Interactions with fungal cell wall polysaccharides determines the salt tolerance of antifungal plant defensins.

[1,2] Mark R Bleackley, [1,2] Charlotte S Dawson, [1]Donovan Garcia Ceron, [1]Jennifer AE Payne, [1,2] Nicole L van der Weerden, [1,2] Marilyn A Anderson

[1] La Trobe Institute for Molecules Science, Bundoora, VIC, Australia
[2] Hexima Ltd, Melbourne, VIC, Australia

Objectives: Host toxicity and the development of fungal resistance threaten the longevity of currently used clinical antifungals. The need for the development of new antifungal molecules to add to the arsenal for control of fungal pathogens is urgent. Naturally occurring antifungal peptides from plants are an attractive option for the development of next generation antifungals. Plants lack the adaptive immune system found in mammals and are thus fully dependent on their arsenal of innate immunity molecules. One of the largest families of plant antifungal peptides are the plant defensins. The plant-defensin fold is extremely stable to extremes in pH and temperature as well as proteolytic degradation. Interactions with lipids and polysaccharides are key components of the mechanisms of many plant defensins. One of the major hurdles in the development of plant defensins for treatment of systemic fungal infections is that most plant defensins are inactive in physiological salt concentrations. Understanding why so many of these molecules lose activity in the presence of salt and identifying members of the plant defensin family that do not lose activity in the presence of salt is crucial for the development of plant defensins as antifungal molecules. Plant defensins bind lipids in the presence of physiological salt [1,2] leading to the hypothesis that the fungal cell wall caused the loss of activity in the presence of salt.

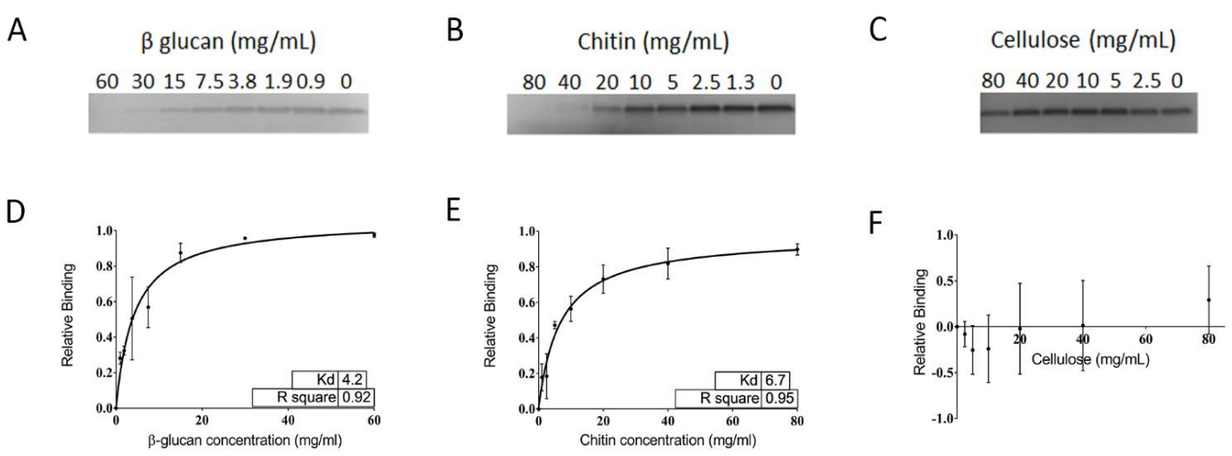

Figure 1. The interaction between NaD1 and β-glucan, chitin and cellulose. Binding of NaD1 to insoluble carbohydrates was assessed by solution depletion assays and SDS-PAGE. Coomassie stained gel images of NaD1 remaining in the supernatant after incubation with increasing amounts of insoluble β-glucan (A), chitin (B) and cellulose (C). The amount of protein in each of the supernatants was quantified via densitometry of the bands and used to generate binding isotherms (D,E and F). The dissociation constants for each pair were calculated using a non-linear regression with a one site binding model. NaD1 binds chitin with a Kd of 6.7 ± 2.4 mg/mL and β glucan with a Kd of 4.2 ± 0.8 mg/mL which when calculated with respect to the molar concentration of each monosaccharide are 33.0 ± 11.8 mM and 25.9 ± 5.0 mM respectively. NaD1 did not bind to cellulose.

