Specific Binding Sites for an Antifungal Plant Defensin from Dahlia (*Dahlia merckii*) on Fungal Cells Are Required for Antifungal Activity

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Dm-AMP1, an antifungal plant defensin from seeds of dahlia (*Dahlia merckii*), was radioactively labeled with t-butoxycarbonyl-[³⁵S]-L-methionine *N*-hydroxy-succinimide. This procedure yielded a [³⁵S]-labeled peptide with unaltered antifungal activity. [³⁵S]Dm-AMP1 was used to assess binding on living cells of the filamentous fungus *Neurospora crassa* and the unicellular fungus *Saccharomyces cerevisiae*. Binding of [³⁵S]Dm-AMP1 to fungal cells was saturable and could be competed for by preincubation with excess, unlabeled Dm-AMP1 as well as with Ah-AMP1 and Ct-AMP1, two plant defensins that are highly homologous to Dm-AMP1. In contrast, binding could not be competed for by more distantly related plant defensins or structurally unrelated antimicrobial peptides. Binding of [³⁵S]Dm-AMP1 to either *N. crassa* or *S. cerevisiae* cells was apparently irreversible. In addition, whole cells and microsomal membrane fractions from two independently obtained *S. cerevisiae* mutants selected for resistance to Dm-AMP1 exhibited severely reduced binding affinity for [³⁵S]Dm-AMP1, compared with wild-type yeast. This finding suggests that binding of Dm-AMP1 to *S. cerevisiae* plasma membranes is required for antifungal activity of this protein.

Plants protect themselves against microbial pathogens by various defense responses such as the modification of plant cell walls and the production of antimicrobial, secondary metabolites (phytoalexins), lytic enzymes such as chitinases and glucanases, and membrane-interacting proteins and peptides (reviewed by Kombrink and Somssich 1995). One particular family of antimicrobial peptides that inhibit growth of a broad range of fungi at micromolar concentrations are the plant defensins (reviewed by Broekaert et al. 1995, 1997). Plant defensins are small (45 to 54 amino acids), basic peptides that share a common, characteristic, three-dimensional folding pattern, stabilized by eight disulfide-linked cysteines. Plant defensins are structurally related to insect defensins

(Broekaert et al. 1995) and even more to the antifungal insect peptide drosomycin (Landon et al. 1997). Based on amino acid sequence homologies, plant defensins can be divided into two main groups (A and B) sharing only 25% similarity (Harrison et al. 1997). In group A, four subfamilies can be distinguished (A1, A2, A3, and A4) with at least 50% similarity within each subfamily. Subfamily A2 contains plant defensins such as Dm-AMP1 from *Dahlia merckii*, Ah-AMP1 from *Aesculus hippocastanum*, and Ct-AMP1 from *Clitoria ternatea*, which share about 70% sequence identity with each other. These plant defensins also have a similar antimicrobial activity spectrum and susceptible fungi show reduced hyphal elongation without marked morphological distortions (Osborn et al. 1995). They have, therefore, been said to be 'nonmorphogenic' in their inhibitory effect on fungi (Broekaert et al. 1995). Subfamily A3 consists of plant defensins from Brassicaceae, including Rs-AFP2 from *Raphanus sativus*. Subfamily A4 so far only contains Hs-AFP1 from *Heuchera sanguinea*. Members of subfamilies A3 and A4 inhibit fungi in a different way than do members of subfamily A2, as they induce tip ballooning and branch formation on susceptible fungi (Osborn et al. 1995). These plant defensins have therefore been termed 'morphogenic' plant defensins (Broekaert et al. 1995).

Treatment of hyphae of *Neurospora crassa* with either of the antifungal plant defensins Rs-AFP2 (subfamily A3) or Dm-AMP1 (subfamily A2) induces an array of relatively rapid membrane responses, including K⁺ efflux, Ca²⁺ uptake, alkalization of the incubation medium, and membrane potential changes (Thevissen et al. 1996). In contrast to insect defensins (Cociancich et al. 1993) and mammalian defensins (Kagan et al. 1990), plant defensins do not form ion-permeable pores in artificial membranes (Thevissen et al. 1996). Furthermore, the existence of specific, high affinity binding sites for the morphogenic plant defensin Hs-AFP1 (subfamily A4) on *N. crassa* hyphae and microsomal membranes was demonstrated (Thevissen et al. 1997). Binding of Hs-AFP1 to these sites was saturable with an apparent K_d of about 30 nM and was partially reversible. In addition, indirect evidence has been obtained suggesting that binding of Hs-AFP1 to this binding site is linked to its antifungal effects. For instance, a single amino acid substitution variant of Rs-AFP2 that lacked antifungal activity (De Samblanx et al. 1997) was unable to compete for this plant defensin binding site, unlike Rs-AFP2 itself

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(Thevissen et al. 1997). In conclusion, it seems likely that binding of Hs-AFP1 with a fungal plasma membrane binding site is the first step in the path leading to fungal growth inhibition.

