The Plant Defensin, NaD1, Enters the Cytoplasm of Fusarium Oxysporum Hyphae*

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The plant defensin, NaD1, from the flowers of Nicotiana alata displays potent antifungal activity against a variety of agronomically important filamentous fungi including Fusarium oxysporum f. sp. vasinfectum (Fov). To understand the mechanism of this antifungal activity, the effect of NaD1 on Fov fungal membranes and the location of NaD1 in treated hyphae was examined using various fluorescence techniques. NaD1 permeabilized fungal plasma membranes via the formation of an aperture with an internal diameter of between 14 and 22 Å. NaD1 bound to the cell walls of all treated hyphae and entered several hyphae, resulting in granulation of the cytoplasm and cell death. These results suggest that the activity of antifungal plant defensins may not be restricted to the hyphal membrane and that they enter cells and affect intracellular targets.

Plants produce a number of cationic peptides for protection against infection by potential microbial pathogens. These include defensins, which are one of the largest families of antimicrobial peptides found in plants. They are particularly abundant in seeds but have also been described in leaves, pods, tubers, fruit, and floral tissues (1, 2). Plant defensins are small (45–54 amino acids), basic proteins with 4–5 disulfide bonds (3). They share structural and functional similarities with defensins from insects (4), mammals (5), and fungi (6). Most plant defensins exhibit antifungal activity; however, antibacterial activity and the inhibition of protein synthesis, α-amylases, and proteases have also been reported (2, 7, 8). Plant defensins, even those with similar activities, share little sequence identity and may act via differing mechanisms. So far, only a limited number of seed defensins have been studied in detail. Defensins from radish (RsAFP2) and dahlia (DmAMP1) interact with specific sphingolipids on fungal plasma membranes and require the presence of these lipids for their antifungal activity. They permeabilize the fungal membrane and induce Ca\(^{2+}\) influx and K\(^+\) efflux, which disrupts the Ca\(^{2+}\) gradient essential for fungal tip growth (9–11). Another plant defensin, MsDef1 from alfalfa, blocks mammalian L-type Ca\(^{2+}\) channels, although interaction with fungal Ca\(^{2+}\) channels has not been demonstrated (12).

Membrane permeabilization is a common activity for many antimicrobial peptides, although the mechanism of permeabilization can differ significantly, and in some cases, remains unclear. A number of models have been suggested, including the barrel-stave pore, toroidal pore, and carpet models (for review, see Ref. 13). Antimicrobial peptides were initially thought to act solely at the plasma membrane, although some exert their cytotoxic effects via interaction with intracellular targets (for review, see Ref. 14). The mammalian proline-rich protein PR-39, for example, enters bacterial cells without disrupting the plasma membrane and inhibits DNA and protein synthesis (15).

NaD1 is an antifungal plant defensin that is expressed at high concentrations in the flowers of the ornamental tobacco, Nicotiana alata (16). In this study, we report the activity of NaD1 against a number of agronomically important fungal pathogens. We investigated the effect of NaD1 on the permeability of the plasma membrane of a susceptible fungus, Fusarium oxysporum f. sp. vasinfectum, using a membrane-impermeable fluorescent dye as well as FITC-labeled 2 dextrans. The location of NaD1 in treated hyphae was examined using immunofluorescence, electron microscopy, and fluorescently tagged protein. The production of reactive oxygen species in response to treatment with NaD1 was also examined using a fluorescent indicator.

EXPERIMENTAL PROCEDURES

Purification of NaD1 from Flowers—Whole N. alata flowers up to the petal coloration stage of flower development were ground to a fine powder and extracted in dilute sulfuric acid as described previously (16). After neutralization with 10 M NaOH and centrifugation, the supernatant was applied to an SP Sepharose column (2.5 × 2.5 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). Bound proteins were eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl. Eluted protein was then separated on a reverse-phase HPLC column (16), and protein concentration was determined using the BCA protein assay (Pierce).

Production of Anti-NaD1 Antiserum—Purified NaD1 (1.5 mg) was conjugated to keyhole limpet hemocyanin (0.5 mg) and injected into a rabbit essentially as described in Ref. 16.

