Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers of *Nicotiana glauca*

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SUMMARY

Defensins are a large family of small, cysteine-rich, basic proteins, produced by most plants and plant tissues. They have a primary function in defence against fungal disease, although other functions have been described. This study reports the isolation and characterization of a class I secreted defensin (NaD2) from the flowers of *Nicotiana glauca*, and compares its antifungal activity with the class II defensin (NaD1) from *N. glauca* flowers, which is stored in the vacuole. NaD2, like all other class I defensins, lacks the C-terminal pro-peptide (CTPP) characteristic of class II defensins. NaD2 is most closely related to Nt-thionin from *N. tabacum* (96% identical) and shares 81% identity with MtDef4 from alfalfa. The concentration required to inhibit *in vitro* fungal growth by 50% (IC₅₀) was assessed for both NaD1 and NaD2 for the biotrophic basidiomycete fungi *Puccinia coronata* f. sp. *avenae* (Pca) and *P. sorghi* (Ps), the necrotrophic pathogenic ascomycetes *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), *F. graminearum* (Fgr), *Verticillium dahliae* (Vd) and *Thielaviopsis basicola* (Tb), and the saprobe *Aspergillus nidulans*. NaD1 was a more potent antifungal molecule than NaD2 against both the biotrophic and necrotrophic fungal pathogens tested. NaD2 was 5–10 times less effective at killing necrotrophs, but only two-fold less effective on *Puccinia* species. A new procedure for testing antifungal proteins is described in this study which is applicable to pathogens with spores that are not amenable to liquid culture, such as rust pathogens. Rusts are the most damaging fungal pathogens of many agronomically important crop species (wheat, barley, oats and soybean). NaD1 and NaD2 inhibited urediniospore germination, germ tube growth and germ tube differentiation (appressoria induction) of both *Puccinia* species tested. NaD1 and NaD2 were fungicidal on *Puccinia* species and produced stunted germ tubes with a granular cytoplasm. When NaD1 and NaD2 were sprayed onto susceptible oat plants prior to the plants being inoculated with crown rust, they reduced the number of pustules per leaf area, as well as the amount of chlorosis induced by infection. Similar to observations *in vitro*, NaD1 was more effective as an antifungal control agent than NaD2. Further investigation revealed that both NaD1 and NaD2 permeabilized the plasma

membranes of *Puccinia* spp. This study provides evidence that both secreted (NaD2) and nonsecreted (NaD1) defensins may be useful for broad-spectrum resistance to pathogens.

INTRODUCTION

The breakdown of crop resistance against phytopathogenic fungi is a perennial problem for plant breeders on a global scale. This is mainly attributed to the planting of crop varieties in genetically uniform monocultures, which makes them more susceptible to pathogens that can reduce yield significantly. The economically important pathogens of cereal species include the biotrophs [stem rust (*Puccinia graminis*), leaf rust (*P. tritici* and *P. hordei*), stripe rust (*P. striiformis*), crown rust (*P. coronata*), powdery mildew (*Blumeria graminis*), smut (*Ustilago tritici*) and bunts (*Tilletia controversa*)] and necrotrophs [leaf blotch (*Phaeosphaeria nodorum*) and ear rot (*Fusarium graminearum*, Fgr)]. Stem rust is the most feared disease, especially in wheat and oats, as severe cases have caused total crop failure. The discovery of a new pathotype of *P. graminis* f. sp. *tritici* in Uganda in 1999 (Ug99) is of particular concern to breeders and a threat to global food security, as it carries new virulence to the major resistance (R) gene Sr31, which renders most of the resistance deployed over the past 50 years ineffective (Pretorius *et al.*, 2000; Singh *et al.*, 2008). Its spread throughout the world's wheat belt, in the Middle East and southern Asia, has been facilitated by the ability of urediniospores to travel large distances on prevailing trade winds (Ayliffe *et al.*, 2008; Hovmöller *et al.*, 2002; Singh *et al.*, 2006). The crown rust pathogen of cultivated oats is the most genetically diverse cereal pathogen as a result of its co-location and inoculum build up on wild oats. In Australasia, the crown rust pathogen has developed virulence to nearly every resistance gene deployed in cultivated oats over previous decades. Novel host mechanisms of resistance are therefore required to provide durable crop protection (Ayliffe *et al.*, 2008; Lay and Anderson, 2005).

Antimicrobial molecules are produced by a diverse range of organisms, including insects (Bulet *et al.*, 1999), plants (Broekaert *et al.*, 1997; Lay and Anderson, 2005), fungi (Mygind *et al.*, 2005) and mammals (Lehrer and Ganz, 1999), for protection against

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infection and damage by potential pathogens. Plants produce several different classes of antimicrobial molecules, including reactive oxygen species, phytoalexins, phytoanticipins and pathogenesis-related proteins (Dangl and Jones, 2001). Recent research efforts have focused on the transgenic introduction of antimicrobial proteins from multiple novel sources into agronomically important crop species (Girgi *et al.*, 2006; Oldach *et al.*, 2001). For example, the incidence of wheat leaf rust (*P. tritici* f. sp. *tritici*) and millet rust (*P. substriata*) was reduced significantly in transgenic wheat and millet plants that expressed afp (antifungal protein from *Aspergillus giganteus*) within the apoplast, relative to wild-type control plants (Girgi *et al.*, 2006; Oldach *et al.*, 2001). Other studies have reported that foliar applications of plant extracts derived from *Tulbaghia violacea* and *Agapanthus africanus* reduced the pustule number and density of leaf rust on barley leaves (Cawood *et al.*, 2010; Barna *et al.*, 2008). However, the active constituents responsible for control remain unknown.