In this study, we have assessed the presence of specific binding sites for Dm-AMP1, a member of the above-mentioned subfamily A2 of nonmorphogenic plant defensins, on the filamentous fungus *N. crassa* and the unicellular fungus *Saccharomyces cerevisiae*. To this end, we have used a ^{35}S -labeled derivative of Dm-AMP1. Our results point to the presence of highly specific binding sites for Dm-AMP1 on fungal cells. Binding of Dm-AMP1 to fungal cells appears to be required for antifungal activity, as yeast mutants selected for resistance to Dm-AMP1 showed drastically reduced binding activity.

RESULTS

Antifungal activity of Dm-AMP1 and its nonradioactive derivative.

Boc- ^{35}S Met-NHS was used to produce a radiolabeled derivative of Dm-AMP1, a nonmorphogenic plant defensin of subfamily A2 from *Dahlia merckii* seed. To test the effect of the derivatization reaction on the antifungal activity of Dm-AMP1, the peptide was allowed to react with nonradioactive Boc-Met-NHS and the specific antifungal activity of the modified peptide was assayed in an antifungal activity assay against four fungal species with different sensitivities to Dm-AMP1. The antifungal activity of modified and unmodified Dm-AMP1 was essentially the same for the four fungi (Table 1). Therefore, it was concluded that the labeling reaction was unlikely to greatly affect binding of modified Dm-AMP1 to its target.

Saturability of binding of ^{35}S Dm-AMP1 to fungi.

^{35}S Dm-AMP1 was found to bind both to hyphae of the filamentous fungus *N. crassa* and to cells of the yeast *S. cerevisiae*. Saturability of the binding of Dm-AMP1 to *N. crassa* hyphae and *S. cerevisiae* cells was examined by incubation with increasing concentrations of ^{35}S Dm-AMP1 (2 to 2,000

nM). As can be seen in Figure 1A and B, saturation curves obtained with either *N. crassa* hyphae or *S. cerevisiae* cells were hyperbolic. Half-maximal binding of ^{35}S Dm-AMP1 to *N. crassa* hyphae occurred at 60 nM, whereas half-maximal binding of ^{35}S Dm-AMP1 to *S. cerevisiae* cells occurred at 250 to 300 nM. Since binding of ^{35}S Dm-AMP1 to fungal cells seems to be irreversible (see below), Scatchard analysis of the specific binding data was not performed.

Reversibility of binding of ^{35}S Dm-AMP1 to fungi.

Association kinetics of binding of Dm-AMP1 to *N. crassa* hyphae and *S. cerevisiae* cells was determined by incubation of ^{35}S Dm-AMP1 for increasing periods of time. Dissociation was initiated after 30 min of incubation by addition of an excess of unlabeled Dm-AMP1. Incubation of ^{35}S Dm-AMP1

Table 1. Antifungal activity of Dm-AMP1, Ah-AMP1, Ct-AMP1, and Dm-AMP1 modified with Boc-Met-NHS (Boc-Met-HsAFP1) on different fungi

Fungus	IC ₅₀ (μM) ^a			
	Dm-AMP1	Boc-Met-Dm-AMP1	Ah-AMP1	Ct-AMP1
<i>Neurospora crassa</i>	2.2 ± 0.8	3.1 ± 0.9	ND ^b	ND
<i>Alternaria brassicicola</i>	2.4 ± 0.5	2.0 ± 0.7	ND	ND
<i>Botrytis cinerea</i>	>80.0	>80.0	ND	ND
<i>Fusarium culmorum</i>	8.0 ± 3.0	9.0 ± 5.0	ND	ND
<i>Saccharomyces cerevisiae</i> (WT)	2.0 ± 0.3	ND	2.3 ± 0.3	2.1 ± 0.2
<i>S. cerevisiae</i> (DM1)	>40.0	ND	>40.0	>40.0
<i>S. cerevisiae</i> (DM2)	>40.0	ND	>40.0	>40.0

^a IC₅₀ values (i.e., antifungal protein concentration required to inhibit 50% of growth) were calculated from dose-response curves with two-fold dilution steps. Data are expressed as averages with standard errors of triplicate measurements.

