Cotton Research & Development

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **MU1C**

Annual Report: ☐ Due 30-September

Progress Report: ☐ Due 31-January

Final Report: ☒ Due 30-September
(or within 3 months of completion of project)

Project Title: Transgenic cotton for the control of Fusarium Wilt

Project Commencement Date: 1/02/02 **Project Completion Date:** 30/06/04

Research Program: 5. Breeding and Biotechnology - Please Select One -

Part 2 – Contact Details

Administrator: Ms Lisa Batten, Grants Officer
Organisation: Melbourne Research and Innovation Office
Postal Address: The University of Melbourne, Victoria, 3010
Ph: 03 8344 2052 **Fax:** 03 9347 6739 **E-mail:** lbatten@unimelb.edu.au

Principal Researcher: Dr Robyn Heath, Senior Research Fellow
Organisation: School of Botany, The University of Melbourne
Postal Address: The University of Melbourne, Victoria, 3010
Ph: 03 8344 3323 **Fax:** 03 8344 4612 **E-mail:** robynlh@unimelb.edu.au

Supervisor: Dr Robyn Heath, Senior Research Fellow
Organisation: School of Botany, The University of Melbourne
Postal Address: The University of Melbourne, Victoria, 3010
Ph: 03 8344 3323 **Fax:** 03 8344 4612 **E-mail:** robynlh@unimelb.edu.au

Researcher 2 Professor David Guest
Organisation: The University of Sydney
Postal Address: Faculty of Agriculture, Food and Natural Resources
John Woolley Building A20 NSW 2006
Ph: 02 9351 2946 **Fax:** 02 9351 4172 **E-mail:** guestd@agric.usyd.edu.au

Signature of Research Provider Representative: _____

Part 3.3 – Final Reports (due 3 months after completion of project)

1. Outline the background to the project

Fusarium wilt is a major disease of cotton and occurs in many of the cotton growing regions of Australia. It is caused by the fungus, *Fusarium oxysporum* f.sp *vasinfectum* (Fov), which was first identified in Australia in 1993. Fov survives in the soil where it enters the roots of cotton seedlings and causes vascular wilt. Fov is readily spread by soil or water and current management methods have focused on slowing the spread of the disease using best hygiene practices.

Fov is difficult to eradicate from the soil and the only option for farmers with soil contaminated with Fov is to grow resistant cotton varieties. However, there are no varieties completely resistant to the Australian isolates of Fov, although several varieties show some tolerance. Increasing the resistance to Fov can be achieved by introducing resistance genes into the plant. This can be done either by backcrossing elite varieties with *Gossypium* species known to carry resistance genes to Fov or by introducing anti-fungal genes by genetic manipulation.

We have focused on the second option and, with funding from Hexima Ltd, have produced several transgenic cotton lines expressing a plant defensin (NaD1). Defensins are a group of antifungal proteins that are present in many plant species and are thought to be part of the natural defense mechanism of plants against pests and pathogens (Lay and Anderson, 2004). Dr Fung Lay at La Trobe University has isolated and characterized a floral plant defensin, NaD1, from ornamental tobacco (*Nicotiana alata*) (Lay et al, 2003). *In vitro* studies show that NaD1 can inhibit germination and hyphal growth of Fov and some other fungal pathogens. Therefore, NaD1 is an excellent candidate for transfer into cotton for control of fungal diseases.

Four transgenic cotton lines expressing NaD1 under the control of the constitutive Cauliflower mosaic virus 35S promoter were made available for this project by Hexima Ltd. These lines had not been fully characterised and homozygous seed was only available for two of the lines. The major part of this project was to assess these lines for increased resistance to Fov and other cotton pathogens compared to the untransformed parent line (cv Coker).

Although the 35S promoter is a strong constitutive promoter and was expected to result in some expression of the NaD1 protein in root tissue, the level may not be high enough to inhibit the growth of the Fov hyphae. Therefore, one of the objectives of this project was designed to assess two tissue specific promoters from *Arabidopsis thaliana*. These promoters had not been tested before in cotton but had the potential to give higher expression of NaD1 in tissue that the Fov hyphae enter.

The major aims of this project were to:

1. Assess the efficacy of the defensin protein, NaD1, against *Fusarium oxysporum*, *Verticillium dahliae* and *Thielaviopsis basicola* in transgenic cotton (Objectives 1-3)
2. Assess new promoters that may be useful for expression of antifungal proteins in transgenic cotton (Objective 4)

This project commenced 1 February 2002 and finished 30 June 2004 when funding from CRDC was withdrawn.

Two scientists, Mr James McKenna (80%) and Ms Maria Rainone (20%) were employed on this grant. Dr Robyn Heath was the Project Supervisor and Professor David Guest provided advice on the Fov and *Verticillium* bioassays. Dr Helen McFadden (CSIRO, Plant Industry, Canberra) performed two of the early Fov bioassays and Dr David Nehl (NSW Department of Primary Industries, Narrabri) conducted the *Thielaviopsis basicola* bioassays.

Since 1 July 2004, Hexima Ltd has continued to support this project through a Research Agreement with the University of Melbourne.

2. List the objectives and the extent to which these objectives have been achieved

The objectives below were designed for a three year program. The shortfall of 8 months has meant that some of the objectives have not been completed.

Objective 1

Characterization of existing transgenic cotton lines expressing NaD1 and assessment of these lines for resistance to Fov

This objective was achieved. Expression levels and gene copy number were determined in three transgenic lines. Initial Fov assessments using the seedling dip assay were unsuccessful and a new method using Fov infected soil was developed. One transgenic line showed increased resistance to Fov in a glasshouse trial.

Objective 2

Detailed studies of Fov and *V. dahliae* infection in transgenic cotton

This objective was partially achieved. *In vitro* assays with purified NaD1 showed that the NaD1 protein can inhibit the growth of *V. dahliae*. The infection process of Fov was studied using light and fluorescent microscopy. Material to be used for immunogold labelling and visualisation with the electron microscope has been prepared.

Objective 3

Assessment of existing transgenic cotton lines expressing NaD1 for resistance to *Thielaviopsis basicola* and *Verticillium dahliae*

The assessment of the transgenic lines for resistance to *Thielaviopsis basicola* was achieved. This work was carried out by Dr David Nehl (report attached).

A new method for inoculation of *V. dahliae* was developed using infected soil. One preliminary glasshouse trial using this method was completed.