Plant defensins are small (45–54 residues), cysteine-rich proteins that are produced by many plant species, as well as numerous plant organs, including leaves, pods, tubers and flowers (Lay and Anderson, 2005; Thomma *et al.*, 2002). Plant defensins can be divided into two major classes. Class I defensins are produced from precursor proteins composed of an endoplasmic reticulum (ER) signal sequence and a positively charged defensin domain of about 47 amino acids. The ER signal directs them into the ER. They travel through the secretory pathway and are secreted into the apoplast. Most of the well-characterized seed defensins belong to this class (van der Weerden and Anderson, 2013). Class II defensins have an additional C-terminal pro-peptide (CTPP) which is negatively charged and is required for vacuolar targeting (Fig. 1A). The class II plant defensin, NaD1, is expressed in the flowers of *N. alata* and is stored in the vacuole. It functions to protect the reproductive organs against attack by necrotrophic fungal pathogens (Lay *et al.*, 2003; van der Weerden *et al.*, 2008). NaD1 binds to the hyphal cell wall of *F. oxysporum* f. sp. *vasinfectum* (Fov), where it interacts with either the glycoprotein or β -glycan layer (van der Weerden *et al.*, 2008, 2010). After passing through the cell wall, NaD1 exerts its antifungal activity by permeabilizing the plasma membrane and entering the cytoplasm of Fov hyphae (van der Weerden *et al.*, 2008). NaD1 forms dimers in solution which are essential for its antifungal activity (Lay *et al.*, 2012).

NaD1 has potent *in vitro* antifungal activity against filamentous ascomycete fungi. The concentration of NaD1 required to inhibit fungal growth by 50% (IC₅₀) is as low as 0.8 μ M for some species (van der Weerden *et al.*, 2008). The ability of NaD1 and secreted defensins to inhibit the growth of obligate biotrophs, which include some of the most agronomically significant basidiomycete pathogens, has not been reported. Here, we report the isolation and characterization of a class I defensin from the flowers of

N. alata (NaD2), and a new bioassay for testing the effect of antifungal molecules on the early developmental stages of obligate biotrophs. The antifungal activity, phenotypic effect and proposed mechanism of activity of NaD1 and NaD2 are reported for two different cereal rust species [*Puccinia coronata* f. sp. *avenae* (Pca) and *P. sorghi* (Ps)], and are compared with the activity against a range of necrotrophic fungi.

RESULTS

Isolation and characterization of a type I defensin from *N. alata*

The term 'defensin' was used as a search query within the sol genomics network database (<http://www.sgn.cornell.edu>) to extract representative solanaceous defensin sequences for primer design and polymerase chain reaction (PCR) amplification of a class I defensin cDNA from *Nicotiana alata*. Representative class I defensin cDNAs derived from *Nicotiana tabacum* [NTS13 (Li and Gray, 1999) and Nt-thionin (Accession No BAA95697)] were selected as candidate sequences and, when aligned, were highly conserved at both the nucleotide and amino acid level (90.9% and 93%, respectively). Primers designed to these defensins amplified a 234-bp fragment from cDNA derived from *N. alata* flowers. This fragment encoded a defensin with a predicted ER signal peptide of 31 amino acids and a mature defensin domain of 47 amino acids, and was named NaD2 (*Nicotiana alata* Defensin 2) (Fig. 1B). The predicted protein lacked the CTPP domain which is characteristic of class II defensins, such as NaD1 (Fig. 1A,B), and shared 96% and 81% identity with Nt-thionin and NTS13, respectively (Fig. 1C). NaD2 contains the eight highly conserved cysteines that define plant defensins, as well as two glycine residues at positions 12 and 33, an aromatic residue at position 10 and a glutamate at position 28, which are present in most, but not all, defensins (van der Weerden and Anderson, 2013). The mass predicted from the cDNA clone (5255.9 Da) was the same as that of the NaD2 protein purified from flowers (5254.2 Da).

NaD2 falls into a group of class I defensins that is widely represented throughout the plant kingdom and includes defensins from lucerne (*Medicago truncatula*, MtDef4) (Sagaram *et al.*, 2011), cowpea (*Vigna unguiculata*, JI-2) (Urdangarin *et al.*, 2000) and African oil palm (*Elaeis guineensis*, EGAD1) (Tregear *et al.*, 2002) (Fig. 1C). NaD2 was 74%–96% identical to defensins from this group, but only 32%–42% identical to solanaceous class II defensins, and less than 30% identical to more distantly related class I defensins from radish and dahlia (Fig. 1C).

Expression of NaD2 in *N. alata* flowers

NaD2 and NaD1 were only expressed in the flowers of *N. alata* and were not detected in stems, leaves or roots (Fig. 2). NaD2, like

Fig. 1 (A) Diagram of precursors encoding class I and class II defensins. Both precursors have endoplasmic reticulum (ER) signal sequences that direct the protein into the secretory pathway. Class I defensins have no additional targeting information and are secreted. Class II defensins have a C-terminal pro-peptide (CTPP). In the solonaceous class II defensins, this pro-peptide targets the defensin to the vacuole, where the CTPP is removed to release the mature domain. Processing sites are indicated by black arrows. (B) Full-length amino acid sequence alignment of NaD2 with the class I defensins Nt-thionin (Accession No AB034956) and NTS13 (Accession No X99403) (*Nicotiana tabacum*) and the class II defensin NaD1 (*N. alata*) (van der Weerden and Anderson, 2013). ER signals are indicated in italics and the C-terminal prodomain of NaD1 is underlined. Processing sites are indicated by an arrow. (C) Amino acid alignment of NaD2 with mature class I defensin sequences from *N. tabacum* (Nt-thionin and NTS13), *Capsicum annuum* (CaDef2), *Medicago truncatula* (MtDef4), *Helianthus annuus* (Jl-2), *Elaeis guineensis* (EGAD1), *Vigna unguiculata* (Cpthio1), *Raphanus sativus* (RsAFP2) and *Dahlia merckii* (DmAMP1), and mature class II defensin sequences from *N. alata* (NaD1), *N. tabacum* (FST), *Petunia hybrida* (PhD1) and *Solanum lycopersicum* (TPP3). Disulphide bonds between the eight conserved cysteines are shown by connecting lines. Loops (L1–L7) are classified as the regions between cysteine residues (van der Weerden and Anderson, 2013).