Reduction and Alkylation of NaD1—Lyophilized NaD1 (500 µg) was dissolved in 400 µl of stock buffer (200 mM Tris-HCl, pH 8.0, 2 mM EDTA, 6 M guanidine-HCl, 0.02% (v/v) Tween 20)

2 The abbreviations used are: FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; ROS, reactive oxygen species.

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before reduction buffer (stock buffer and 15 mM dithiothreitol) was added (44 μl), and the reaction was incubated at 40 °C for 4.5 h. The reaction mixture was cooled to room temperature before iodoacetic acid (0.5 M in 1 M NaOH, 55 μl) was added, and the incubation was continued in the dark for 30 min at room temperature. A Nanosep omega® spin column (3000 molecular mass cut off, PALL Life Sciences) was used to remove salts, dithiothreitol, and iodoacetic acid. The protein concentration was determined using the BCA protein assay (Pierce).

**Fungal Growth Inhibition Assays—Antifungal activity against F. oxysporum f. sp. vasinfectum** (DPI), Queensland, Australia), F. oxysporum f. sp. vasinfectum (DPI), Queensland, Australia), *Thielaviopsis basicola* (gift from Helen McFadden, CSIRO Plant Industry, Black Mountain, Australia), *Leptosphaeria maculans* (gift from David Nehl, New South Wales DPI, Narrabri, Australia), *Verticillium dahliae* (gift from Helen McFadden, CSIRO Plant Industry, Black Mountain, Australia), *Aspergillus nidulans* (gift from Michael Hynes, Genetics Department, University of Melbourne, Victoria, Australia), and *Aspergillus niger* (gift from Helen McFadden, CSIRO Plant Industry, Black Mountain, Australia) was assessed essentially as described in Lay et al. 16.

Spores were isolated from sporulating cultures growing in half-strength potato dextrose broth (Fov and T. basicola), Czapek-Dox broth (V. dahliae) (Difco Laboratories), or 10% (v/v) clarified V8 media (L. maculans and A. nidulans) by filtration through sterile muslin. Spore concentrations were determined using a hemocytometer and adjusted to 5 × 10⁴ spores/ml in the appropriate growth media. Spore suspensions (80 μl) were added to the wells of sterile 96-well flat-bottomed microtiter plates along with 20 μl of filter sterilized (0.22-μm syringe filter; Millipore) NaD1 or water to give final protein concentrations of 0–10 μM. The plates were shaken briefly and placed in the dark at 25 °C until the optical density at 595 nm of the water control reached ~0.2 (24–72 h depending on growth rate). Hyphal growth was determined by measuring optical density at 595 nm using a microtiter plate reader (SpectraMax Pro M2; Molecular Devices). Each test was performed in quadruplicate.

**Yeast Growth Inhibition Assays—** The effect of NaD1 on the growth of the yeast strains Candida albicans, Pichia pastoris, and Saccharomyces cerevisiae was assayed in microtiter plates. Cells were grown in YPD for 48 h and then counted using a hemocytometer and diluted to a concentration of 5 × 10⁴ cells/ml in fresh YPD. Cell suspension (100 μl) containing 0–10 μM NaD1 was added to the wells of a 96-well microtiter plate and incubated for 48 h at 30 °C. Growth was determined by measuring the optical density at 595 nm using a microtiter plate reader. Each test was performed in quadruplicate.

**Bacterial Growth Inhibition Assays—** The effect of NaD1 on the growth of the bacterial strains Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus cereus was assayed in microtiter plates. Cells were grown overnight in Luria-Bertani broth and diluted to a concentration of 1 × 10⁴ cells/ml. Ten microliters of the diluted overnight culture was then added to 190 μl of LB containing 0–10 μM NaD1. Plates were incubated at 37 °C without shaking for 16 h, and growth was determined by measuring optical density at 595 nm. Each sample was performed in quadruplicate.

**Mammalian Cell Growth Inhibition Assays—** HeLa cells were seeded at 50% in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal calf serum with or without NaD1 (10 μM) at 37 °C under an atmosphere of 5% CO₂ and 95% air in a 60-mm Petri dish. Cells were incubated for 48 h at 37 °C before they were stained with trypan blue (Sigma) to check viability and counted using a hemocytometer.