Objective 4

Production and assessment of new transgenic cotton lines transformed with gene constructs containing tissue specific promoters

This objective was partially achieved. A new gene construct incorporating the CesA promoter was prepared. Two transformation experiments using this construct were established and embryos from the experiments are currently being produced.

3. Detail the methodology and justify the methodology used

3.1. Characterization of existing transgenic cotton lines expressing NaD1

3.1.1 Production of homozygous seed

Four transgenic cotton lines were provided by Hexima Ltd. The lines (cv Coker) contained the NaD1 gene under the control of the 35S promoter. Two lines were homozygous (35.9.1 and 35.105.1), while two lines were hemizygous (35.125.1 and 48.287.1). To produce homozygous lines of 35.125.1 and 48.287.1, seed from hemizygous plants were germinated on media containing 10 ug/ml kanamycin and the surviving plants grown in the glasshouse. Seed from these plants were then grown on media containing 10 ug/ml kanamycin. If 100% of the seed survived in the presence of kanamycin the plant was considered homozygous.

3.1.2 Detection of NaD1 in transgenic cotton

NaD1 was detected by immunoblot analysis. Cotton leaf tissue (50 mg) was extracted in 2 X sample loading buffer (NuPage) with 10% mercaptoethanol. Protein concentrations were determined using the RC DC protein assay kit (BioRad). Proteins were separated on a 4-12% gradient acrylamide gel (NuPage) and transferred to 0.22 micron nitrocellulose. Membranes were blocked in 3% BSA in TBS for 1 h and then incubated in anti-NaD1 antisera (Lay et al 2003) overnight at room temperature. Membranes were washed in 1 X TBS 0.05% Tween 20 and then incubated with HRP-conjugated goat-anti-rabbit IgG (1:100,000) for 1 h. The membranes were washed, incubated in substrate (Supersignal West Pico, Pierce) and exposed to film (Hyperfilm ECL, Amersham).

3.1.3 Determination of copy number

The number of copies of the NaD1 gene in each of the transgenic line was determined by Southern blot analysis. Genomic DNA was extracted from cotton leaves using the DNeasy^{tm} plant mini kit (Qiagen), digested with *Bgl* II or *Bcl* I and then loaded onto a 0.8% agarose in 1 X TBE gel and run overnight at 40V. Before blotting, the DNA gel was treated in 0.2M HCl for 10 min, 1.5M NaCl, 0.5M NaOH for 20 min and 1.0M Tris-Cl pH 7.5, 1.5M NaCl for 20 min. Gels were blotted overnight in 10 X SSC onto Hybond-N+ (Amersham) membrane. Membranes were cross-linked by exposure to UV light (1200kj). Membranes were pre-hybridised for 1 to 2 h at 42˚C in a solution of 50% formamide, 4 X SSPE, 5 X Denhardts, 0.5% SDS and 0.1mg/ml Herring sperm DNA. Membranes were probed overnight in the pre-hybridisation solution at 42˚C. The probe was produced using the Prime-a-gene labelling kit (Promega) with ^{32}p DCTP and NaD1 cDNA as template. Membranes were washed twice in 2 X SSC, 0.1% SDS for 30 min at 42˚C and exposed to film (Fujifilm).

3.2. *Fusarium oxysporum* seedling bioassays

3.2.1 Seedling dip assay

Isolates (24500 VCG 01111, 24595 VCG 01111, 24598 VCG 01112, 24599 VCG 01112) were provided by Wayne O'Neil (Department of Primary Industries, Qld). Initial work with the isolates suggested that isolate 24500 VCG 01111 was the most virulent and in all further assays this isolate was used. Glycerol stocks were prepared by mixing 800µL of microconidia (produced on carnation leaf agar plates) with 200µL of sterile 80% glycerol. Stocks were stored at -80˚C.

Cultures of Fov were prepared by infecting 1/4 strength potato dextrose broth (PDB) and incubating the cultures for 2 weeks at 26˚C. Microconidia were collected by filtering through two layers of sterile tissues (Kleenex). Spores were diluted in 1/4 X PDB (Difco) to a concentration of 1 X 10^7 Spores/mL. Fourteen day old cotton seedlings grown in a vermiculite/perlite/sand mix, were removed from the soil and the root systems of the plants dipped in the spore solution for 15 min. Control plants were dipped in 1/4 X PDB without Fov. Seedlings were transferred to pots containing the vermiculite/perlite/sand mix, and allowed to grow for a further 6 weeks in the glasshouse at approximately 28˚C. Plants were

assessed weekly for height and disease symptoms. The temperature in the glasshouse was monitored either by data logger or rotary temperature monitor.

3.2.2 Infected soil bioassay

Cultures of Fov were prepared in 1/4 X PDB and grown for approximately 1 week at 26ºC. The culture (5 to 10 mL) was used to infect autoclaved hulled millet which was then grown for 2 to 3 weeks at room temperature. The infected millet was incorporated into a pasteurised peat based soil mix at 1% (v/v), by vigorous mixing in a 200 L compost tumbler. The infected soil was transferred to plastic containers (10 L of mix per 13.5 L container). Control soil contained uninfected millet. Seed was sown directly into the containers, 12 seeds per box in a 3 X 4 array. The same number of seed of each line was sown randomly in each box, and the boxes were rotated and moved weekly to reduce variation that may occur due to positional effects in the glasshouse. The height of plants was measured weekly and the plants were scored three times a week for disease symptoms. At the end of the experiment (12 weeks) the plants were destructively harvested and scored for disease.

Statistical analysis of the disease scores was completed using ordinal logistic regression. The analysis was carried out by Dr Ian Gordon, Director, Statistical Consulting Centre, The University of Melbourne.

3.3. *In vitro* bioassays

3.3.1 *Verticillium dahliae*

Microconidia were produced by infecting 1/4 X Czapek-Dox broth (Difco) with *V. dahliae* and incubating at 26°C. The isolate was provided by Dr Helen McFadden (VCG-4B, original source: Dr Stephen Allen, CSD Ltd). The microconidia were collected by filtering through two layers of sterile tissues (Kleenex) and stored in glycerol at -80˚C.

Microconidia (5 X 10^4 spores/mL) were germinated in media containing purified NaD1 or ovalbumin as a control. Assays were performed in microtitre plates (Greiner) and the growth of hyphae determined by measuring the absorbance at 590 nm from 0 to approximately 48 hours. Five concentrations of NaD1 or ovalbumin (2, 5, 10, 15 and 20 ug/mL) were used in the assays.