^b Not determined.

Fig. 1. Saturability of binding of ^{35}S Dm-AMP1 to *Neurospora crassa* hyphae and *Saccharomyces cerevisiae* cells. Increasing amounts of ^{35}S Dm-AMP1, diluted with unlabeled Dm-AMP1 to a final specific radioactivity of 0.015 TBq/mmol, were added to either (A) *N. crassa* hyphae or (B) *S. cerevisiae* cells in the absence (squares, total binding) or presence of 25 μM unlabeled Dm-AMP1 (circles, nonspecific binding). Specific binding (triangles) was determined as the difference between total binding and nonspecific binding. Data correspond to one representative experiment out of three. Data are expressed as means ± standard error of three independent determinations.

with *N. crassa* hyphae resulted in rapid binding of the radioligand; half-maximal binding occurred within 5 min (Fig. 2A). Dissociation of radiolabeled Dm-AMP1 from its binding sites on hyphae in the presence of excess, unlabeled Dm-AMP1 was not observed even after 2 h, indicating either that [35 S]Dm-AMP1 binding to hyphae is irreversible or that dissociation is very slow. Nonspecific binding, determined by addition of 50 μ M unlabeled Dm-AMP1, remained at about 15 to 20% of total binding throughout the experiment.

To confirm the apparent irreversible nature of Dm-AMP1 binding, the following experiment was set up. Two identical sets of *N. crassa* hyphae were allowed to bind [35 S]Dm-AMP1 for 1 h, whereafter a ninefold excess of binding buffer

(lacking Dm-AMP1) was added to one set of *N. crassa* hyphae. After another 2 h of incubation, binding of [35 S]Dm-AMP1 to both sets was compared and found not to differ significantly (results not shown). This indicates that 10-fold dilution of free [35 S]Dm-AMP1 had not entailed the establishment of a new binding equilibrium.

Very similar results were obtained when *S. cerevisiae* cells were used instead of *N. crassa* hyphae. As can be seen in Figure 2B, association of [35 S]Dm-AMP1 to its binding sites on *S. cerevisiae* cells did not reach a saturation level after 2 h of incubation. Dissociation of radiolabeled Dm-AMP1 from its binding sites on yeast cells in the presence of a 1,250-fold excess of unlabeled Dm-AMP1 was not observed even after 2 h, indicating that [35 S]Dm-AMP1 binding to *S. cerevisiae* cells is irreversible. Nonspecific binding, determined by addition of 50 μ M unlabeled Dm-AMP1, remained at about 10 to 15% of total binding throughout the experiment.

Fig. 2. Kinetics of binding and dissociation of [35 S]Dm-AMP1 to *Neurospora crassa* hyphae and *Saccharomyces cerevisiae* cells. Association kinetics for Dm-AMP1 (■) binding to (A) *N. crassa* hyphae and (B) *S. cerevisiae* cells were determined by addition of [35 S]Dm-AMP1 (40 nM) at time 0 and incubation of samples for increasing time intervals. Dissociation kinetics of Dm-AMP1 binding (▲) were initiated 30 min after addition of [35 S]Dm-AMP1 by addition of 50 μ M unlabeled Dm-AMP1 (arrow). Nonspecific binding of [35 S]Dm-AMP1 (●) was measured by addition of 50 μ M unlabeled Dm-AMP1, simultaneously with [35 S]Dm-AMP1 at time 0. Data are averages of duplicate measurements and correspond to one representative experiment out of three.

Specificity of binding of 35 S-labeled Dm-AMP1 to fungi.

To investigate whether binding of Dm-AMP1 to fungi was competitive, *N. crassa* hyphae or *S. cerevisiae* cells were preincubated with increasing concentrations of native, unlabeled Dm-AMP1 for 15 min prior to addition of radiolabeled Dm-AMP1. Binding of [35 S]Dm-AMP1 on both *N. crassa* hyphae and *S. cerevisiae* cells could efficiently be competed for by preincubation with excess, unlabeled Dm-AMP1. Concentrations of unlabeled Dm-AMP1 required for 50% inhibition of binding were about 0.4 and 3 μ M for *N. crassa* and *S. cerevisiae*, respectively (Fig. 3A, B).