NaD1, was most abundant in the sepals, but NaD2 was less prominent within the ovaries relative to NaD1 (Table 1). NaD2 was most abundant at stage I of floral development (initial bud stage) and gradually declined as a percentage of total protein as the flower matured (Table 1). NaD2 was less abundant than NaD1 at all stages of floral maturity and within all different floral organs tested, as measured using enzyme-linked immunosorbent analysis (ELISA) on total protein extracts from *N. alata* flowers (Table 1).

Antifungal activity of *N. alata* defensins

NaD1 and NaD2 were purified from flowers and tested for their *in vitro* (in liquid growth media) antifungal activity against a broad range of filamentous ascomycete pathogens including *Fov*, *Fgr*, *Verticillium dahliae* (Vd), *Thielaviopsis basicola* (Tb) and the saprobe *Aspergillus nidulans* (An) (Table 2). NaD1 was significantly more effective than NaD2 (4–14 times higher IC₅₀) at killing

Fig. 2 Expression of defensins in *Nicotiana alata*. Proteins extracted from flowers (F), stems (S), leaves (L) and roots (R) and separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Defensins were detected by immunoblotting with antibodies raised against NaD2 (A) or NaD1 (B). M, SeeBlue® plus two prestained protein standard (Life Technologies) size markers. The NaD2 and NaD1 defensins were only detected in flowers. Recombinant defensins (rNaD1 and rNaD2) were used as positive controls.

all of these pathogens (nonbiotrophic ascomycetes) (Table 2). The IC_{50} for NaD1 was less than or equal to $1 \mu\text{M}$ for all five ascomycetes. In contrast, NaD2 was most active against *Fgr* ($IC_{50} = 2 \mu\text{M}$) and was less active against *Fov*, *Tb* and *An* ($IC_{50} = 5\text{--}7 \mu\text{M}$). NaD2 did not inhibit the growth of *Vd* at the concentrations tested.

Development of a bioassay to test the effects of *N. alata* defensins on rust fungi

An *in vitro* bioassay was developed to assess the effect of antifungal proteins on spore germination, germ tube growth and differentiation of pathogens with hydrophobic spores (not amenable to liquid culture). Wetting agents were trialled; however, these interfered with the bioassay (data not shown). The bioassay described here is based on the inoculation of urediniospores onto 1% water agar, followed by the excision of 7-mm-diameter plugs of equal volume, which were arrayed onto microscope slides. In all cases, fresh urediniospores from living plants exhibited optimal viability, that is, had a germination frequency of >85%.

Antifungal effect of *N. alata* defensins on urediniospore germination

NaD1 and NaD2 inhibited the germination of both of the *Puccinia* rust pathogens tested (Fig. 3A,B). The most dramatic inhibition was for *Pca* urediniospores treated with $10 \mu\text{M}$ NaD1 (Fig. 3A). The

IC_{50} values for NaD1 and NaD2 against both *Puccinia* rust pathogens are shown in Table 2. Urediniospores which germinated in the treated sample were stunted and did not resemble normal germ tubes, as observed in the controls (Fig. 3A). NaD1 was more effective against *Ps* than *Pca* at the high concentrations tested, especially at $5 \mu\text{M}$ (Fig. 3B). NaD2 had higher IC_{50} values than NaD1, but still inhibited germination significantly relative to the negative control protein, NaPI (Heath *et al.*, 1997) (Fig. 3B). NaPI had no effect on urediniospore germination and spores appeared to have the same germination frequency as the double-distilled H_2O (dd H_2O) control (Fig. 3B).

Effect of *N. alata* defensins on germ tube growth and appressorium formation

Both NaD1 (Figs 4 and 5) and NaD2 (Fig. 5) arrested germ tube elongation when they were added to *Pca* and *Ps* germings, 80 min after germination. Growth arrest from NaD1 treatment was assessed 4 h after the addition of defensin. Growth arrest occurred in 70% of total scored germings for both *Ps* and *Pca* (Fig. 4B). The defensins appeared to be fungicidal as the growth arrest of *Puccinia* germ tubes was persistent 24 h after application of either NaD1 or NaD2. NaD1 treatment resulted in the stunting of germ tubes and a granular appearance of the cytoplasm in spores and germ tubes of both *Pca* and *Ps* (Fig. 4A). NaD2 produced the same effect (Fig. 5).

Four hours after spore germination, the germ tubes of *Pca* were subjected to a heat treatment for 2 h (30°C) in the presence of 0.5 mM *trans*-2-hexen-1-ol in dd H_2O to induce appressoria formation on fresh agar plugs. When NaD1 was applied to plugs that had been treated for appressorial induction 4 h after spore germination, a significant reduction (66.6%) in the number of appressoria was observed relative to the water control (Fig. 4C).

Permeabilization of rust germ tubes by *N. alata* defensins

The extent to which NaD1 and NaD2 permeabilized the plasma membrane of *Ps* germ tubes was measured using the fluorescent nucleic acid dye, SYTOX green (Life Technologies, Grand Island, NY, USA) (Fig. 5). SYTOX green dye cannot pass through an intact plasma membrane and only binds to nucleic acids of cells with compromised membranes. SYTOX green fluorescence was detected 15 min after application of the defensins to the germ tubes of both *Puccinia* species, but was not detected in the urediniospore (Fig. 5) (data not shown for *Pca*).