3.3.2 Microscopy for bioassays

Fov or *V. dahliae* (5 x 10^4 spores/mL) were grown in flat bottom, 12 well plates (Greiner Bio-one) in the presence of ovalbumin or NaD1 (15ug/mL).

Fov hyphae were observed directly through the bottom of the wells with a Leica MZFLIII dissecting microscope. Hyphae were not fixed or stained.

To observe *V. dahliae*, sterilized glass cover slips were placed in the bottom of the wells at the start of the incubation. After 48 hours, the cover slips were removed and the hyphae fixed for 1 h in 2.5% glutaraldehyde, 0.06M PIPES pH 7.0 followed by 3 X 10 min washes in PIPES. The hyphae were then post-fixed in 1% osmium tetroxide followed by 3 X 15 min washes in PIPES. Samples were then dehydrated in ethanol, critical point dried and sputter coated in gold before viewing with a scanning electron microscope (Jeol JSM840A).

3.4. Microscopy for infected plant tissue

3.4.1 Fluorescent microscopy

Tissue was stained with the fluorescent dye 3,3' dihexloxacarbocyanin iodide (DioC6) for 1 to 10 minutes. A 0.8 mg/mL stock of DioC6 in ethanol was diluted 10 X in water before use. Samples were viewed with a Leica MZFLIII dissecting microscope at excitation of 450-490nm.

3.4.2 Light microscopy

Samples were stained by boiling for 5 min in Trypan blue solution and then destaining by boiling in 2.5 mg/mL chloral hydrate. Before use, a Trypan blue stock was prepared by dissolving 10 mg of Trypan blue in 10 mL lactic acid, 10 g phenol, 10 mL glycerol and 10 mL water. The Trypan blue solution was prepared by mixing the stock Trypan blue 1:1 with 100% ethanol. Samples were visualized on an Olympus BH2 light microscope.

3.4.3 Transmission Electron Microscopy

Samples of hypocotyl and root from infected and uninfected cotton lines were prepared using two different methods: a conventional fixation method (i) and freeze-substitution (ii).

(i) Tissue was fixed under vacuum in 4% glutaraldehyde in PBS pH 7.2. Samples were post-fixed in 1% osmium tetroxide, imbedded in LR white resin, sectioned to 80nm and placed on gold grids. Samples were post-stained in uranyl acetate and triple lead citrate.

(ii) Tissue was frozen using a Leica EM High Pressure Freezer (Wien) at 2.7 x 10^5 kPa and at an approximate rate of –10,000°C/sec. After freezing, samples were stored under liquid nitrogen prior to freeze-substitution in a Leica EM automated freeze-substitution unit. The frozen tissue was freeze-substituted in 0.1% uranyl acetate in ethanol at -90°C for 72 h and the temperature raised to -50°C at 6°C/hour. The tissue was rinsed in three, 30 minute changes of ethanol. The temperature was raised to -25°C and the samples were then infiltrated with a graded series of LR White resin in ethanol consisting of 25% resin (8 hours), 50% resin (overnight), 75% resin (8 hours) and three times in 100% resin (overnight, 8 hours, overnight). The samples were polymerised overnight in gelatine capsules in a 65°C oven. The polymerised resin blocks were cut into ultra thin sections using a Leica Ultracut R microtome and collected on formvar coated 200 mesh gold grids. The grids were stained for 20 min in 2% aqueous uranyl acetate and then 5 min in triple lead citrate. Samples were viewed on a Philips Bio Twin Transmission Electron Microscope.

3.5. *Thielaviopsis basicola* bioassays

The bioassays were conducted by David Nehl, NSW Department of Primary Industries, Narrabri. Seeds were germinated in soil naturally infested with *Thielaviopsis basicola*. Emergence was recorded at 11 days after sowing. Growth of the plants, incidence of *T. basicola* and mycorrhizal development was measured at 21 and 42 days after sowing. Further details are provided in Dr Nehl's report (attached).

3.6. *Verticillium dahliae* bioassays

An infected soil assay based on the Fov assay was used (Section 3.2.2) except that the infected millet was incorporated into the soil at 3% (v/v). The *V. dahliae* isolate (VCG-4B) was provided by H. McFadden. The height of plants was measured weekly and the plants were scored three times a week for disease symptoms. At the end of the experiment (6 to 8 weeks) the plants were destructively harvested and scored for disease.

3.7. Production of new transgenic cotton lines

Gene constructs were produced in the binary vector pBIN19 (Bevan 1984). The NaD1 gene (Lay et al 2003) was amplified by PCR using gene specific primers. The NaD1 gene along with the CesA4 promoter (Doblin et al., 2002) or the pXero2 promoter (provided by Professor Roger Parish, La Trobe University), were cloned into the *Eco*RI site of pBIN19. Both promoters are derived from *Arabidopsis thaliana.* The new gene constructs were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation, and the presence of the plasmid confirmed by gel electrophoresis and PCR.

The transformation protocol was essentially that of Umbeck (1992). Cultures of *Agrobacterium* were grown to 2 X 10^6 cells per mL and used to infect hypocotyl sections of cv Coker. Embryogenic callus was selected on media containing kanamycin.

4. Detail and discuss the results including the statistical analysis of results

4.1. Characterization of existing transgenic cotton lines expressing NaD1

4.1.1 Production of homozygous seed

At the start of the project only two lines, 35.9.1 and 35.105.1, were homozygous. These lines were used in the early Fov bioassays. The other two lines, 35.125.1 and 48.287.1, were hemizygous and it took several months to produce homozygous seed. Line 35.125.1 seed was obtained first and this line was included in the later Fov bioassay but not the *Verticillium* bioassay. Line 48.287.1 was only included in the *Verticillium* bioassay.

4.1.2 Expression of NaD1

The expression of NaD1 in homozygous lines 35.9.1, 35.105.1 and 35.125.1 was determined by immunoblot analysis (Figure 1). The highest expression of NaD1 was in the hypocotyl and lowest expression was in the roots. Line 35.9.1 had a slightly higher level of expression than the other 2 lines.