In order to further assess the specificity of binding of 35 S-labeled Dm-AMP1, the ability of different antifungal peptides to compete with [35 S]Dm-AMP1 was tested, both on *N. crassa* hyphae and *S. cerevisiae* cells. The antifungal peptides used in this experiment were Ah-AMP1 and Ct-AMP1, plant defensins isolated from seed of horse chestnut (*Aesculus hippocastanum*) and *Clitoria ternatea*, respectively, that show 68 and 72% sequence identity to Dm-AMP1, respectively (Osborn et al. 1995); Rs-AFP2, isolated from seed of radish (*Raphanus sativus*) (Terras et al. 1992) and Hs-AFP1, isolated from seed of *Heuchera sanguinea* (Osborn et al. 1995), which are plant defensins with 50 and 46% sequence identity to Dm-AMP1, respectively; Ace-AMP1, isolated from onion (*Allium cepa*) seed and structurally related to lipid transfer proteins (Cammue et al. 1995); Ac-AMP1, isolated from amaranth (*Amaranthus caudatus*) seed and belonging to the hevein-type antimicrobial peptides (Broekaert et al. 1992, 1997); Mj-AMP1, isolated from *Mirabilis jalapa* seed and belonging to the knottin-type antimicrobial peptides (Cammue et al. 1992; Broekaert et al. 1997); and Ib-AMP4, a 20 amino acid antimicrobial peptide with two disulfide bridges isolated from *Impatiens balsamina* seed (Tailor et al. 1997). [35 S]Dm-AMP1 binding to either *N. crassa* hyphae or *S. cerevisiae* cells could only be competed for by members of the A2 subfamily of plant defensins (Dm-AMP1, Ah-AMP1, and Ct-AMP1) but not by the plant defensins belonging to subfamilies A3 and A4 (Rs-AFP2 and Hs-AFP1, respectively) nor by the structurally unrelated antimicrobial peptides (Ace-AMP1, Ac-AMP1, Mj-AMP1, and Ib-AMP4) (Fig. 4A, B).

It has previously been shown that plant defensins lose their antifungal activity upon reduction of their disulfide bridges (Terras et al. 1992) as well as upon addition of divalent

cations to the growth medium (Terras et al. 1993; Osborn et al. 1995; Thevissen et al. 1996). Reduced Dm-AFP1 at 40 μ M was unable to compete with binding of [35 S]Dm-AFP1 to *N. crassa* hyphae. Addition of 20 mM MgCl₂ to the binding assay reduced binding of [35 S]Dm-AFP1 to *N. crassa* hyphae by over 93% (results not shown).

Isolation and characterization of *S. cerevisiae* mutants resistant to Dm-AMP1.

By a selection procedure performed in liquid medium containing 5 μ M Dm-AMP1, two independent Dm-AMP1-resistant *S. cerevisiae* colonies could be isolated (DM1 and DM2). The antifungal activity of Dm-AMP1 on wild-type *S. cerevisiae* and on the yeast mutants resistant to Dm-AMP1 was assessed (Table 1). Wild-type yeast was found to be sensitive to Dm-AMP1 at concentrations above 1 to 2 μ M,

whereas the Dm-AMP1-resistant yeast mutants were resistant up to 40 μ M Dm-AMP1. In addition, the resistant mutants showed comparable resistance levels to Ah-AMP1 and Ct-AMP1 (Table 1), two nonmorphogenic plant defensins belonging, as does Dm-AMP1, to subfamily A2.

The Dm-AMP1-resistant yeast mutants might have acquired resistance to Dm-AMP1 due to overproduction of Dm-AMP1-degrading proteases. In order to assess this possibility, wild-type yeast and the Dm-AMP1-resistant yeast mutants (DM1 and DM2) were incubated with Dm-AMP1 for 24 h at 30°C. After this incubation, the antifungal activity of the supernatants containing Dm-AMP1 were assessed against the test fungus *N. crassa*. No substantial differences in antifungal activity were observed, indicating that the resistant yeast strains did not preferentially degrade Dm-AMP1 (results not shown). We also tested the susceptibility of DM1, DM2, and wild-type

Fig. 3. Competitivity of binding of [35 S]Dm-AMP1 to *Neurospora crassa* hyphae and *Saccharomyces cerevisiae* cells. Binding of [35 S]Dm-AMP1 (40 nM) to (A) *N. crassa* hyphae and (B) *S. cerevisiae* cells in the presence of increasing amounts of unlabeled Dm-AMP1 is shown. Unlabeled Dm-AMP1 was added 15 min prior to addition of [35 S]Dm-AMP1. Reaction volume of the assay was 100 μ l. Data are means $\pm$ standard error of three independent determinations.