Effects of NaD1 and NaD2 as foliar agents against oat crown rust

The foliar application ($200 \mu\text{g/mL}$) of both NaD1 and NaD2 on crown rust-susceptible oat plants resulted in reduced pustule

Table 1 Percentage of NaD2 and NaD1 in total protein extracts derived from different stages of development and floral organs from *Nicotiana alata* flowers. Total soluble protein extracted from *N. alata* flowers was analysed by enzyme-linked immunosorbent analysis (ELISA). The relative level of defensins to total soluble protein decreased as the flowers reached maturity.

Floral developmental stage	NaD2 (%)	NaD1 (%)	<i>Nicotiana alata</i> floral organ	NaD2 (%)	NaD1 (%)
I	0.35	2.3	Petals	0.04	0.14
II	0.26	1.8	Sepals	0.08	0.38
III	0.28	1.6	Anthers	0.01	0.13
IV	0.17	1.6	Styles	0.02	0.13
V	0.08	0.4	Stigmas	0.02	0.05
			Ovaries	0.02	0.23

Table 2 The concentrations of NaD1 and NaD2 required to reduce fungal growth by 50% (IC_{50}) of the necrotrophic fungal species *Aspergillus nidulans* and the obligate biotrophic rust species *Puccinia coronata* f. sp. *avenae* and *Puccinia sorghi*. IC_{50} values (NaD1) against fungal species within the Ascomycota were sourced from van der Weerden *et al.* (2008).

Microbial classification	Organism tested	IC ₅₀ (μM)		IC ₅₀ NaD2/IC ₅₀ NaD1
		NaD1	NaD2	
Fungi				
Ascomycota	<i>Fusarium oxysporum</i> f. sp. <i>vasinfectum</i>	1	5	5
	<i>Fusarium graminearum</i>	0.5	2	4
	<i>Verticillium dahlia</i>	0.75	>10	>13.5
	<i>Thielaviopsis basicola</i>	1	7	7
	<i>Aspergillus nidulans</i>	0.8	5	6.3
Basidiomycota	<i>Puccinia coronata</i> f. sp <i>avenae</i>	2.5	4	1.6
	<i>Puccinia sorghi</i>	2	5	2.5

frequency and increased photosynthetic area relative to the oat leaves sprayed with water prior to inoculation (Fig. 6). As observed during antifungal activity assays, NaD1 was more effective than NaD2 at reducing the pustule number per leaf area (70% versus 36%) and maintaining the photosynthetic area, relative to control plants that were severely infected and chlorotic at 10 days post-infection (Fig. 6).

DISCUSSION

Plant defensins have been isolated from a number of diverse ornamental and crop species, and have been tested for their antifungal properties on several agronomically significant fungal pathogens (for a review, see van der Weerden and Anderson, 2013). The potent antifungal activity of NaD1, a class II defensin from *N. alata* flowers, has been reported against numerous filamentous ascomycete fungi, including *Fov*, *Fgr*, *Leptosphaeria maculans* and *Botrytis cinerea* (Lay *et al.*, 2003; van der Weerden *et al.*, 2008). The class I defensin NaD2, characterized in this study, is also produced by the flowers of *N. alata*, but is a weaker (2–10 times) antifungal molecule than NaD1, especially on necrotrophic fungal pathogens. The IC_{50} values obtained for NaD2 on necrotrophic ascomycete pathogens were similar to those of other well-characterized class I plant defensins, such as DmAMP1 (*Dahlia merckii*) (Thevissen *et al.*, 2000) and MtDef4 (*M. truncatula*) (Ramamoorthy *et al.*, 2007), whereas NaD1 showed similar inhibitory activity to that reported for the radish defensin RsAFP2 (Terras *et al.*, 1992).

NaD2 falls into a subgroup of class I defensins that is widely represented throughout the plant kingdom. Members of this group can be found in Arabidopsis, sunflower, alfalfa and the African oil palm, as well as other solanaceous species, such as capsicum and petunia (van der Weerden and Anderson, 2013). Several of these defensins have antifungal activity (Tregear *et al.*, 2002; Urdangarin *et al.*, 2000), although the mechanism of antifungal activity has only been studied in detail for one of these, MtDef4. MtDef4 permeabilizes the membrane of target hyphae, but does not require the presence of the sphingolipid glucosylceramide (GlcCer) for activity (Ramamoorthy *et al.*, 2007). The residues in loop five, which form part of the γ -core motif, have been implicated in the antifungal activity of plant defensins (Yount and Yeaman, 2004). Residues in this loop have been reported to be crucial for the antifungal activity of RsAFP2, MsDef1 and MtDef4 (De Samblanx *et al.*, 1997; Sagaram *et al.*, 2011; Schaaper *et al.*, 2001; Spelbrink *et al.*, 2004). The γ -core motif of MtDef4 has been demonstrated to be largely responsible for antifungal activity. When Sagaram and co-workers grafted the γ -core motif of MtDef4 onto MsDef1 (a similar class I defensin with weaker antifungal activity), the hybrid molecule showed antifungal activity against *Fgr* that was equivalent to MtDef4 (Sagaram *et al.*, 2011). Furthermore, the hybrid molecule retained activity against an MsDef1-resistant mutant of *Fgr* that lacked GlcCer (Sagaram *et al.*, 2011). NaD2 and MtDef4 share a high degree of similarity and may therefore share a similar mode of action. Based on the sequence similarity between MtDef4 and NaD2, it is also likely that the γ -core motif is involved in the antifungal activity of NaD2.

Fig. 3 The inhibitory effects of defensin on urediniospore germination. (A) Micrographs of the effect of double-distilled water (ddH₂O) (i) on urediniospore germination of *Puccinia coronata* f. sp. *avenae* (*Pca*) compared with treatment with 10 μM NaD1 (ii). Scale bar, 20 μm . (B) Graph representation of the effect of NaD1 and NaD2 on urediniospore germination of two rust pathogens *Pca* and *Puccinia sorghi* (*Ps*). Full and broken lines represent *Pca* and *Ps*, respectively, and squares, triangles and diamonds represent assays using NaD1, NaD2 and NaPI protein treatments, respectively. All experiments were repeated three times and at least 100 urediniospores were scored on each agar plug. (C) Graphic representation of germ tube growth (μm) on 1% water agar plates versus time (min) after germination. In all cases, error bars represent the standard error of the mean of four biological replicates.