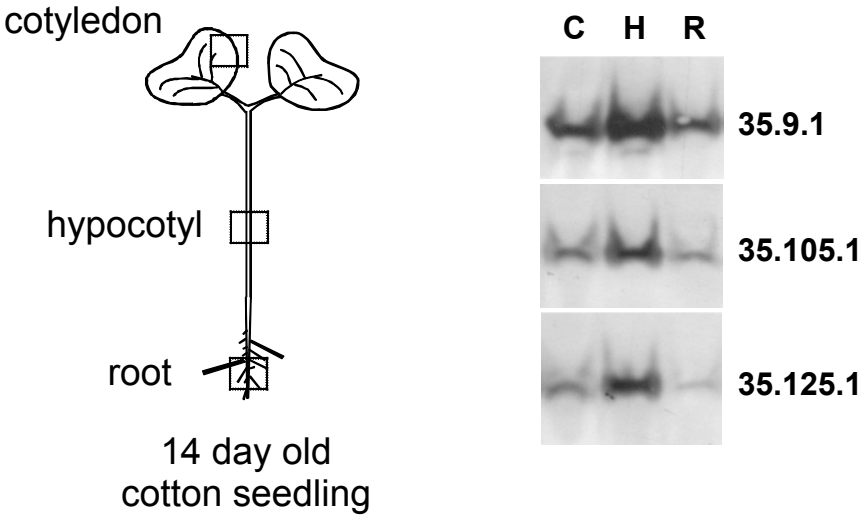


Figure 1 Expression of NaD1 in plant tissues from 14 day old seedling by immunoblot analysis. H = hypocotyl, C = cotyledon, R = roots. 20 ug of total soluble protein was loaded per lane.

4.1.3 Gene copy number

The four transgenic cotton lines (35.9.1, 35.105.1, 35.125.1 and 48.287.1) were analysed to determine whether a single copy of the NaD1 gene had been inserted.

Single bands were identified for lines 35.9.1, 35.125.1 and 48.287.1 when the DNA was digested with *Bgl* II restriction enzyme (Figure 2A), suggesting that these lines had a single copy of NaD1.

It is unclear whether line 35.105.1 has one or two copies of the NaD1 gene. When genomic DNA was digested with *Bgl* II, one major band and one minor band was observed (Figure 2A), while only one band was observed when the DNA was digested with *Bcl* I (Figure 2B). As this line did not perform well in the fungal bioassays, we decided not to pursue further analysis to determine copy number.

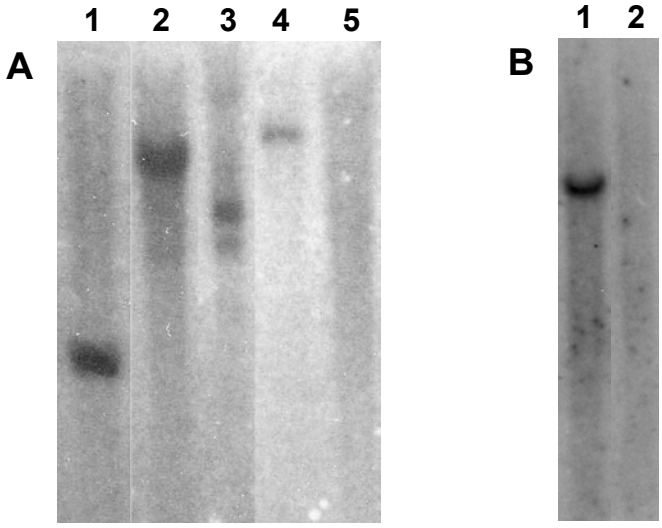


Figure 2. Southern hybridisation of transgenic cotton lines. **A:** 10 ug of genomic DNA digested with *Bgl* ll. Lane 1: 35.9.1, lane 2: 35.125.1, lane 3: 35.105.1, lane 4: 48.287.1, lane 5: untransformed Coker. **B:** 10 ug of genomic DNA digested with *Bcl* I. Lane 1: 35.105.1, lane 2: untransformed Coker

4.2. Assessment of existing transgenic cotton lines expressing NaD1 for resistance to Fov

4.2.1 Assessment of lines 35.9.1 and 35.105.1 using the Fov root dip bioassay

(i) CSIRO glasshouse trials

During the first 12 months of the project only limited PC2/pathology glasshouse space was available at the University of Melbourne. Dr Helen McFadden at CSIRO, Plant Industry, Canberra, kindly agreed to assess the lines using the Fov root dip bioassay. Only the two homozygous lines: 35.9.1 and 35.105.1 were assayed. Plant height, vascular browning index (VBI) and foliar symptoms were measured.

Two trials were completed (Tables 1 and 2). Trial 1 had a high disease incidence and trial 2 a low disease incidence. In both experiments the resistant varieties DeltaEmerald and Sicot 189 (trial 1 only) had lower disease symptoms than the susceptible variety Siokra 1-4. The untransformed Coker control had similar disease symptoms (low VBI and high %plant height) to the resistant varieties. The results for Coker were unexpected as this variety is considered highly susceptible to Fov. The Coker seed was slow to germinate which may have affected the results.

The two transgenic lines showed no increase in resistance to Fov compared to the parent untransformed Coker. This was confirmed by statistical analysis of the VBI data using the Chi test (H. McFadden, data not shown).

Table 1. Results of trial 1. 24 plants per line were tested. #plant height includes dead plants. *The vascular browning index (VBI) is the average score for plants using the following scale: 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, NT-not tested.

Trial 1						
	Siokra 1-4	Sicot 189	D. Emerald	Coker	35.9.1	35.105.1
No. dead plants	11	3	6	9	14	9
No. living plants	13	21	18	12	10	15
%living plants	54%	88%	75%	57%	42%	63%
%plant height#	35%	43%	45%	47%	40%	36%
VBI*	4.6	4.1	3.8	3.7	4.8	4.5

Table 2. Results of trial 2. 30 plants per line were tested. #plant height includes dead plants. *The vascular browning index (VBI) is the average score for plants using the following scale: 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, NT-not tested.

Trial 2					
	Siokra 1-4	D. Emerald	Coker	35.9.1	35.105.1
No. dead plants	1	2	1	1	5
No. living plants	28	28	29	29	25
%living plants	97%	93%	97%	97%	83%
%plant height#	70%	86%	93%	76%	69%
VBI*	2.0	1.4	1.4	1.6	2.4

(ii) University of Melbourne glasshouse trials

Four small glasshouse trials using the root dip assay were completed at the University of Melbourne. Conditions for these trials were not optimal: space was very limited and the glasshouse temperature was high (average day temperature of 28°C and an average night temperature of 20°C). A different method of scoring for disease was used compared to the CSIRO glasshouse trials (see table legends). Only the two homozygous transgenic lines; 35.9.1 and 35.105.1 were assayed. Due to lack of space, the susceptible line Siokra 1-4 was not tested.