**Membrane Permeabilization Assay—** Fov hyphae were grown in half-strength potato dextrose broth from a starting concentration of 5 × 10⁴ spores/ml for 18 h at 25 °C. Hyphae were incubated with 2 μM reduced and alkylated NaD1 (NaD1_R&L) or an equivalent volume of water for 2 h at room temperature with gentle agitation. SYTOX green (Molecular Probes) was added (final concentration of 0.5 μM), and the hyphae were allowed to stand for 10 min. Fluorescence of hyphae in microtiter trays was measured using a fluorometer (SpectraMax M2, Molecular Devices) with excitation and emission wavelengths of 488 and 538 nm, respectively, or visualized using an Olympus BX50 fluorescence microscope with an MWIB filter (excitation wavelength 460–490 nm).

**FITC Dextran Assay—** Fov hyphae were grown as described above and incubated with NaD1 (0–50 μM) for 2 h at room temperature with gentle agitation. Hyphae were washed twice for 10 min with half-strength potato dextrose broth to remove NaD1 before FITC dextran of either 4 kDa (FD-4, Sigma) or 10 kDa (FD-10, Sigma) were added to a final concentration of 1 mg/ml. Hyphae were incubated for a further 30 min at room temperature and then washed twice with half-strength potato dextrose broth to remove excess dextrans. Hyphae were visualized using an Olympus BX50 fluorescence microscope with an MWIB filter (excitation wavelength 460–490 nm). The assay was also repeated with dextrans being added at the same time as NaD1 to identify transient pores.

**Immunofluorescence Detection of NaD1 on hyphal Cell Wall—** Fov hyphae were grown as described above and incubated with NaD1 or water for 2 h at room temperature with gentle agitation. Hyphae were washed twice in 0.6 M KCl and fixed in 4% (w/v) formaldehyde for 30 min at room temperature with gentle shaking. Hyphae were washed twice in 0.6 M KCl before they were air-dried onto poly-L-lysine (Sigma)-coated coverslips. Coverslips were placed in 6-well microtiter plates and covered in blocking buffer (1 mg/ml bovine serum albumin, 0.2% (v/v) Triton X-100 in PBS) for 1 h. Blocking buffer was then removed, and hyphae were immersed in Protein-A purified anti-NaD1 primary antibody (1/200 dilution) for 1 h. Hyphae were washed (3 × 10 min) with blocking buffer before the addition of an Alexa Fluor® 568-labeled secondary antibody (goat anti-rabbit IgG, 1/200 dilution, Molecular Probes). After 1 h, hyphae were washed with blocking buffer (3 × 15 min) and placed onto glass slides with Slow Fade® antifade solution (Molecular Probes) for visualization by fluorescence microscopy using an Olympus BX50 fluorescence microscope with an MNG2 filter (excitation wavelength 530–550 nm).

**Isolation of NaD1 from Treated Hyphae—** Fov hyphae were grown as described previously prior to the addition of NaD1 (10 μM final concentration) to 1 ml of culture. Samples (100 μl) were collected after 0, 5, 10, 30, 60, 90, and 120 min. Hyphae were collected by centrifugation (10 min, 10,000 × g), and the
supernatant was stored at –20 °C for analysis. Hyphae were washed (2 × 10 min) with KCl (0.6 M) to remove any ionically bound protein before they were resuspended in 50 mM CAPS buffer (pH 10.0) containing 10 mM dithiothreitol for 20 min. Hyphae were collected by centrifugation, and the supernatant, containing cell wall proteins, was collected for analysis. The pellet (containing cells) was resuspended in water, and the cells were lysed using glass beads (Sigma, 60 mg) and vortexing (3 × 10 min). Cellular debris was removed by centrifugation (16,000 × g, 10 min), and the supernatant (containing cytoplasmic contents) was collected for analysis. All samples were analyzed by SDS-PAGE and immunoblotting with the anti-NaD1 antibody.