Fig. 4. Inhibition of binding of [35 S]Dm-AMP1 to *Neurospora crassa* hyphae and *Saccharomyces cerevisiae* cells by different antifungal peptides. [35 S]Dm-AMP1 (40 nM) was incubated with (A) *N. crassa* hyphae and (B) *S. cerevisiae* cells, together with increasing amounts of Dm-AMP1 (—●—), Ah-AMP1 (—■—), Ct-AMP1 (—▲—), Rs-AFP2 (—○—), Hs-AFP1 (—□—), Ac-AMP1 (—△—), Mj-AMP2 (—◇—) and Ib-AMP4 (—○—). Unlabeled proteins were added 15 min prior to addition of [35 S]Dm-AMP1. Data are averages of triplicate measurements and correspond to one representative experiment out of three.

yeast to three different antifungal drugs (verapamil, carbonyl-cyanide *m*-chlorophenylhydrazone, and carbendazim), but found no difference between the mutants and wild-type yeast (results not shown).

Binding of [³⁵S]Dm-AMP1 to resistant *S. cerevisiae* mutants.

In order to investigate whether the Dm-AMP1-resistant mutants would be affected in their binding capacity for Dm-AMP1, binding assays with [³⁵S]Dm-AMP1 were performed on the mutants DM1 and DM2 and wild-type yeast strain. In these comparative binding assays, the Dm-AMP1-resistant mutants were both found to bind about 10-fold less [³⁵S]Dm-AMP1 than wild-type yeast cells (Fig. 5A). Binding of [³⁵S]Dm-AMP1 to DM1 and DM2 was about as high as binding to yeast cells preincubated with an excess of 50 μ M unlabeled Dm-AMP1, indicating that most of the residual binding to DM1 and DM2 results from nonspecific binding.

To exclude the possibility that reduced Dm-AMP1 binding by the Dm-AMP1-resistant mutants was due to alterations in their cell wall composition or structure, [³⁵S]Dm-AMP1 binding to plasma membrane fractions derived from wild-type and Dm-AMP1-resistant yeast mutants was assessed. As can be seen in Figure 5B, the binding capacity of wild-type plasma membranes for [³⁵S]Dm-AMP1 was about sevenfold higher, compared with that of plasma membranes derived from the resistant mutants.

DISCUSSION

In the present study, we have examined the binding of Dm-AMP1, a member of the A2 subfamily of plant defensins, to living *N. crassa* and *S. cerevisiae* cells. To this end, Dm-AMP1 was subjected to a labeling procedure consisting of the derivatization of primary amino groups with Boc-Met-NHS. The resulting modified peptide had essentially the same antifungal activity against four fungal species tested, indicating that the modifications had not affected side chains that are important for interaction with the fungal target.

Binding of radiolabeled Dm-AMP1 to *N. crassa* hyphae and *S. cerevisiae* cells appeared to be specific and saturable, and could be competed for by preincubation with excess unlabeled Dm-AMP1. However, no competition for binding sites occurred when the excess, unlabeled Dm-AMP1 was added 30 min after the labeled peptide, indicating that the binding is irreversible. This is in contrast to binding of Hs-AFP1 to *N. crassa* hyphae, which was shown to be at least partially reversible (Thevisen et al. 1997). Binding of radiolabeled Dm-AMP1 (subfamily A2) could also be competed for by preincubation with the subfamily A2 plant defensins Ah-AMP1 and Ct-AMP1, but not by subfamily A3 or A4 plant defensins. In contrast, binding of Hs-AFP1 (subfamily A4) could only be efficiently competed for by itself and to a much lesser extent by the subfamily A2 and A3 plant defensins (Thevisen et al. 1997). None of the structurally unrelated peptides tested showed any significant competition for the binding sites of Dm-AMP1 (Fig. 4) or Hs-AFP1 (Thevisen et al. 1997). These results indicate that the binding sites for plant defensins are highly specific and, furthermore, that the binding sites for subfamily A2 plant defensins and subfamily A4 plant defensins in *N. crassa* are distinct. They might, however, be structurally related.

