The potential of the antifungal protein NaD1 for control of fusarium wilt and verticillium wilt

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1. Introduction and background

Two of the major fungal diseases of cotton in Australia are fusarium and verticillium wilts caused by the fungus *Fusarium oxysporum* f.sp vasinfectum (*Fov*) and *Verticillium dahliae* respectively. Both pathogens infect seedlings via the root and cause wilting, stunted growth and for *Fov*, death of some plants. In Australia *Fov* is the major cause of crop losses due to fungal infection, however, the incidence of verticillium wilt has increased recently due to the planting of susceptible varieties (Johnson and Nehl, 2004).

We have been studying NaD1, a naturally occurring antifungal protein from the ornamental tobacco (*Nicotiana alata*) (Lay et al., 2003). NaD1 is a member of the plant defensin family, a group of small, basic peptides with similar 3-D structures (Thomma et al., 2002). Plant defensins have a range of antifungal activities. Several plant defensins, when expressed in transgenic plants, enhance resistance to pathogen attack (Bart et al., 2002), and field trials by Monsanto, of transgenic potato expressing an alfalfa defensin, have demonstrated resistance to *Verticillium dahliae* in some lines (Gao et al., 2000).

In a program funded by Hexima Ltd, we have produced transgenic cotton plants expressing NaD1 under the control of the 35S promoter. Four lines, carrying single copies of the gene, were selected for further analysis. Expression of NaD1 was high in most plant tissues tested.

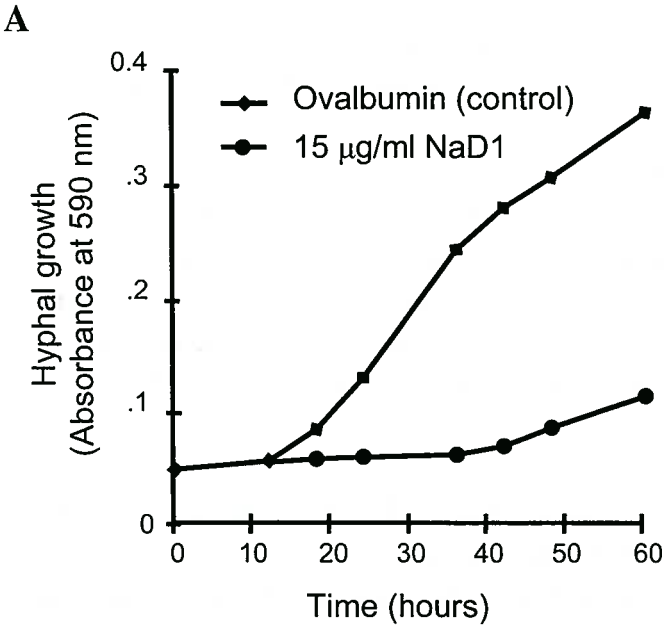
2. *In vitro* bioassays

Microconidia were germinated in media (1 X 10^4 spores/ml) containing purified NaD1 or ovalbumin as a control. Assays were performed in microtitre plates and the growth of hyphae determined by measuring the absorbance at 590 nm from 0 to approximately 60 hours. Five concentrations of NaD1 or ovalbumin (2, 5, 10, 15 and 20 □g/ml) were used in the assays.

NaD1 inhibited spore germination and hyphal growth of both *Fov* (Fig. 1A) and *V. dahliae* in *in-vitro* assays (Fig. 2A). Partial inhibition of fungal growth was observed at around 10 □g/ml and close to 100% inhibition was obtained at 15 □g/ml or higher. These results are very similar to those published for other plant defensins and related pathogens (Osborn et al., 1995).

The inhibited fungi were also observed under a light microscope (Fig. 1B and Fig. 2B). No major morphological changes were observed, although *Fov* grown in the presence of NaD1 did show some increased branching and shorter hyphae.

Figure 1. *In vitro* inhibition of *Fov* VCG01111 hyphal growth by NaD1. **A**: Time course
B: Appearance at 24 h (X 25 magnification)