Labeling of NaD1 with a Bimane Fluorescent Tag—Lyophilized NaD1 was dissolved in 0.1 M MES buffer (pH 5.0) to a final concentration of 2 mM. The fluorescent tag bimane amine (Molecular Probes) was added to a final concentration of 10 mM along with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (final concentration 2 mM). The reaction was incubated at room temperature for 2 h with gentle stirring before centrifugation (10 min, 13,000 rpm) to remove any precipitated protein. A Nanosep omega® spin column (3000 molecular mass cut off, Pall Life Sciences) was used to remove salts, unbound bimane amine, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide from the labeled protein. Protein concentration was determined using the BCA protein assay.

Fluorescence Microscopy of NaD1-Bimane—Hyphae grown for 18 h as described above were treated with NaD1-bimane (2 μM) for between 30 min and 6 h. Hyphae were then visualized by fluorescence microscopy using an Olympus BX50 fluorescence microscope with an MWU filter (excitation wavelength of 330–385 nm).

Fixation and Immunogold Labeling for Electron Microscopy—Hyphae that had been treated with 2 μM NaD1 as described above were washed three times in PBS before fixation in 4% (w/v) paraformaldehyde in PBS for 1 h at 4 °C. Hyphae were again washed three times in PBS before dehydration in a standard ethanol series (15 min each, 50, 70, and 90% ethanol, 3 × 15 min 100% ethanol). Hyphae were then infiltrated with LR White resin (ProSciTech) for 1 h at room temperature followed by 18 h at 4 °C, 1 h at room temperature, and 24 h at 60 °C. Fresh LR White was used at each step. Ultrathin sections were cut and placed on Formvar-coated gold grids. Grids were blocked with PBS containing 8% (w/v) bovine serum albumin and 1% (v/v) Triton X-100 for 1 h and labeled with Protein-A purified anti-NaD1 antibodies (2 μg/ml) for 1 h. Grids were washed in blocking solution (3 × 10 min) and labeled with 15 nm gold particle-labeled goat anti-rabbit antibodies (ProSciTech) for 1 h. Grids were washed again in blocking solution (3 × 10 min) and then water (15 min) before being air-dried. A JEOL JEM2010HC x e80 KV transmission electron microscope was used to examine labeled grids, and pictures were taken on Kodak EM film (ProSciTech) and developed in a dark room before scanning on a Hewlett Packard Scanjet 5P scanner.

Detection of Reactive Oxygen Species—Fov hyphae were grown as described above and incubated with 5 μg/ml dihydroorhodamine 123 (Sigma) for 2 h followed by extensive washing with growth medium. Hyphae were then treated with NaD1 (2 μM) or water for 1 h before being washed with 0.6 M KCl.

| Cell type                        | NaD1 IC<sub>50</sub> μM |
|----------------------------------|-------------------------|
| F. oxysporum f. sp. vasinfectum  | 1.0                     |
| L. maculans                     | 0.80                    |
| A. nidulans                     | 0.80                    |
| V. dahliae                      | 0.75                    |
| T. basicola                     | 0.80                    |
| C. albicans                     | >10                     |
| S. cerevisiae                   | >10                     |
| P. pastoris                     | >10                     |
| S. aureus                       | >10                     |
| B. cereus                       | >10                     |
| E. coli                         | >10                     |
| P. aeruginosa                   | >10                     |
| HeLa cells                      | >10                     |

Antifungal Activity of NaD1—NaD1 was tested in vitro against the filamentous fungi F. oxysporum f. sp. vasinfectum (Fov), V. dahliae, T. basicola, A. nidulans, and Leptospheria maculans. At 1 μM, NaD1 retarded the growth of Fov and L. maculans by 50%, whereas V. dahliae, T. basicola, and A. nidulans were all inhibited by ~65%. At 5 μM NaD1, the growth of all five species was inhibited by more than 90%. NaD1 had no effect on the growth of the yeast strains S. cerevisiae, C. albicans, and P. pastoris or the Gram-negative and Gram-positive bacterial strains tested (Table 1). Additionally, it was not toxic to mammalian HeLa cells. The addition of 1 mM Ca<sup>2+</sup> to the growth medium abolished activity and reduction, and alkylation of NaD1 rendered the protein inactive (data not shown), demonstrating that the three-dimensional structure of the protein is essential for activity.