Due to the irreversible nature of Dm-AMP1 binding to fungal cells, it was not possible to determine a dissociation constant. The apparently irreversible binding of [³⁵S]Dm-AMP1 to fungal cells could be the result of a low rate constant for dissociation. If this is the case, then the dissociation half-time for binding of [³⁵S]Dm-AMP1 to *N. crassa* hyphae has to be higher than 120 min, as can be concluded from the experimental data in Figure 2A. Alternatively, irreversibility could be due to binding site-mediated insertion of [³⁵S]Dm-AMP1 into the plasma membrane. In this case, the

Fig. 5. [³⁵S]Dm-AMP1 binding capacity of wild-type yeast and Dm-AMP1-resistant yeast mutants. **A**, [³⁵S]Dm-AMP1 (40 nM) was incubated with wild-type yeast cells or Dm-AMP1-resistant mutant yeast cells DM1 or DM2. **B**, [³⁵S]Dm-AMP1 (240 nM) was incubated with plasma membrane fractions derived from wild-type or mutant DM1 or DM2 yeast cells. Total ATPase activities of the plasma membrane fractions from wild-type, DM1, and DM2 yeast cells were 36, 42, and 32 U per mg of protein, respectively. Gray bars and white bars indicate binding without or with preincubation, respectively, with an excess of 50 μ M unlabeled Dm-AMP1 for 15 min prior to addition of radiolabeled Dm-AMP1. Data are averages of triplicate measurements and correspond to one representative experiment out of three.

plant defensin binding site could serve as an anchor point allowing the plant defensins to insert into or translocate across the plasma membrane, presumably accompanied by the formation of porelike structures permeable to ions or other solutes. The latter hypothesis is supported by our observations that plant defensins including Dm-AMP1 cause a weak but measurable permeabilization of *N. crassa* and *S. cerevisiae* cells to the fluorescent compound SYTOX Green at plant defensin concentrations equivalent to those required for fungal growth inhibition (Thevissen et al., *in press*). Binding site-mediated insertion in plasma membranes has previously been proposed to explain the antimicrobial activity of a number of antimicrobial proteins (Lakey et al. 1994; Schmitt and Compain, 1995; Chikindas et al. 1993; Van Belkum, 1991; Brotz et al. 1998), but this explanation has so far not gained wide recognition.

Binding sites on *S. cerevisiae* cells for Dm-AMP1 appear to share some characteristics with those of *N. crassa* hyphae. In both cases, binding of [³⁵S]Dm-AMP1 was saturable and could be competed for by subfamily A2 plant defensins, but not by subfamily A3 and A4 plant defensins or unrelated proteins. Furthermore, binding of [³⁵S]Dm-AMP1 was found to be irreversible for both *S. cerevisiae* cells and *N. crassa* hyphae. It is therefore conceivable that a structurally related receptor for subfamily A2 plant defensins might occur in a variety of different fungi. Interestingly, plasma membrane fractions derived from two independently obtained *S. cerevisiae* mutants, which can grow at Dm-AMP1 concentrations completely suppressing growth of a wild-type strain, exhibit a substantially decreased binding capacity for [³⁵S]Dm-AMP1, compared with plasma membrane fractions derived from wild-type yeast. This finding provides strong support to the idea that binding of Dm-AMP1 to its binding site on the *S. cerevisiae* plasma membrane is linked to its antifungal effect. The genes affected in the DM1 and DM2 mutants are likely candidates for genes encoding or controlling the Dm-AMP1 receptor site. Genetic evidence has been obtained indicating that the mutations in DM1 and DM2 affect the same nuclear gene (K. Thevissen and W. F. Broekaert, *unpublished results*). We are currently focusing our efforts on the cloning of this gene in the hope that this will further advance our knowledge of the mode of action of plant defensins.

MATERIALS AND METHODS

Materials.

The antifungal peptides Dm-AMP1, Ah-AMP1, Ct-AMP1, and Hs-AFP1 were isolated as described previously by Osborn et al. (1995). Rs-AFP2, Ace-AMP1, Ac-AMP1, Mj-AMP2, and Ib-AMP4 were isolated as described previously by Terras et al. (1992), Cammue et al. (1995), Broekaert et al. (1992), Cammue et al. (1992), and Tailor et al. (1997), respectively. t-Butoxycarbonyl-L-methionine *N*-hydroxysuccinimidylester (Boc-Met-NHS) was obtained from Bachem (Bubendorf, Switzerland) and Boc-[³⁵S]Met-NHS from Amersham International (Slough, UK). Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and β -glucuronidase were purchased from Sigma (St. Louis, MO). All other reagents were of reagent grade and were obtained from commercial sources.