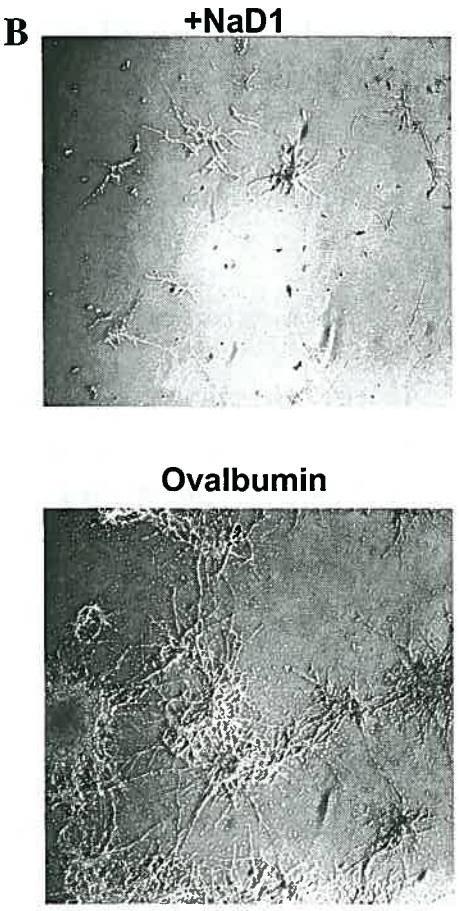
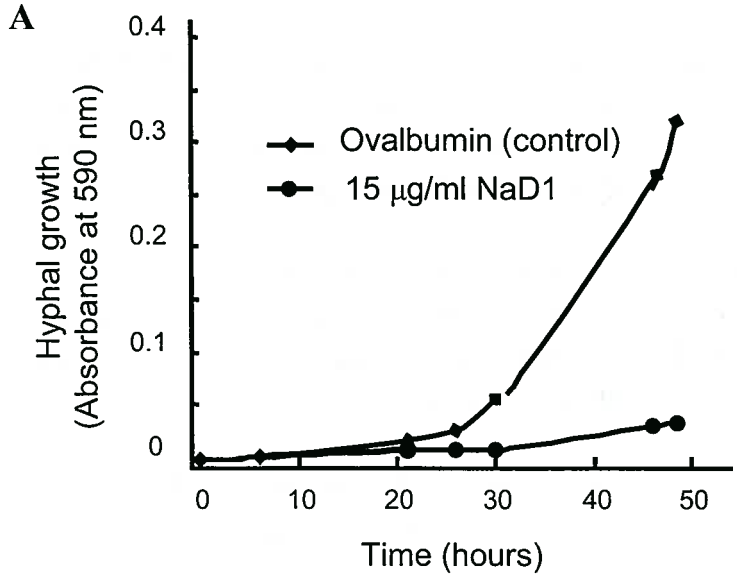
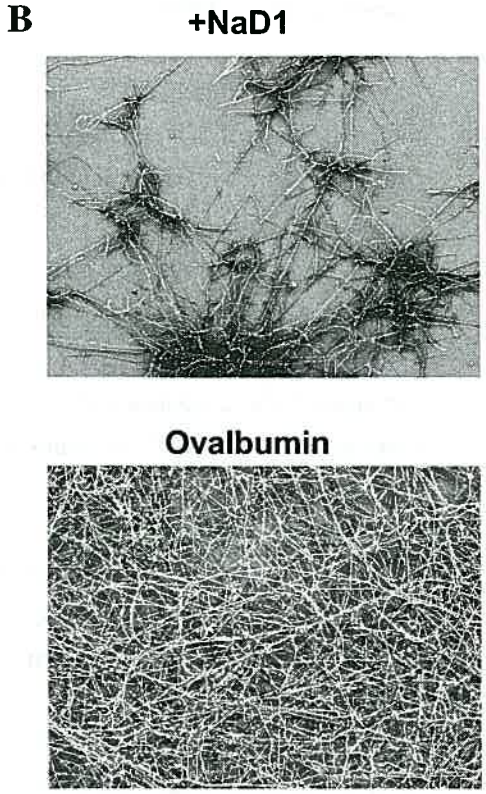


Figure 2. *In vitro* inhibition of *Verticillium dahliae* hyphal growth by NaD1. **A**: Time course
B: Appearance at 48 h (X 1,000 magnification)





3. Transgenic plant bioassays

We have developed an infected soil assay to assess the transgenic cotton lines for resistance to *Fov* and *Verticillium dahliae*. Millet infected with fungus was incorporated into a peat based soil mix at a concentration of 1% v/v (*Fov*) or 3% v/v (*V. dahliae*) and transferred to large plastic containers in the glasshouse.

Three control varieties were included in each trial: a resistant variety (Sicot 189 for *Fov* and Sicala V2 for *V. dahliae*), a susceptible variety (Siokra 1-4) and the untransformed parent (Coker 315). Seeds were randomised within each container.

The height and condition of the plants was recorded weekly and the disease scored 8-11 weeks after sowing by measuring the vascular browning at the time of harvest (see legend Table 1).

Fov bioassay

Several bioassays have been performed with *Fov*. Experiments set up in the cooler months consistently result in higher disease pressure and a better separation of the resistant and susceptible varieties. One transgenic cotton line, 35.125.1, has resistance to *Fov* and we are continuing to assess this line.

The results presented in Table 1 are from an experiment performed from mid June to late August, 2003. Sixty seed (30 uninfected and 30 infected) were planted for each variety or transgenic line. As expected the resistant variety Sicot 189 had the highest number of surviving plants (60%) and a low disease score (3.4) while the susceptible variety Siokra 1-4 had substantially fewer surviving plants (24%) and a higher disease score (4.6). Untransformed Coker was intermediate with the controls. Importantly, transgenic line 2 (35.125.1) rated better than the Coker parent and had similar plant survival and disease score to Sicot 189.

	Siokra 1-4	Coker	Sicot 189	Transgenic line 1	Transgenic line 2
No. dead plants	19	13	10	18	13
No. living plants	6	12	15	12	17
% living plants	24%	48%	60%	40%	57%
Disease score*	4.6	3.8	3.4	4	3.3

Table 1. *Fov* bioassay with transgenic cotton

*Average of all plants that germinated. 0 = no symptoms, 1 = vascular browning to base of stem, 2 = vascular browning to cotyledons, 3 = vascular browning past cotyledons, 4 = vascular browning to true leaves, 5 = dead

Future plans

Further assessment of the transgenic lines is in progress. The priority is to increase the number of replicates for statistical analysis and to complete the assays in the colder months when the disease pressure is higher.

Work by Christina Hall at the University of Melbourne on the entry of *Fov* into the cotton root suggests that it will be important to target the NaD1 protein to specific regions of the root (see poster at this conference). The original transgenic cotton lines were transformed with the NaD1 gene under control of the constitutive 35S promoter. These lines express moderate levels of NaD1 in the roots however we do not know whether the hyphae make direct contact with the protein. To answer this question we are currently studying the infection process using electron microscopy and gold labeling to detect NaD1. We are also producing new transgenic cotton lines with the NaD1 gene under the control of a different promoter that may give better expression of NaD1 in the root tissue.

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