the mutant GIPC profiles. Dimannosylinositol- and trimannosylinositolphosphorylceramides isolated and characterized from *Aspergillus nidulans* (Levery, S.B., unpublished data) were used as a reference to indicate relative polarities of the *N. crassa* GIPCs.

Unlike *S. cerevisiae*, most fungi synthesize not only GIPC but also neutral glycosylceramides and steryl glucosides (Levery et al., 2002). Glycosylceramides were recently shown to play a key role in the sensitivity of the yeast *P. pastoris* to RsAFP2 (Thomma et al., 2003; Thevissen, K., unpublished data). Therefore, neutral lipid fractions of *N. crassa* WT, MUT16, and MUT24, comprising mainly glucosylceramides and steryl glucosides, were analysed. In Fig. 3B, an HPTLC of neutral lipid fractions extracted from WT, MUT16, and MUT 24 strains of *N. crassa* (lanes 1–3, respectively) is shown. Three orcinol$^+$ bands were observed for each strain. Of these, components having Rf values characteristic for steryl β-glucoside (GlcSte) and β-glucosylceramide (GlcCer) were identified by comparison with standard compounds (see bands marked in Fig. 3). The third orcinol$^+$ band, having the lowest Rf value, did not co-migrate with any standard compound. Preliminary NMR and MS analysis of the unknown low Rf component indicated that it is not a glycolipid, but rather a small oligosaccharide fragment (data not shown).

Interestingly, although the amounts of GlcCer were similar in all three strains, both mutants expressed considerably more GlcSte than the *N. crassa* WT. Preliminary NMR and MS analysis of the putative GlcCer components of WT and mutant *N. crassa* strains indicated that the GlcCer components are 2-hydroxy-fatty-*N*-acyl-β-D-glucopyranosyl-(*4E,8E*)-9-methyl-4,8-sphingadienines. While the fatty-*N*-acyl moiety of the WT GlcCer were essentially (>90%) *E*-Δ(3)-unsaturated and composed of 2-hydroxy-(3*E*)-octadecenoic acid, those of the mutant GlcCer were 100% saturated and composed of 2-hydroxy-palmitic acid. Preliminary characterization by ^{1}H-NMR of the putative GlcSte components from the *N. crassa* WT and mutant strains and comparisons with published ^{1}H-NMR data suggested that the GlcSte components were ergosterol-β-D-glucopyranoside (data not shown). Among the higher Rf components (low polarity, orcinol/hexose^{-}) observed in the HPTLC, an abundant brownish stained band appeared in the WT profile, but was strongly reduced in the mutant profiles. The reciprocal expression of this band with respect to GlcSte suggests that it may represent free sterol.

4. Discussion

In the present study, we have isolated several plant defensin-resistant *N. crassa* mutants, displaying different cross-resistance profiles towards other families of structurally different antimicrobial plant proteins. To test whether resistance towards plant defensins is due to the absence of a specific class of membrane targets, two *N. crassa* mutants only displaying resistance against plant defensins were further characterized with respect to plant defensin-induced membrane permeabilization and complex lipid components. We demonstrate that the plant defensins induce significantly less permeabilization of membranes of *N. crassa* mutants MUT16 and MUT24 as compared to membranes of *N. crassa* WT. For *N. crassa* WT, a significant correlation between plant defensin-induced permeabilization and their antifungal activity was observed. Additionally, clear differences exist in the lipid profiles of *N. crassa* WT and mutants.

Analysis of the acidic lipid fraction revealed a different, more highly glycosylated GIPC structure in the *N. crassa* mutants in comparison with the *N. crassa* WT GIPC. This finding suggests that GIPC biosynthesis in the mutants has been directed into an alternative pathway favoring synthesis of more highly glycosylated GIPC. This could involve either extension of the WT core structure by activation of one or more glycosyl chain extending/branching enzymes in the mutants, or switching to synthesis of a core structure that is a more suitable substrate for chain extending/branching enzymes. Such glycosyltransferases could be pre-existing in the WT, but their GIPC products would not normally be observed in the absence of the alternate core substrate.

Analysis of the neutral lipid fraction revealed structurally different GlcCer and altered levels of GlcSte in the *N. crassa* mutants in comparison with the *N. crassa* WT. GlcCer of *N. crassa* WT and mutants are 2-hydroxy-fatty-*N*-acyl-β-D-glucopyranosyl-(*4E,8E*)-9-methyl-4,8-sphingadienines. 9-Methyl-4,8-sphingadienine is the sphingobase present in glucosylceramides from various fungal and yeast species such as *P. pastoris*, *Candida albicans* (Sakaki et al., 2001), *Cryptococcus neoformans* (Levery et al., 2000; Rodrigues et al., 2000), *Aspergillus fumigatus* (Boas et al., 1994; Toledo et al., 1999), and various *Fusarium* species (Duarte et al., 1998). However, WT and mutant GlcCer differ remarkably in several structural features: while the fatty-*N*-acyl moiety in the WT GlcCer is mostly *E*-Δ(3)-unsaturated (>90%) and composed of 2-hydroxy-3-(*E*)-octadecenoic acid (C18), those of the mutant GlcCer are essentially 100% saturated and composed of 2-hydroxy-hexadecanoic acid (C16). The occurrence of *E*-Δ(3)-unsaturation has so far been reported only in fungi belonging to Euascomycetes, and does not appear to be expressed in Saccharomycetales and Basidiomycetes (Levery et al., 2000; Toledo et al., 2001). It seems that in the plant defensin-resistant *N. crassa* mutants, the *E*-Δ(3)-desaturase activity has in some way been deleted or inactivated. In addition, the almost complete switch of predominant fatty acid chain length forms in the *N. crassa* mutants, namely from C18 to C16, is remarkable. The occurrence of 2-hydroxypalmitic acid as the major fatty acid in fungal GlcCer is particularly common in edible mushrooms and other Basidiomycetes (Fogedal et al., 1986; Kawai and Ikeda, 1982, 1983, 1985; Mizushina et al., 1998; Sawabe et al., 1994). GlcCer consisting of 2-hydroxypalmitic acid are not common in Euascomycetes, but not unknown either: this form has been reported to occur as a major GlcCer fraction of some species, including *Magnaporthe grisea* (Koga et al., 1998), a *Pachybasium* sp. (Sitrin et al., 1988), and *Pseudallescheria boydii* (Pinto et al., 2002). Although the amounts of GlcCer are similar in all three *N. crassa* strains, both mutants express considerably more GlcSte than the WT. GlcSte in the WT and mutants was characterized as ergosterol-β-D-glucopyranoside. This glycolipid has been isolated and characterized as the major sterol glycoside from a number of fungi, including *P. pastoris* (Sakaki et al., 2001) and a transgenic *S. cerevisiae* strain expressing the *P. pastoris* sterol-β-D-glucosyltransferase (Warnecke et al., 1999).