Binding of NaD1 to the Cell Walls of Fov—Potential binding of NaD1 to the cell surface of Fov was investigated using immunofluorescence with an antibody raised to NaD1 isolated from N. alata flowers. NaD1 bound along the entire length of the cell surface in hyphae treated with 2 μM NaD1 (Fig. 1). No background fluorescence was detected in controls in which the NaD1 was omitted. Another control employed another ~6-kDa cysteine-rich protein and its corresponding specific antibody. This protein, C1, is a proteinase inhibitor from the flowers of N. alata and does not retard the growth of Fov hyphae (16). C1 did not bind to the hyphae. To determine whether NaD1 was binding to the cell wall or the plasma membrane, the cell wall of treated hyphae was solubilized and analyzed by SDS-PAGE and immunoblotting. NaD1 was present in these samples (Fig. 2), suggesting that the interaction of NaD1 with the cell surface was due, at least in part, to binding to the cell wall and that it was not restricted to the plasma membrane. Binding to the cell wall occurred rapidly (within 5 min) and increased over the next 60 min. In the presence of 1 mM Ca<sup>2+</sup>, no binding of NaD1 to the cell wall was detected (data not shown).
Membrane Permeabilization by NaD1

SYTOX Green Assay—Permeabilization of the Fov hyphae by NaD1 was measured using the fluorescent dye SYTOX green. SYTOX green fluorescence increases substantially upon binding to nucleic acids, but the dye only enters cells when the plasma membrane is compromised. At a very low non-inhibitory concentration of NaD1 (0.1 μM), a small degree of permeabilization could be seen (Fig. 3). The nuclei of these permeabilized hyphae appeared intact, and the cytoplasm appeared unaltered (Fig. 4). NaD1-induced permeabilization increased in a concentration-dependent manner (Fig. 3). At higher concentrations of NaD1, the SYTOX green formed a diffuse pattern of fluorescence across the cell. The nuclei were no longer intact, and the cytoplasm of all permeabilized hyphae appeared granular (Fig. 4). The entry of SYTOX green into the Fov hyphae occurred within 10 min of the addition of NaD1. No permeabilization was observed in hyphae treated with reduced and alkylated NaD1 (NaD1R&A).

Predicted Aperture Size—In an effort to determine whether NaD1 forms an aperture of a distinct size or merely destabilizes the plasma membrane as predicted in the carpet model, NaD1-treated hyphae were incubated with FITC-labeled dextrans of either 4 kDa (average globular diameter of 14 Å) or 10 kDa (average globular diameter of 23 Å). Dextrans of 4 kDa entered hyphae at the same NaD1 concentration that led to SYTOX green uptake (molecular mass ~650 Da), whereas 10 kDa dextrans were excluded even at very high concentrations of NaD1 (Fig. 5). To examine whether aperture formation by NaD1 was transient or relatively stable, the assay was conducted in two ways. Dextrans were either added at the same time as NaD1 or added after removal of unbound NaD1 by extensive washing. The 4-kDa FITC-dextran was able to enter under both conditions.

Entry of NaD1 into Fungal Hyphae

Immunoblotting of Cytoplasmic Contents—Cytoplasmic contents of hyphae treated with NaD1 were examined after various time points by immunoblotting. NaD1 appeared in the cytoplasm after 30 min, and the amount present increased up until 60 min. NaD1 appeared in the cytoplasmic contents after 30 min. Purified NaD1 (1 μg) was used as a control.

Electron Microscopy—Hyphae treated with either NaD1 (10 μM) or water for 2 h were washed, fixed, and sectioned for immunogold labeling using the anti-NaD1 antibody and visualized by electron microscopy. In the NaD1-treated sections, there were a number of hyphae with granulated cytoplasm. Interestingly, NaD1 was abundant inside these hyphae but was absent from hyphae that appeared healthy (Fig. 6, a and b). Labeling was not observed when NaD1-treated hyphae with granular cytoplasm were probed with preimmune antibody.
Hyphae that were not exposed to NaD1 had no signs of granulation and did not bind the anti-NaD1 antibody (Fig. 6c).