Only two experiments are reported here (trial 3 and trial 4). The other two trials had poor seed germination and very low levels of Fov infection.

The disease incidence was low in both trials (Tables 3 and 4). The untransformed Coker control had the highest disease score and the transgenic lines were similar to the resistant variety DeltaEmerald. The results for Coker were different to the CSIRO glasshouse trials

where Coker was more resistant to Fov. As the same source of seed was used, it is possible that the different glasshouse conditions may have affected the performance of Coker.

Table 3. Results of trial 3. 15 plants per line were tested. #Plant height is the average height of surviving inoculated plants compared to the average height of surviving mock inoculated plants. *The average disease score was determined by recording vascular browning in cross sections at the base of the stem six weeks after infection. 0: no browning, 1: <5% browning, 2: 5-20% browning, 3: 20-40% browning, 4: dead.

Trial 3				
	D. Emerald	Coker	35.9.1	35.105.1
No. dead plants	2	5	1	2
No. living plants	13	10	14	13
% living plants	87%	67%	93%	87%
% plant height of surviving plants#	85	80	116	97
Disease score*	0.9	1.7	0.7	0.9

Table 4. Results of trial 4. 30 plants per line were tested. #Plant height is the average height of surviving inoculated plants compared to the average height of surviving mock inoculated plants. *Disease score is the average score based on the following scale: 0 = no infection visible at base of stem, 1 = visible infection at base of the stem, 2 = visible infection to the level of cotyledons, 3 = visible infection to the true leaves, 4 = dead. NT = not tested.

Trial 4			
	D. Emerald	Coker	35.9.1
No. dead plants	0	14	7
No. living plants	30	16	23
% living plants	100%	53%	77%
% plant height of surviving plants#	70	67	74
Disease score*	0.4	2.1	1.6

The conclusion from this data and from the trials done in Canberra is that the transgenic lines 35.9.1 and 35.105.1 do not appear to have any significant increase in resistance to Fov compared to the untransformed Coker parent.

4.2.2 Assessment of lines 35.9.5, 35.105.1 and 35.125.1 using the Fov infected soil bioassay

Due to poor disease incidence and variable results using the root dip assay in the experiments performed at the University of Melbourne, we decided to develop an infected soil bioassay for all further trials. This assay more closely follows the infection process in the field, as seed is planted directly into infected soil.

We used millet to grow the Fov and then thoroughly mixed the infected millet into the soil using a compost tumbler (Figure 5). The infected soil was then transferred to containers in which the cotton seed was germinated. We were fortunate to gain access to a further PC2 glasshouse at the University of Melbourne in which the day temperature was set at 25ºC. We also carried out the experiment in the cooler months so that conditions were more favourable for high disease levels.

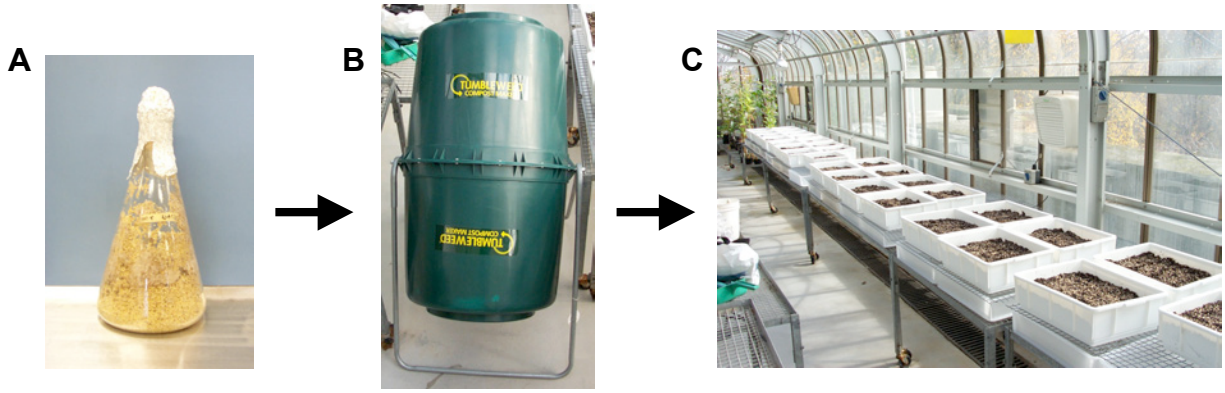


Figure 5. Infected soil bioassay used at the University of Melbourne. A: millet infected with Fov, B: compost tumbler used to mix the soil and millet, C: containers with infected soil in the University of Melbourne glasshouse.

One experiment using the infected soil bioassay was completed (Table 5). Two transgenic lines were tested: 35.9.1 which had been assessed in the seedling dip assay and 35.125.1 in which homozygous seed had recently become available. Controls were Siokra 1-4 (susceptible), Sicot 189 (resistant) and untransformed Coker. Thirty infected and 30 uninfected plants of each variety/line were grown for 12 weeks. Height and symptom development was measured throughout the trial (Figure 6 and Table 5) and the disease score was determined by destructive sampling at the end of the trial (Table 5).

The disease incidence was high in this trial and it was interesting to follow the progress of the disease over the 12 weeks (Figure 6). The susceptible variety Siokra 1-4 was first to show wilting at day 16. All other lines started to show wilt symptoms between days 24 and 27. The untransformed Coker and transgenic line 35.9.1 continued to develop symptoms and eventually reached similar levels to Siokra 1-4 (about 82% of plants showed wilt symptoms). However the resistant line Sicot 189 and the transgenic line 35.125.1 only showed symptoms in about 60% of the plants after 12 weeks.