Microorganisms.

Filamentous fungi were grown on six cereal agar and spores were harvested as described by Broekaert et al. (1990). Spore stocks in 20% (vol/vol) glycerol were at a final concentration of 2×10^7 spores per ml. Yeast (*Saccharomyces cerevisiae*) was grown and stored following standard protocols (Ausubel et al. 1993). The fungal strains used in this study were *Neurospora crassa* FGSC 2489, *Botrytis cinerea* MUCL 30158, *Fusarium culmorum* IMI 180420, *Alternaria brassicicola* MUCL 20297, and *Saccharomyces cerevisiae* W303-1A (genotype: *MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*).

Antifungal activity assay.

Antifungal activity of protein samples against filamentous fungi was assayed by microspectrophotometry as described previously (Broekaert et al. 1990; Terras et al. 1992). Briefly, in a well of a 96-well microplate, 10 μ l of the protein sample was mixed with 90 μ l of potato dextrose broth (12 g/liter; Difco, Detroit, MI) containing fungal spores that had been pregerminated for 16 h. Growth was recorded after 48 h of further incubation at 22°C. The absorbance at 595 nm served as a measure for microbial growth (Terras et al. 1992). IC₅₀ values (i.e., the concentration of the antifungal protein required to inhibit 50% of the fungal growth) were calculated from dose-response curves with twofold dilution steps (Terras et al. 1992).

Antifungal activity of protein samples against *S. cerevisiae* was determined in a manner analogous to that for the antifungal activity against filamentous fungi. Briefly, in a well of a 96-well microplate, 15 μ l of the protein sample was mixed with 138 μ l of minimal medium (0.8 g of CSM per liter, BIO 101, La Jolla, CA; 6.5 g of yeast nitrogen base without amino acids per liter, Difco; 20 g of glucose per liter) and 2 μ l of an overnight culture of yeast cells. The microplates were incubated at 30°C without shaking, and the absorbance at 595 nm was recorded after 20 h of incubation.

Preparation of microsomal membranes from *S. cerevisiae* cells.

Microsomal membranes were isolated from *N. crassa* as previously described (Thevissen et al. 1996). Isolation of microsomal membranes from *S. cerevisiae* cells was done in a manner analogous to that for *N. crassa*. Yeast cells were grown overnight in YPD (10 g of yeast extract per liter; 20 g of peptone per liter, 20 g of glucose per liter) at 30°C and were harvested (absorbance at 600 nm = 1.10) by centrifugation at $3,500 \times g$ for 10 min. After resuspension of the pellet in homogenization buffer (50 mM Tris, pH 7.5; 5 mM EDTA; 1 mM EGTA; 0.3 M sucrose, 5 mg of bovine serum albumin per ml), the cell suspension was lysed by one passage through a high pressure cell disrupter (Z series; Constant Systems, Warwick, UK). The suspension of disrupted cells was subjected to a series of subsequent centrifugation steps: $4,000 \times g$ for 10 min (with recovery of the supernatant); $15,000 \times g$ for 20 min (with recovery of the supernatant); $100,000 \times g$ for 60 min (with recovery of the pellet). The final pellet was suspended in 20 ml of wash buffer (1 mM Tris-EGTA; 10% [vol/vol] glycerol, pH 7) and washed in a glass homogenizer. The suspension was centrifuged at $100,000 \times g$ for 60 min and the pellet was resuspended in a very small volume of wash buffer

(approximately 1 ml). Aliquots were flash frozen in liquid nitrogen and stored at -80°C . This fraction contains microsomes that are mainly derived from the plasma membrane and the endoplasmic reticulum. Total protein in the membrane fractions was determined after addition of sodium dodecyl sulfate to a final concentration of 0.5% (wt/vol), with the bicinchoninic acid assay (Smith et al. 1985) with bovine serum albumin as standard. Total ATPase activity of the membrane fractions was determined as described by Bowman and Bowman (1988) and units are expressed as μmol of phosphate released in 1 h at 30°C .

Preparation of ^{35}S -labeled Dm-AMP1.

Dm-AMP1 was radiolabeled with Boc- ^{35}S Met-NHS at 30 TBq/mmol (Amersham International) as described previously for Hs-AFP1 (Thevissen et al. 1997). Specific activities of the labeled Dm-AMP1 preparations were typically around 1 TBq/mmol. Cold-labeled Dm-AMP1 was prepared as described previously for Hs-AFP1 (Thevissen et al. 1997).