**Labeling of NaD1 with the Fluorophore Bimane**—Uptake of NaD1 into living hyphae was observed using NaD1 that had been labeled with the fluorophore bimane-amine. The NaD1 labeled in this manner retained full antifungal activity (Fig. 7A). NaD1-bimane was taken up by hyphae within 30 min, and after 2 h, hyphae that had internalized NaD1 developed granular cytoplasm (Fig. 7B).

**Production of Reactive Oxygen Species**

To further examine the process of cell death, the production of reactive oxygen species (ROS) was investigated in hyphae treated with NaD1. The non-fluorescent molecule dihydrorhodamine 123 was preloaded into hyphae, which were then treated with NaD1. Upon production of reactive oxygen species, dihydrorhodamine 123 is oxidized to the fluorescent molecule rhodamine 123. A concentration-dependent accumulation of intracellular ROS was observed in Fov hyphae following exposure to NaD1 (Fig. 8).

**DISCUSSION**

NaD1 is expressed at high levels in the flowers of *N. alata* where it functions to protect the reproductive organs against damage from potential pathogens. We have previously reported the antifungal activity of NaD1 against the filamentous fungi *F. oxysporum f. sp. dianthi* (Race 2) and *Botrytis cinerea* (16). To determine whether NaD1 acts specifically on filamentous fungi, we tested the effect of NaD1 on other filamentous fungi as well as yeast, bacterial, and mammalian cells. NaD1 inhibited the growth of all the filamentous fungi tested at low concentrations (IC₅₀ < 1 μM) but did not affect the growth of any of the yeast or Gram-positive or Gram-negative bacteria tested. NaD1 was also not toxic to HeLa cells at the highest concentration tested. Therefore, NaD1 appears to be predominantly specific for filamentous fungi, unlike the defensin RsAFP2, which is active against *C. albicans* and *P. pastoris* (17), and DmAMP1, which is active against *S. cerevisiae* (11). Disruption of disulfide bonds resulted in a loss of antifungal activity, indicating that the three-dimensional structure of NaD1 is required for activity. This is in contrast to the mammalian α- and β-defensins, which retain antibacterial activity upon removal of their cysteine residues (18, 19).

In an effort to elucidate its mechanism of action, the effect of NaD1 on plasma membranes and its location within treated Fov hyphae was investigated using various fluorescent techniques. Most studies to date have examined the interaction of antimicrobial peptides with membranes using artificial bilayers and often use very high peptidelipid ratios (20), which may not be...
representative of in vivo activities. In this study, the interaction of NaD1 with intact fungal hyphae was examined.

The first step in antimicrobial peptide killing must be attraction of the peptide to the pathogen, and the first point of contact is the cell wall. The antifungal protein from tobacco, osmotin, for example, requires the presence of particular mannoproteins in the cell wall of S. cerevisiae for its activity (21). The cell wall of C. albicans also contains proteins that are essential for the internalization and antifungal activity of the human protein histatin 5 (22, 23). This suggests that the cell wall may play an essential role in the activity of antifungal proteins.

The binding of NaD1 to the cell wall of Fov hyphae was studied using immunofluorescence of whole cells and immunoblotting of solubilized cell wall fractions. NaD1 bound to the cell wall of all hyphae. The inability of another small, cysteine-rich protein to bind indicated that the interaction of NaD1 with the hyphal walls was specific. The addition of divalent cations to the growth medium is known to inhibit the activity of many antimicrobial peptides, including defensins (1). These conditions prevented growth inhibition as well as binding of NaD1 to Fov hyphal walls. Specific cations are believed to prevent the binding of osmotin to mannoproteins by competing for binding sites (24). A similar effect may occur here, although no evidence exists for this as yet.

Killing of cells by antimicrobial peptides has been proposed to occur in two main ways: (i) through disruption of the plasma membrane leading to leakage of cytoplasmic contents or (ii) through interaction with intracellular targets (reviewed in Ref. 13). This study demonstrated that NaD1 permeabilized the plasma membrane of susceptible hyphae in a dose-dependent manner that correlates with growth inhibition. At non-inhibitory concentrations of NaD1, some permeabilization was detected, and the cytoplasm of permeabilized hyphae appeared normal under the light microscope. However, at higher, inhibitory concentrations of NaD1, permeabilized hyphae had significant cytoplasmic granulation, and the SYTOX green fluorescence pattern was much more diffuse across the cell, indicating that the nuclei were not intact. This suggests that
NaD1-induced permeabilization is required for growth inhibition, although it may not be sufficient to induce cell death.