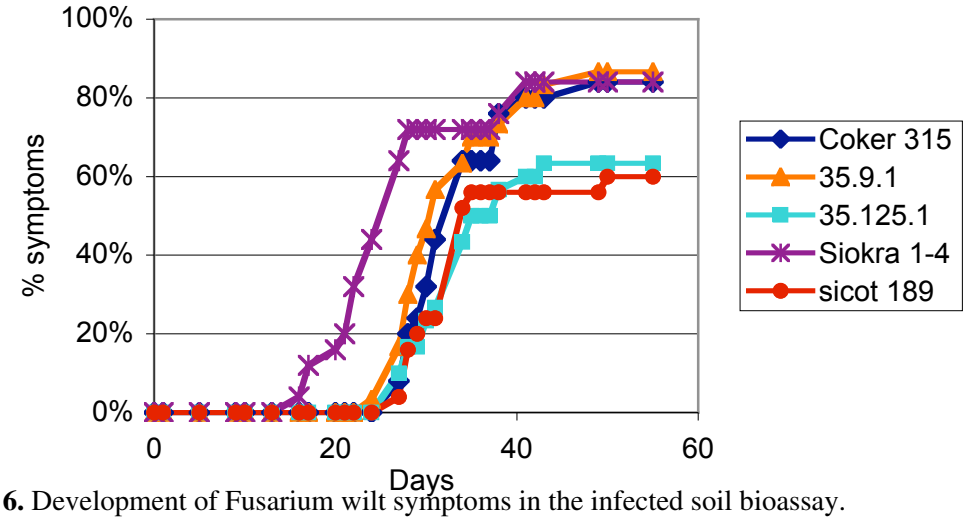


Figure 6. Development of Fusarium wilt symptoms in the infected soil bioassay.

Table 5. Results of trial 5. 30 plants per line were tested. #Plant height is the average height of surviving inoculated plants compared to the average height of surviving mock inoculated plants. *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.

Trial 5					
	Siokra 1-4	Sicot 189	Coker	35.9.1	35.125.1
No. dead plants	19	10	13	18	13
No. living plants	6	15	12	12	17
% living plants	24%	60%	48%	40%	57%
% plant height of surviving plants#	75 %	80 %	93 %	83 %	95 %
Disease score*	4.6	3.4	3.8	4	3.3

The susceptible variety Siokra 1-4 had the highest disease score and the resistant variety Sicot 189 and the transgenic line 35.125.1 had the lowest disease scores. The untransformed Coker control had a disease score intermediate between the resistant and susceptible varieties.

Statistical analysis of this trial showed some promising trends. The resistant line Sicot 189, untransformed Coker and the transgenic line 35.125.1 all had lower levels of infection than the susceptible line Siokra 1-4 and these results were significant (P = 0.05 or lower). Unfortunately, due to the small sample size, there was no statistical significance between the best performing transgenic line (35.125.1) and Coker (P = 0.4).

This experiment has recently been repeated at the University of Melbourne with funding support from Hexima Ltd. The transgenic line 35.125.1, Siokra 1-4 and Sicot 189 were assessed using the infected soil bioassay and a large number of plants were infected per line. The results from this trial confirmed the data collected for trial 5. Importantly, there was a significant difference between the disease score of transgenic line 35.125.1 and the parent Coker (P = 0.04). This latest trial strongly suggests that the presence of the NaD1 gene has resulted in an increase in resistance to Fov.

4.3. Detailed studies of Fov and *V. dahliae* infection in the presence of NaD1

4.3.1 *In vitro* bioassays

NaD1 has previously been shown to inhibit spore germination and hyphal growth of Fov *in vitro* (Lay et al., 2003). As plant defensins are known to inhibit a range of filamentous fungi we performed an *in vitro* assay with purified NaD1 and *V. dahliae* (Figure 7). Partial inhibition of fungal growth was observed at 10 ug/mL and close to 100% inhibition was obtained at 15 ug/mL. These results were very similar to those for Fov and NaD1 (Lay et al., 2003) and for similar *in vitro* assays published for other plant defensins and related pathogens (Osborn et al., 1995).

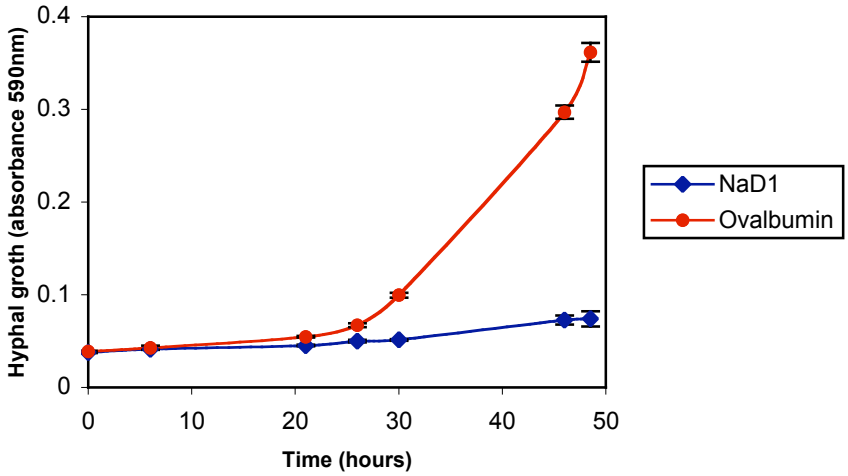
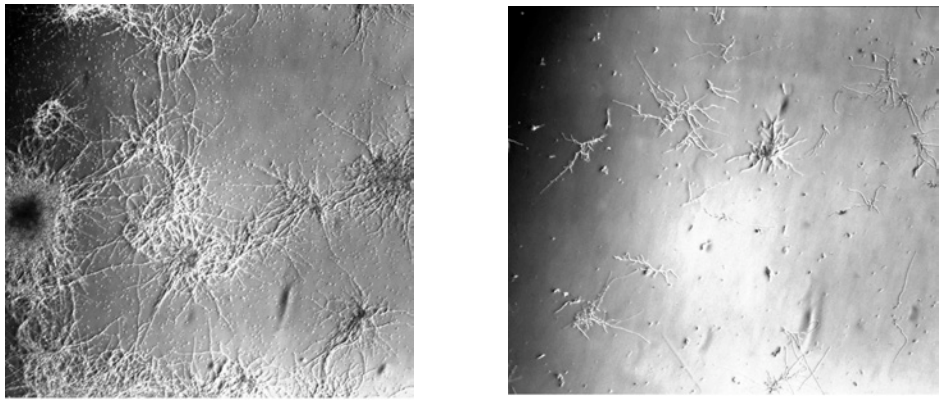


Figure 7. Time course of *Verticillium dahliae* hyphal growth grown *in vitro* in the presence of 15 ug/mL of NaD1 or 15 ug/mL ovalbumin control.

Fov and *V. dahliae* hyphae were observed under the microscope to determine whether the presence of NaD1 affected the hyphal morphology.