A bioassay on artificial media was developed to assess the effect of defensins on different developmental stages of obligate biotrophs, such as the cereal-infecting *Puccinia* rusts. As a result of obligate biotrophy and the hydrophobicity of rust urediniospores, the defensins could not be assessed using aqueous assays performed in microtitre plates. Water agar plugs mounted on microscope slides supported urediniospore hydration and efficient application of the defensins. Field-derived isolates of rust spores are often not only genetically diverse, but can differ in virulence to their hosts (Anikster and Wahl, 1979; Burdon, 1987; Dracatos *et al.*, 2008, 2009; McDonald and Linde, 2002; Park *et al.*, 2011;

Potter *et al.*, 1990). This may explain the observation that some urediniospores germinated faster than others, or appeared to be less susceptible to defensin treatment. NaD1 inhibited rust germination at comparatively higher concentrations ($\text{IC}_{50} < 2.5 \mu\text{M}$) than the ascomycete fungi ($\text{IC}_{50} < 1 \mu\text{M}$), such as *Fov*, *Fgr* and *L. maculans* (Lay *et al.*, 2003; van der Weerden *et al.*, 2008). Higher IC_{50} inhibitory concentrations observed for the *Puccinia* species tested in this study may be a result of absorption and subsequent dilution of NaD1 within the water agar matrix.

Both defensins inhibited the germination of urediniospores and elongation of germ tubes, and were toxic against both *Puccinia* spp.

Fig. 4 (A) Micrograph images of 10 μM NaD1-treated germlings of *Puccinia coronata* f. sp. *avenae* (*Pca*) (iii), *Puccinia sorghi* (*Ps*) (iv) and their corresponding water controls (i) and (ii). Arrows denote examples of stunted germlings with granular cytoplasm. Scale bar, 20 μm. (B) The effect of 10 μM NaD1 treatment on germ tube growth from germinated *Pca* and *Ps* (white) urediniospores relative to untreated controls (black). Germlings were classified as stunted if, 4 h after defensin treatment, their length was less than twice the diameter of the urediniospore. The percentage of stunted germ tubes was calculated as the ratio of stunted to total germ tubes multiplied by 100. In all cases, error bars represent the standard error of the mean of four replicates. All experiments were repeated three times and at least 100 urediniospores were scored on each agar plug. (C) The effect of NaD1 treatment, applied 4 h after urediniospore germination, on the differentiation of *Pca* germlings, as measured by appressorium production on artificial media. Induction of appressoria was performed by treatment with 0.5 mM *trans*-2-hexen-1-ol together with heating at 30 °C for 2 h. Appressorium production was the most reproducible measurement of differentiation for experimental purposes using this method.

tested. Further evidence of the fungicidal activity of the defensins includes the observations of tip swelling and granulation of the cytoplasm in the germ tubes of *Ps* and *Pca*, respectively. In this study, both NaD1 and NaD2 permeabilized the fungal plasma membrane of the *Puccinia* spp. tested. NaD1 and NaD2 were also able to protect oat seedlings from heavy infection by *Pca* spores when the defensins were applied to the foliage as a spray.

Defensins are encoded by large multi-gene families (300 in *Arabidopsis thaliana* and *Medicago* spp.) (Silverstein *et al.*, 2005, 2006). It is likely that different defensins have evolved in plant species to provide protection against pathogens with different mechanisms of invasion and with diverse cell wall composition. Both NaD1 and NaD2 were able to inhibit the *in vitro* germination and germ tube growth of *Ps* and *Pca*, and NaD1 inhibited germ

Fig. 5 Micrographs of *Puccinia sorghi* (*Ps*) germlings treated with SYTOX green dye and double-distilled water (ddH₂O) (i and ii), 10 μ M NaD1 (iii and iv) and 10 μ M NaD2 (v and vi). Note that fluorescence caused by SYTOX green uptake is only visible in *Ps* germ tubes that have been exposed to both SYTOX green and NaD1 or NaD2 treatment. Images were viewed under white light (i, iii and v) and under a 460–490-nm MWIB filter (ii, iv and vi). Scale bar, 20 μ m.

tube differentiation. Although NaD2 had lower antifungal activity than NaD1 on necrotrophic fungi (4–14 times less active), the difference was less marked against *Puccinia* species, where it was two-fold less active than NaD1. Based on the presence of an ER signal peptide, NaD2 is likely to be secreted into the apoplast and would therefore be more likely to come into contact with biotrophic pathogens, such as rust fungi, than would NaD1, which is targeted to the vacuole. A recent study by Kaur *et al.* (2012) demonstrated that defensins targeted to the apoplast, but not to intracellular compartments, can provide protection against the obligate biotroph *Hyaloperonospora arabidopsidis*. As a consequence, NaD2 could be well suited for *in planta* expression. The stunting of germ tubes on exposure to defensin, reported by Kaur *et al.* (2012), was similar to that observed in this study.