Although permeabilization of membranes has been reported for many antimicrobial peptides, the mechanisms of permeabilization can be very different, and in many instances, remains elusive. Various models have been suggested, including the barrel-stave model, which involves the formation of a pore by the oligomerization of amphipathic peptides to form a hydrophilic channel; the toroidal pore model, in which the pore includes lipid head groups to stabilize the high positive charge of the peptides; and the carpet model, whereby layering of the plasma membrane with positively charged protein causes destabilization in a detergent-like manner (13). NaD1 allowed entry of a 4-kDa dextran (average globular diameter of 14 Å) into hyphae but not a 10-kDa dextran (average globular diameter of 23 Å). The aperture was still present after extensive washing of the hyphae. This suggested that NaD1 formed a relatively stable aperture with an internal diameter of between 14 and 23 Å. Furthermore, the size of this apparent pore was not affected by NaD1 concentration, unlike other antimicrobial peptides such as melittin, which exhibits concentration-dependent increases in pore size (25).

It is still unknown whether permeabilization by NaD1 causes cell death by inducing leakage of cytoplasmic contents, by ion fluxes, or by facilitating entry of NaD1 into cells to access intracellular targets. In this study, the ability of NaD1 to enter Fov cells was monitored by electron microscopy and fluorescently labeled protein. Both of these approaches demonstrated that NaD1 was able to enter and accumulate at high concentrations inside hyphae. Internalization of NaD1 was observed 30 min after addition to the hyphae and increased up until 60 min. This was confirmed by immunoblotting of cytoplasmic contents.

Furthermore, the cytoplasm of hyphae that had taken up NaD1 became granular. Hyphae that did not take up NaD1 did not show signs of cytoplasmic granulation, indicating that NaD1 internalization may be an essential step in the cell killing process. This may also indicate the presence of an intracellular target for NaD1. The predicted size of the aperture induced by NaD1 (14–23 Å) and the diameter of NaD1 itself (~18 Å) suggests that NaD1 could be internalized through an aperture it creates in the membrane. This is interesting because other peptides, such as buforin II, are believed to cross plasma membranes by temporarily inserting into the membrane before translocating across and not by forming long-lived channels (26). Recently, another plant defensin, Psd1 from peas, has also been reported to enter the hyphae of the filamentous fungus Neurospora crassa. Psd1 travels to the nucleus, where it interacts with the cell cycle control protein cyclin F and halts the cell cycle (27). In contrast, NaD1, which has only 27% amino acid sequence identity with Psd1, does not appear to enter the nucleus and hence is likely to act via a different mechanism.

In this study, hyphae treated with NaD1 also produced reactive oxygen species, which suggests that the cells were undergoing a process of cell death. In this case, ROS accumulation may cause membrane damage, leading to organelle breakdown. Interestingly, ROS production was not seen at concentrations below that required for growth inhibition, even those at which membrane permeabilization was observed. This further supports the idea that membrane permeabilization, whereas required, may not be sufficient to cause cell death. The plant defensin RsAFP2 also induces ROS, and sequestration of ROS can inhibit RsAFP2 activity (28).

Taken together, we propose a model for the antifungal activity of NaD1 against Fov. NaD1 binding to the cell wall occurs first followed by rapid permeabilization of the fungal plasma membrane, allowing entry of NaD1 into hyphal cells. Whether death then occurs due to membrane leakage or interaction of NaD1 with intracellular targets, which induces programmed cell death, is still unknown; however, the production of ROS does suggest an apoptosis-like phenotype.

Plant defensins are encoded by multigene families. For example, over 300 putative defensin-like genes have been identified in Arabidopsis (29) and Medicago (30). Although some function in defense, others have a role in development (31), pollen recognition (32), and metal tolerance (33). Our observation that NaD1 can enter fungal cells points to the possibility that some plant defensins may enter plant cells and affect signaling pathways. Although we have no evidence for this, it is interesting to speculate that certain defensins may be able to transit the membranes of plant cells and affect tissue development after interaction with a specific intracellular target.

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