Binding assays.

For binding studies with *N. crassa* hyphae, *N. crassa* was grown at an inoculum density of 3×10^5 spores per ml as described previously (Thevissen et al. 1996). The medium consisted of half-strength potato dextrose broth (12 g/l; Difco). After 20 h of incubation at 22°C , 80- μl aliquots of the *N. crassa* culture were incubated, unless stated otherwise, with 4 pmol of ^{35}S Dm-AMP1 (approximately 1.08 TBq/mmol) either with or without appropriate concentrations of competing antifungal peptides in a total volume of 100 μl in siliconized polypropylene microcentrifuge tubes (Sigma) at 22°C . Total protein in the *N. crassa* cultures was measured after homogenization by high speed reciprocal shaking in the presence of 10% (wt/vol) 400- to 600- μm glass beads (Sigma) in a Fast-prep apparatus (Bio 101/Savant, Farmingdale, NY). Protein measurement was done by the bicinchoninic acid method (Smith et al. 1985) with bovine serum albumin as standard. Total protein content in *N. crassa* cultures was approximately 1.8 mg protein per ml. After incubation for the time periods indicated, the samples were transferred to wells of a Multi-Screen Durapore 96-well filtration plate (0.65 μm pore size; Millipore, Bedford, MA). After filtration with a MultiScreen vacuum filtration manifold (Millipore), harvested hyphae were washed three times with 100 μl of ice-cold binding buffer (20 mM MES [morpholineethanesulfonic acid], 50 mM KCl, 1 mM MgCl_2 , pH 6.2). Filters with the hyphae or microsomal membranes were punched out manually with Multiscreen punch tips (Millipore), suspended in scintillation cocktail (Ready Safe scintillation cocktail; Analis, Gent, Belgium), and counted for ^{35}S in a liquid scintillation counter. Nonspecific binding was determined as the amount of radioactivity bound in the presence of 25 or 50 μM unlabeled Dm-AMP1. Specific binding was calculated by subtracting nonspecific binding from total binding.

For binding studies with *S. cerevisiae* cells, a *S. cerevisiae* culture was grown overnight at 30°C (absorbance at 600 nm = 1.00). The growth medium consisted of half-strength minimal medium (0.4 g of CSM per liter, BIO 101; 3.25 g of yeast nitrogen base per liter, Difco; 10 g of glucose per liter). Binding assays were performed in a manner analogous to that with *N. crassa*.

For binding studies with *S. cerevisiae* microsomal membranes, 2- μl aliquots of a membrane suspension containing 1.6 mg of protein per ml were incubated with 4 pmol of ^{35}S Dm-AMP1 in a total volume of 100 μl of binding buffer (20 mM MES, 50 mM KCl, 1 mM MgCl_2 , pH 6.2) in siliconized polypropylene microcentrifuge tubes at 4°C .

Reduction of Dm-AMP1.

Reduction of Dm-AMP1 was performed by the addition of 100 mM Tris-HCl (pH 8) and 100 mM dithioerythritol (DTE), followed by incubation at 45°C for 1 h. Reagents were removed on a disposable reversed phase C₈ silica column (Bond Elut, 500 mg solid phase; Varian, Harbor City, CA). The C₈ silica column was subsequently rinsed with 6 ml of distilled water containing 0.1% (vol/vol) trifluoroacetic acid. The reduced peptide was eluted from the latter column with 5 ml of 50% (vol/vol) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid. Protein fractions (monitored by measurement of the absorbance at 280 nm) were vacuum dried and redissolved in distilled water.

Isolation of Dm-AMP1-resistant *S. cerevisiae* mutants.

For the isolation of *S. cerevisiae* mutants resistant to Dm-AMP1, an overnight culture of wild-type *S. cerevisiae* in minimal medium (0.8 g of CSM per liter, BIO 101; 6.5 g of yeast nitrogen base without amino acids per liter, Difco; 20 g of glucose per liter) was diluted 100-fold with fresh minimal medium, supplemented with 5 μM Dm-AMP1, to a cell density of approximately 10^6 to $2 \cdot 10^6$ cells per ml. This culture was transferred to a 96-well microplate in 100- μl aliquots. After 2 days of incubation at 30°C , independent Dm-AMP1-resistant colonies could be isolated. This selection procedure combines the advantage of selection in liquid medium (stringent selection, minimal amount of Dm-AMP1 needed) with the advantage of selection on solid medium (growth of individual colonies).

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