Almost 20 unique plant defensins have been transformed into various plants (for examples, see review by De Coninck *et al.*, 2013). Given the relative success of producing transgenic plants with defensins and the lapse of time since the first defensin

transgenic plant was produced with enhanced fungal resistance (Terras *et al.*, 1995), it is interesting that none have been commercialized to date. One example of a class I defensin that showed particular promise was alfAFP (also known as MsDef1) from alfalfa (Gao *et al.*, 2000). Constitutive expression of alfAFP in potatoes conferred enhanced resistance (approximately six-fold reduction in fungal load in the transgenic versus nontransgenic plants) to early dying disease caused by *Vd*. Furthermore, the protection conferred by alfAFP was maintained under both glasshouse and field conditions over several years at different geographical sites (Gao *et al.*, 2000). However, plants expressing alfAFP also showed a reduction in potato tuber size (Seale and Vordtriede, 2010). Perhaps this and other unreported undesirable agronomic effects of transgene expression may have limited the commercial application of plant defensins for disease control in crop plants. Recently, a class II defensin from petunia flowers was used to produce transgenic banana resistant to *Fusarium oxysporum* f. sp. *Cubense* (Ghag *et al.*, 2012). These banana plants were pheno-

Fig. 6 Images of whole oat plants (A) and oat leaves (B) sprayed with NaD2 (left and top), NaD1 (centre) or water (right and bottom) prior to inoculation with *Puccinia coronata* spores. (C) Percentage reduction in pustules per square centimetre of infected leaves for NaD2-treated (black) and NaD1-treated (white) plants relative to water-treated leaves (control) (grey). NaD1 and NaD2 reduced the number of pustules per square centimetre by 70% and 37%, respectively.

typically normal; however, banana plants expressing the petunia defensin mature domain in the absence of the CTPP were severely stunted (Ghag *et al.*, 2012). This indicates that vacuolar targeted class II defensins may have less phytotoxic effects than their class I counterparts, making them a superior choice for the production of commercially viable transgenic crops.

In this study, we report the isolation and characterization of a type I defensin from *N. alata* (NaD2) and comparison of the antifungal activity of both NaD1 and NaD2 on necrotrophic and biotrophic pathogens. We have determined that defensins are able to kill rust urediniospores and germ tubes, and prevent the differentiation of rust germings. Foliar application of defensins also reduces the infection of oat plants by *Pca*. Further research is required to determine whether transgenic crop plants expressing either NaD1 or NaD2 will provide resistance to rust fungi in the field.

EXPERIMENTAL PROCEDURES

Cloning and PCR amplification of NaD2

Total RNA was extracted from immature stigmas (pre-anthesis) of *N. alata* flowers using TRIzol®, essentially as described by the manufacturer (Life Technologies). The RNA was precipitated with diethyl-polycarbonate (DEPC)-treated lithium chloride (final concentration of 2 M), collected by centrifugation and washed with 70% ethanol that had been pre-treated with DEPC. The quality of the RNA was assessed by gel electrophoresis (1% agarose). cDNA was synthesized from total RNA using a Superscript® III First Strand Synthesis Kit (Life Technologies). Any remaining RNA was removed by incubation with 2 U RNase H (Life Technologies) at 37 °C for 20 min.

Forward (GGATCCATGGCAAACCTCCATGCCG) and reverse (GAGCTCTTAGCAAGGCTGGTACAGA) primers were designed on the basis of the *Nt*-thionin cDNA sequence (Accession No AB034956) to amplify the coding sequence of the *N. alata* homologue of *Nt*-thionin (ER signal sequence and mature domain) and included both *Bam*HI and *Sac*I restriction sites at the 5' ends (italics). The NaD2 cDNA was amplified in a 50-μL PCR that contained 100 ng of stigma cDNA, Thermapol buffer (New England Biolabs, Ipswich, MA, USA), 0.2 mM deoxynucleoside triphosphate (dNTP) mix (Promega, Madison, WI, USA), 2.5 U *Taq* DNA polymerase (New England Biolabs) and 0.4 μM of forward and reverse primers. The PCR cycling conditions were as follows: 94 °C for 5 min for the initial denaturation step; 30 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min; and a final extension period of 72 °C for 10 min. PCR products were separated using gel electrophoresis and purified using the Wizard® SV clean-up system (Promega), cloned into the pCR® 2.1-TOPO® vector (Life Technologies) and transformed into *Escherichia coli* TOP10 cells (Life Technologies), according to the manufacturer's instructions. Plasmid DNA was extracted using the QIAprep® Spin Miniprep kit (Qiagen, Venlo, Netherlands) and positive clones were confirmed by sequence analysis using both M13 forward and reverse primers.

Isolation of floral defensins

NaD1 and NaD2 were extracted from flowers essentially as described in van der Weerden *et al.* (2008). The proteins were eluted as distinct peaks on reverse-phase high-performance liquid chromatography (RP-HPLC), and the identity and purity of each protein were confirmed by mass spectroscopy.

Expression of recombinant NaD1 and NaD2 (rNaD1 and rNaD2) in *Pichia pastoris*

Expression of rNaD1 and rNaD2 was performed in the methylotrophic yeast *Pichia pastoris*. A DNA fragment encoding the mature NaD1 protein was amplified by PCR using the following forward and reverse primers: forward (**CTCGAGAAAAGAGCTAGAGAATG**), which added the *XhoI* restriction endonuclease site (bold) and the KEX2 cleavage site (italics); reverse (**GCGGCCGCTTACATGGCTTAGTAC**), which added a *NotI* restriction endonuclease site (bold) and a stop codon (italics). Primers were designed to remove the STE13 protease site between the KEX2 cleavage site and NaD1 to prevent the addition of glutamic acid–alanine (Glu–Ala) repeats at the N-terminus of NaD1. An Ala was added to the N-terminus to ensure efficient cleavage by KEX2. A similar approach was used for NaD2 with the following forward and reverse primers: forward **CTCGAGAAAAGAGCTAGAACTTGCGAG** (*XhoI* restriction site in bold, KEX2 cleavage site in italics) and reverse **GCGGCCGCTTAGCAAGGCTGGT** (*NotI* site in bold, stop codon in italics). Further cloning and transformation were performed essentially as described by the manufacturer (Life Technologies). Electrocompetent *Pichia pastoris* GS115 cells (Life Technologies) were prepared as described by Chang *et al.* (2005), and linearized DNA was transformed into these cells using standard protocols. NaD1 and NaD2 were then expressed in buffered minimal media according to the manufacturer's instructions and purified via cation-exchange chromatography. Eluted proteins were subjected to RP-HPLC using a 40-min linear gradient as described by Lay *et al.* (2003). Protein peaks were collected and analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-NaD1 and anti-NaD2 antibodies (see below). Fractions containing the protein of interest were lyophilized and resuspended in sterile MilliQ ultrapure water (Merck Millipore, Billerica, MA, USA). The protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

Production of anti-NaD1 and anti-NaD2 antisera

Polyclonal antibodies were raised against purified NaD1 or NaD2 (1.5 mg) that had been conjugated to keyhole limpet haemocyanin (0.5 mg) and injected into a rabbit, essentially as described by Lay *et al.* (2003).