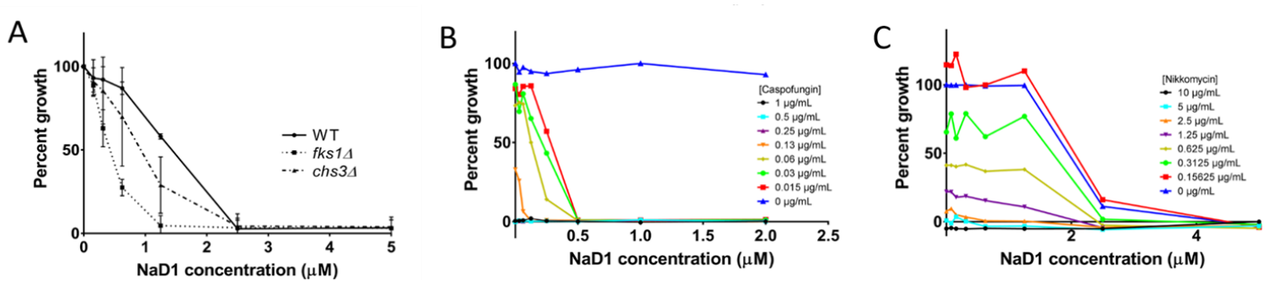

Figure 2. β-glucan and not chitin levels are responsible for the protective qualities of the fungal cell wall against plant defensins. The contributions of chitin and β-glucan towards the protective function of the fungal cell wall were assessed by modulating the level of each polysaccharide both chemically and genetically. (A) A *S. cerevisiae* strain with a deletion in the major 1,3-β-glucan synthase subunit *fks1Δ* was more sensitive to NaD1 than wildtype or a strain lacking the major chitin synthase *chs3Δ*. Similarly, the 1,3-β-glucan synthase inhibitor caspofungin acted in synergy with NaD1 against *C. albicans* (B) while the chitin synthase inhibitor Nikkomycin Z did not (C).

1. Poon, I., et al., *Phosphoinositide-mediated oligomerization of a defensin induces cell lysis.* Elife, 2014. **3**: p. e01808.
2. Payne, J.A., et al., *The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4, 5 bisphosphate.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2016.

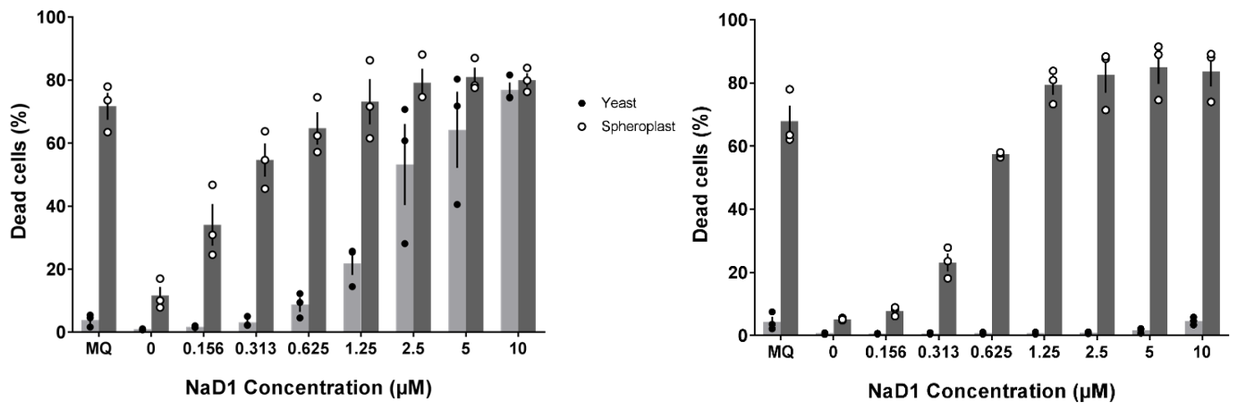

Figure 3. The cell wall protects yeast against the antifungal activity of NaD1 and is responsible for the lack of activity in the presence of NaCl. (A) Intact yeast cells and spheroplasts were treated with a range of concentrations of NaD1 and cell death was monitored by flow cytometry using the cell viability stain SYTOX green. Cell death of spheroplasts occurred at much lower defensin concentrations than that required to kill intact yeast cells. (B) When the experiment was performed with the addition of 100mM NaCl activity against spheroplasts was retained but activity against yeast with intact cell walls was lost. Data is the average of three independent experiments, values for individual experiments are represented as filled (yeast) or empty (spheroplast) dots, error bars are standard error of the mean.