Whether the changes in membrane lipid composition in *N. crassa* mutants MUT16 and MUT24 are correlated with decreased plant defensin-induced membrane permeabilization and resistance towards different plant

defensins cannot be stated yet, although there is strong evidence to support this hypothesis. First, it was shown that DmAMP1-susceptibility of different yeast strains is linked to the presence of $M(IP)_2C$ in their membranes (Im et al., 2003; Thevissen et al., 2000a). GIPC such as $M(IP)_2C$ are believed to act as the binding site for DmAMP1 (Thevissen et al., 2000a). Our present results demonstrate that DmAMP1-resistant *N. crassa* mutants have different GIPC profiles as compared to *N. crassa* WT. The novel GIPC identified in *N. crassa* mutants MUT16 and MUT24 might be the result of a rescue pathway, switched on upon (a) mutation(s). Second, it was shown that RsAFP2-sensitivity of yeast strains is linked to the presence of GlcCer and that RsAFP2 interacts directly with fungal GlcCer (Thomma et al., 2003; Thevissen, K., unpublished data). GlcCer are membrane compounds that are structurally related to GIPC. Our present observation that membranes of RsAFP2-resistant *N. crassa* mutants have different GlcCer structures compared to *N. crassa* WT supports the finding that GlcCer play an important role in host sensitivity towards RsAFP2. A same key role for GlcCer was demonstrated for the antifungal activity of heliomicin, an insect peptide with a 3-D-structure and activity spectrum comparable to that of RsAFP2 (Thevissen, K., unpublished data). Indeed, both *N. crassa* mutants MUT16 and MUT24 are at least 500-fold more resistant to heliomicin as compared to their corresponding WT. The observations that the selected *N. crassa* mutants have an altered GlcCer profile and, in addition, have lost the ability to form ascospores and display reduced growth correspond to the finding that inhibition of UDP-Glc:ceramide glucosyltransferase in *Aspergillus* species affects spore germination, cell cycle, and hyphal growth (Levery et al., 2002).

Information on the activity of DmAMP1 and RsAFP2 indicates that the specific structure of sphingolipids in the fungal plasmamembrane, namely GIPC and GlcCer, respectively, might be crucial in determining permeability, binding and resistance towards these plant defensins. In case of Hs-AFP1, neither GIPC nor GlcCer seems to be involved in its mode of action (Thevissen, K., unpublished data). Possibly, the higher level of steryl glucosides (GlcSte) in *N. crassa* mutants MUT16 and MUT24 as compared to *N. crassa* WT might explain their increased resistance to Hs-AFP1. In the yeast *P. pastoris*, Ugt51p catalyses the biosynthesis of GlcSte and a lack of this enzyme leads to reduced levels of GlcSte (Warnecke et al., 1997). A *P. pastoris* Δ*ugt51* strain, lacking the enzyme UDP-glucose:sterol glucosyltransferase, was found to be 4-fold more sensitive towards Hs-AFP1 as compared to *P. pastoris* WT (Thevissen, K., unpublished data). Therefore, high amounts of GlcSte in the membrane seem to be linked with increased resistance towards Hs-AFP1. The precise role of GlcSte in the path leading to growth inhibition by Hs-AFP1 remains to be elucidated. Previously, it was shown that GlcSte accumulate under stress conditions in *P. pastoris* (Sakaki et al., 2001), the slime mould *Physarum polycephalum* (Murakami-Murofushi et al., 1997) and in cultured human fibroblasts (Kunimoto et al., 2000). The observation of stress-induced GlcSte accumulation in different organisms suggests the involvement of these glycolipids in stress responses. The accumulation of GlcSte in *N. crassa* mutants might therefore be a general response to RsAFP2 selection-stress.

In conclusion, membranes of the *N. crassa* mutants with broad resistance towards different members of the plant defensin family characterized in this study contain structurally different glucosylceramides, novel glycosphingolipids, and an altered level of steryl glucosides. Detailed structure elucidation of these novel membrane compounds will not only give more insight in the mode of action of plant defensins but also in the biosynthesis of complex lipids present in fungal membranes.

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