SDS-PAGE and immunoblot analysis

Soluble protein (30 µg) from whole flowers and from all other tissues (200 mg) was analysed for expression using both α -NaD1 and α -NaD2 antibodies, as described by Lay *et al.*, 2003.

Quantification of NaD1 and NaD2 levels using ELISA

Prior to use in an ELISA, both the α -NaD1 and α -NaD2 antibodies were biotinylated. Protein A-purified antibodies (2 mg) were passed through a PD-10 column (GE healthcare Bio-Sciences, Uppsala, Sweden) to exchange the glycine with phosphate-buffered saline (PBS) before they

were lyophilized on a DYNAVAC FD5 freeze drier. The antibodies were dissolved in 1 mL PBS, pH 7.5, and incubated on ice for 2 h at a molar ratio of 1:20 with sulfo-NHS-LC-Biotin (Pierce). The concentration of biotin-labelled antibody was estimated by absorbance at 280 nm. The α -NaD2 (100 ng per well) and α -NaD1 (50 ng per well) antibodies were absorbed to the base of the wells of a 96-well microtitre plate by incubation at 4 °C in a humid box overnight. The wells were then washed with PBS (4 × 2 min) and blocked with 3% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA; ELISA grade) in PBS for 2 h at room temperature, and then rewashed as described above. Protein extracts were prepared by grinding 100 mg of liquid nitrogen-frozen tissue in a mixer mill (2 × 10 s, frequency 30) before the addition of 900 µL of 2% insoluble polyvinylpyrrolidone (PVPP) (Sigma-Aldrich) in PBST [PBS; 0.05% (v/v) Tween-20]. Protein extracts (100 µL) were added to the wells and the plates were incubated for 2 h at 25 °C. NaD1 and NaD2 protein standards (100 µL, 0.1–0.0015 ng/µL) were prepared in PBS and added to the wells at the same time as the protein extracts. Excess extract was removed by washing with PBS (4 × 2 min) prior to the addition of α -NaD2 (150 ng per well) or α -NaD1 (50 ng per well) biotin-labelled antibodies in PBS. The plates were then incubated for 1 h at 25 °C. Excess antibody was removed by again washing with PBST (4 × 2 min) prior to the addition of NeutrAvidin horseradish peroxidase (HRP)-conjugate (0.1 µL/well in 100 µL PBST, Thermo Fisher Scientific). Plates were incubated for 1 h at 25 °C before washing with PBST (4 × 2 min) and water (2 × 2 min). The ImmunoPure OPD substrate (Thermo Fisher Scientific) was prepared according to the manufacturer's instructions, and 100 µL of substrate solution were added to each well. Plates were allowed to develop for 5 min before the reaction was stopped by the addition of 50 µL of 2.5 M sulphuric acid. Absorbance at 490 nm was measured using a SpectraMax M5e microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA).

Fungal isolates

Field isolates (mixture of genotypes) of oat crown rust (*Pca*) were provided by Dr Philip Keane (La Trobe University, Melbourne, Vic., Australia) and common maize leaf rust (*Ps*) urediniospores were provided by Dr Tony Pryor (CSIRO Plant Industries, Canberra, Australia). All bioassays were performed, where possible, with freshly harvested urediniospores.

Five other fungal isolates were assessed for sensitivity to both NaD1 and NaD2: *Fgr* (Australian isolate CS3005; provided by CSIRO Plant Industry, St Lucia, Qld, Australia); *Fov* (Australian isolate VCG01111 isolated from cotton; provided by Wayne O'Neill, Farming Systems Institute, DPI, Qld, Australia); *Vd* (provided by Helen McFadden, CSIRO Plant Industry, Black Mountain, Australia); *Tb* (provided by Dr David Nehl, NSW Department of Primary Industry, Narrabri, Australia); *An* (strain A850; provided by Professor Michelle Momany, University of Georgia, Athens, GA, USA).

Antifungal bioassays on ascomycetes

Antifungal bioassays were prepared essentially as described by van der Weerden *et al.* (2008).

Production of rust spores for *in vitro* bioassays

Rust spores were prepared essentially as described by Dracatos *et al.* (2010). Separate spore suspensions were prepared for *Pca* and *Ps* depending on the host to be inoculated. Briefly, urediniospore suspensions were prepared containing 5×10^6 spores/mL in 25 mL of a stock solution (one drop of Tween-20 in 100 mL of tap water) and decanted into a 500-mL conical glass flask attached to a Venturi atomizer. Spore suspensions were mixed vigorously and the atomizer was used to spray inoculate susceptible 6-week-old oat plants (*Avena sativa* cv. Swan, provided by Dr Philip Keane La Trobe University) or maize seedlings (Pioneer 33V15, provided by Dr Joe Kochman, Department of Primary Industry, Toowoomba, Qld, Australia). Plants were inoculated at 17.00 h, followed by an incubation period of 16 h in the dark at 20 °C within a dew chamber, before plants were placed in the glasshouse at 25 °C.