Polysaccharide		NaD1	HXP4	NaD2	TsD10	DmAMP1
B-glucan	K_d (mM)	25.9 ± 5.0	30.2 ± 12.0	58.6 ± 11.7	202.5 ± 25.9	NB
	R-squared	0.92	0.94	0.88	0.87	NB
Chitin	K_d (mM)	33.0 ± 11.8	6.9 ± 3.0	7.9 ± 2.0	NB	NB
	R-squared	0.95	0.96	0.97	NB	NB

Table 1. Quantitation of the interaction between plant defensins and fungal cell wall polysaccharides. Binding was assessed using binding isotherms as presented in Figure 1 for NaD1. NB indicates combinations where no binding was observed. K_d values are calculated based on the concentration of the monosaccharide units as the polysaccharides used were heterogeneous and are presented ± SEM from three independent replicates. DmAMP1 did not interact with β-glucan or chitin.

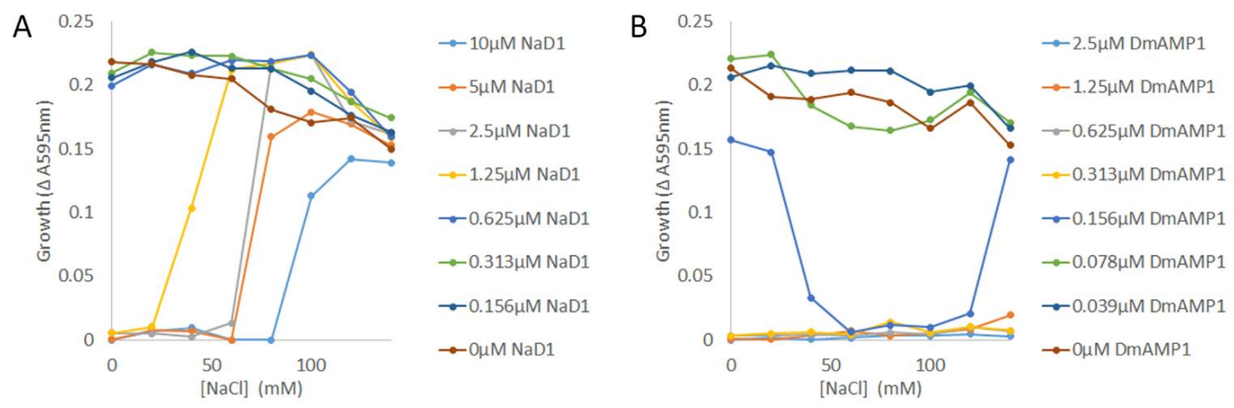

Figure 4. Unlike NaD1, DmAMP1 does not lose activity at higher salt concentrations. *S. cerevisiae* was grown in ½ PDB with increasing concentrations of salt and plant defensins in a checkerboard pattern. Growth at each defensin concentration was plotted as a function of salt concentration. (A) The activity of NaD1 against *S. cerevisiae* was inhibited by increasing concentrations of salt as indicated by the increase in absorbance at 595 nm in the 1.25, 2.5, 5 and 10 μM traces as the NaCl concentration approached 100 mM. (B) DmAMP1 activity was not inhibited by the NaCl as indicated by the lack of increase in absorbance at 595 nm in the 0.313, 0.625, 1.25 and 2.5 μM traces. Intermediate concentrations of NaCl improved the growth inhibition by 0.156 μM DmAMP1. Data is representative of three independent replicates.

Conclusion: Interaction between plant defensins and fungal cell wall carbohydrates is a potential mechanism for the loss of antifungal activity in elevated salt concentrations. Future work will focus on further assessing the potential for these molecules to be developed as antifungals by looking at their effects on various mammalian cell types and efficacy at clearing fungal infections in animal models. The differences in the mechanisms of salt-tolerant and intolerant defensins will also be investigated.

m.bleackley@latrobe.edu.au