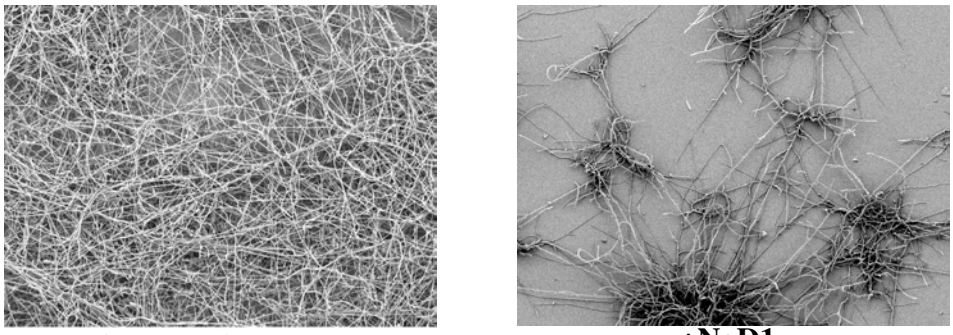
Fov hyphae showed some morphological changes when viewed under a dissecting microscope (Figure 8). In the presence of NaD1, the Fov hyphae had increased branching and were slightly shorter than the hyphae grown in the presence of ovalbumin. Similar observations have been reported for other plant defensins and filamentous fungi.



Ovalbumin control **+NaD1**

Figure 8. Fov hyphae grown *in vitro* for 24 hours in the presence of either 15 ug/mL NaD1 or 15 ug/mL ovalbumin control. 25 X magnification.

V. dahliae hyphae showed no morphological differences when viewed with the scanning electron microscope (Figure 9).



Ovalbumin control **+NaD1**

Figure 9. *V. dahliae* hyphae grown in vitro for 48 hours in the presence of either 15 ug/mL NaD1 or 15 ug/mL ovalbumin control. 1,000 X magnification.

4.3.4 Fov infection in transgenic plants

There were two aims for this part of the project:
(i) To determine the location of NaD1 in the roots and vascular tissue of existing transgenic cotton lines and to determine whether the Fov hyphae would come into contact with the NaD1 protein.
(ii) To visualise any effects NaD1 has on Fov hyphal penetration or growth during infection of transgenic plants.

Localization studies

NaD1 has a putative vacuolar targeting sequence and in *Nicotiana alata,* NaD1 is located in the vacuole (Lay et al., 2003). As discussed previously, the existing transgenic lines produce NaD1 under the control of the constitutive 35S promoter. NaD1 is located in the vacuole in leaves of line 35.9.1 (R. Heath unpublished data) and NaD1 was shown by immunoblot analysis to be present in the roots of three transgenic lines (35.9.1, 35.105.1 and 35.125.1) (Section 5.1.2).

Root and stem tissue of several lines (infected and uninfected) have been prepared for this study. We have prepared samples using both conventional fixing and freeze substitution. When viewed under the EM, the freeze substitution samples gave excellent sub-cellular details and the pathogen could easily be observed (Figure 10).

The samples are ready for immuno-gold labelling which will be done when resources are available.

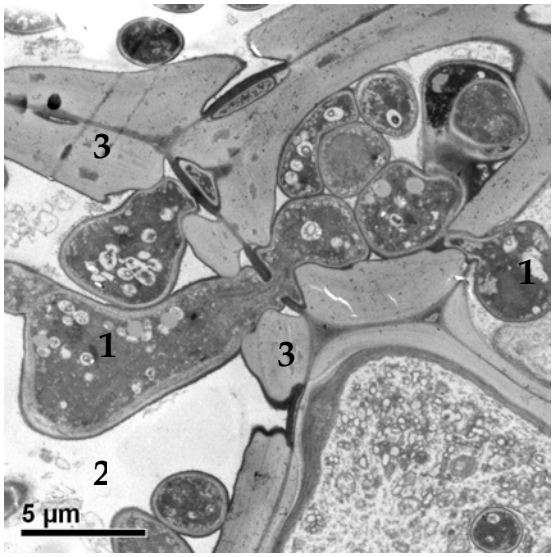


Figure 10. Cross-section of infected cotton stem at 4200 X magnification. 1: Movement of Fov through the xylem pits, 2: xylem vessels, 3: highly lignified secondary cell wall of the xylem vessels.

Fov hyphal penetration and growth

Fov enters the plant via the root. After initial penetration of the epidermis (Figure 11), the fungal hyphae travels to the vascular tissue both intercellular and intracellular. Once the hyphae reaches the vascular tissues, there is uncontrolled proliferation of the fungus (Figure 12). The pathogen moves through the pits in the xylem walls, eventually resulting in collapse of the vascular system and the characteristic wilt symptoms.

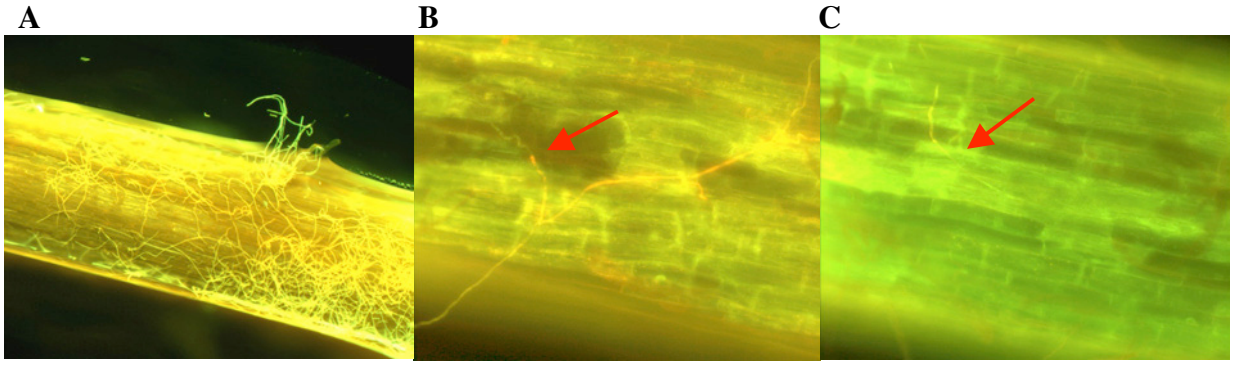


Figure 11. Visualisation of the early colonization of the Fov pathogen using the fluorescent dye DioC6. **A:** Colonization of the surface for the root 100X magnification. **B & C:** penetration of the fungal hyphae between (intercellular) the root epidermal cells (denoted by the red arrows) 200 X magnification.