In vitro bioassay to determine the effect of NaD1 and NaD2 on *Puccinia* urediniospore germination and germ tube growth

Both NaD1 and NaD2 that had been isolated from *N. alata* flowers were assessed for their inhibitory effect on urediniospore germination (*Pca* and *Ps*). The germ tube growth of *Pca* and *Ps*, after exposure to NaD1, was also assessed, as was the ability of the germ tubes to differentiate and produce appressoria (*Pca*) in the presence of NaD1. A 6-kDa serine proteinase inhibitor protein from *N. alata* (NaPI) with no reported antifungal activity (Heath *et al.*, 1997) was used as a negative control. Preparation of the bioassay involved the collection of fresh urediniospores from the surface of pustules on oat leaves with a fine wet paintbrush, and spreading of the urediniospores onto 9-cm-diameter Petri dishes containing 10 mL of 1% water agar. An inverted 1-mL sterile pipette tip was used to extract water agar plugs with the inoculum (diameter, 7 mm) from the centre of the plate. Plugs were placed on a microscope slide using a sterile scalpel. Urediniospores were left to hydrate for 20 and 75 min in the dark at 20 °C for germination and germ tube growth assays, respectively.

For the assessment of urediniospore germination, plugs treated with a total of six different protein concentrations (10, 5, 2, 1, 0.5 and 0.1 µM), as well as water as a nonprotein-treated control, were included on each slide (10 µL per plug). Four replicate slides were used per experiment and each experiment was repeated three times. Plates were then left to incubate in the dark at 20 °C for 150 min (when the inoculated control plate started to produce germ tubes that were >80 µm in length) (Fig. 3C). The germination of urediniospores was measured by counting spores versus germlings (spores with germ tubes >40 µm in length) under a light microscope under 100× magnification. Four experimental replicates were used and two readings were taken per replicate from the centre of the inoculated water agar plugs. In all cases, at least 100 urediniospores and/or germ tubes were scored for each treatment replicate. The mean germination percentage (%) was calculated for each treatment and plotted against the protein concentration (µM). The defensin concentration that inhibited growth by 50% (IC₅₀) was then determined from germination inhibition plots. Rust germ tubes were scored as stunted if their length was less than twice the diameter of the spore and nonstunted when their appearance was the same as that of the untreated control (e.g. >40 µm in length).

Measurement of the effect of NaD1 on crown rust appressoria induced on artificial media

Appressoria were induced on 1% water agar plugs by a modified method previously described by Wiethölter *et al.* (2003). This method is based on the presence of a humid environment in combination with both physical (heat shock) and chemical (*trans*-2-hexen-1-ol) (Sigma-Aldrich) stimuli. Crown rust urediniospores (2 mg) were spread evenly onto a Petri dish (containing 10 mL of 1% water agar) using a fine paintbrush, and six plugs were removed as described previously and placed on the lid of a 5-cm Petri dish (repeated for four Petri dishes to give a total of 24 plugs). Spores were left to germinate for 90 min at 20 °C, and then 5 mL of 0.5 mM *trans*-2-hexen-1-ol were added to the bases of the Petri dishes, and the lids with plugs were placed on the Petri dish bases and sealed with Parafilm, followed by heat treatment at 30 °C for 2 h. NaD1 (10 µL of 10 µM) was applied 4 h post-germination and water was applied as a control. All steps were performed in the dark. Appressoria numbers were counted (as a percentage of germinated spores) after 24 h by examining eight individual plugs per treatment under an Olympus BX50 microscope (Olympus, Tokyo, Japan).

Membrane permeabilization assay

Ps and *Pca* urediniospores were brushed onto water agar and incubated at 20 °C for 1 h in the dark. Agar plugs with germinated spores, with germ tubes of approximately 60 µm in length, were treated in duplicate with either 10 µL of 10 µM NaD1 or NaD2 containing 0.5 µM SYTOX green in dimethylsulphoxide (DMSO) (Molecular Probes), which was applied in a darkened room and left to stand for 10 min. In all cases, a distilled water control containing 0.5 µM SYTOX green and no defensin and a second control with 10 µM NaD1 or NaD2 and no SYTOX green were included in the bioassay. Images were captured with an Olympus BX50 fluorescence microscope using an excitation wavelength of 460–490 nm and an MWIB filter.

Measurement of the effect of NaD1 and NaD2 on crown rust infection of oat plants

Oat seeds (cv. Swan) were sown in 9-cm pots (14 seeds/pot) filled with a 2:1 mixture of pine bark and river sand. An initial dose of the water-soluble fertilizer Aquasol (10 g/10 L of tap water) was applied before sowing. After 7 days, both Aquasol and urea were applied (10 g/10 L of tap water). Lyophilized defensin (1.2 mg) was resuspended in sterile MilliQ water to 1 mg/mL, diluted to 200 µg/mL and then 3 mL of each treatment (NaD1, NaD2 and water) was sprayed separately onto 8-day-old seedlings (eight seedlings per pot × four seedling pots per treatment) using a hydrocarbon pressure pack. Forty five minutes later, the seedlings were inoculated with a pathotype of *Pca* that is virulent on Swan by the spraying (5×10^6 spores/mL) of urediniospores suspended in light mineral oil (Isopar-L, ExxonMobil Chemical, Houston, TX, USA) using a hydrocarbon pressure pack. Inoculated plants were incubated overnight in a dew chamber at 20 °C in the dark, before being transferred to a growth cabinet (22–23 °C). Plants were subjected to ambient natural light regimes. Representative photographs of each treatment were taken at 10 days post-infection (both

whole plants and leaf sections), and the mean pustule number per square centimetre of leaf area was calculated on the basis of eight measurements (two leaves per pot) per treatment.

ACKNOWLEDGEMENTS

We thank Dr Phillip Keane for assistance with microscopy and Dr Tony Pryor and Professor Robert Park for the provision of rust isolates used in this study.

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