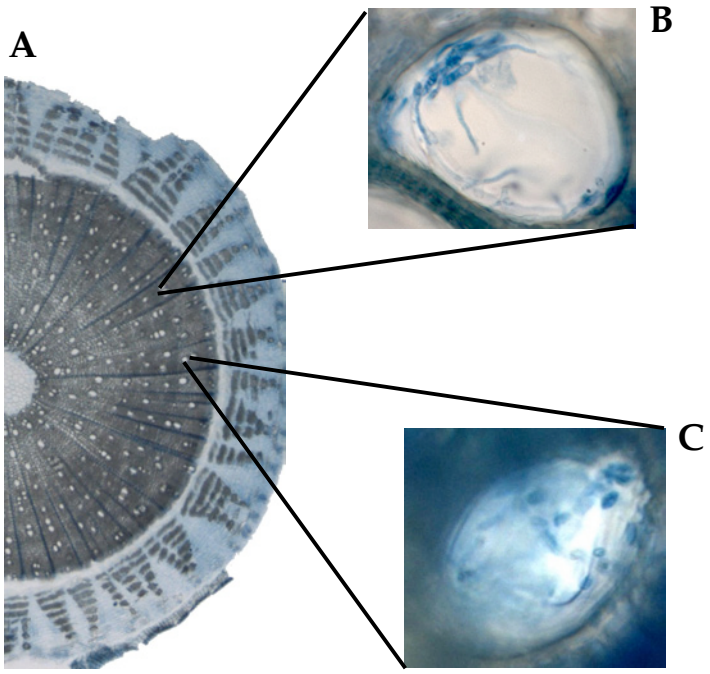


Figure 10. A: Cross section of a infected cotton stem at 40 X magnification stained by trypan blue, **B & C**: 200 X magnification of cotton xylem vessels showing the proliferation of the Fov pathogen in the vascular tissues.

We have tried several methods to visualise Fov in plant tissue and found staining with the fluorescent dye DioC6 or trypan blue provided good results. We had planned to use these methods to compare infected and non-infected tissue, however time limitations prevented us from completing this part of the project.

4.4. Assessment of existing transgenic cotton lines expressing NaD1 for resistance to *Thielaviopsis basicola*

Assessment of three of the transgenic cotton lines for resistance to black root rot *Thielaviopsis basicola* was carried out by Dr David Nehl at the Australian Cotton Research Institute. Line A was untransformed Coker, Line B was transgenic line 35.9.1, Line C was transgenic line 35.105.1 and Line D was transgenic line 35.125.1. Two experiments were performed in the PC2 glasshouse. The results are presented in the attached report.

Some differences in susceptibility to black root rot were observed, although these differences were not consistent for Lines B and C. Results with Line D (35.125.1) suggest that this line has enhanced resistance to black root rot and further assessment is recommended. Note that line 35.125.1 is the line that also showed enhanced resistance to Fov.

There was no significant difference in arbuscular mycorrhizal colonisation between the Coker and the 3 transgenic lines. This finding is important for regulatory approval if the NaD1 gene is to be assessed under field conditions.

4.5. Assessment of existing transgenic cotton lines expressing NaD1 for resistance to *Verticillium dahliae*

A *V. dahliae* bioassay using the infected soil bioassay developed for Fov was used to assess the transgenic lines. Only one preliminary experiment was completed (Table 6). Three transgenic lines were tested: 35.9.1, 35.125.1 and 48.287.1. Controls were Siokra 1-4 (susceptible), Sicala V2 (resistant) and untransformed Coker. Thirty infected and 30 uninfected plants of each variety/line were grown for 12 weeks. Height and symptom development was measured throughout the trial and the disease score was determined by destructive sampling at the end of the trial (Table 6).

The disease pressure was very low and all plants survived and looked healthy. This trial was conducted over the summer months and the temperature in the glasshouse was high. It is likely that the conditions favoured plant growth instead of pathogen invasion. No conclusions can be drawn from this experiment as the disease scores were too low.

Further bioassays are planned for the cooler months when lower temperatures can be maintained.

Table 6. Results of *V. dahliae* bioassay. #Plant height is the average height of surviving inoculated plants compared to the average height of surviving mock inoculated plants. *Disease scores is based on a 0 to 5 ranking system of vascular browning where 0 is no incidence and 5 is death.

	Siokra 1-4	Sicala V2	Coker	35.9.1	35.125.1	48.287.1
% plant height of surviving plants#	92.8	100.4	97.7	94.4	99.5	96.9
Disease score*	0.9	0.00	0.3	0.4	0.6	0.5

4.6. Production and assessment of new transgenic cotton lines transformed with gene constructs containing tissue specific promoters

Two gene constructs, one with the pXero2 promoter and one with the CesA promoter were produced. Each construct was transferred into *Agrobacterium tumefaciens* and used in two transformation experiments with hypocotyl explants of cv Coker. However, subsequent sequencing of the gene constructs confirmed that an error with one of the NaD1 primers used in the early stages of production of the constructs had occurred. This meant that the NaD1 gene would not be translated correctly. The constructs and transformation experiments were destroyed.

A new gene construct incorporating the CesA promoter was prepared once the error had been discovered. The construct was sequenced and the correct sequence confirmed. Two transformation experiments using this construct were established and are progressing well. These experiments will be completed with support from Hexima Ltd.

5. Provide a conclusion as to research outcomes compared with objectives. What are the "take home messages"?

We have been studying the biological activity of NaD1, a plant-derived antifungal protein. The major findings of the project were:

- One transgenic cotton line (35.125.1) expressing NaD1 under the control of the 35S promoter has shown enhanced resistance to Fusarium wilt (*Fusarium oxysporum*) in a glasshouse bioassay.
- Preliminary studies suggest that line 35.125.1 also has enhanced resistance to black root rot (*Thielaviopsis basicola*) in glasshouse trials but does not affect mycorrhizal colonisation.
- Purified NaD1 inhibits *V. dahliae* in an *in vitro* assay.

6. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?

If NaD1 proves to be successful in providing increased resistance to cotton pathogens then the next step would be to introduce the gene into commercial cotton varieties. The adoption of new cotton varieties with increased resistance to Fov infection would result in a reduction in spread of Fov, higher yields and less cost in disease management. There is also the potential for increased profit which would benefit both the grower and the local community.

7. Provide a summary of the project ensuring the following areas are addressed:

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses etc.)

This is the first time that a plant defensin has been shown to have increased resistance to cotton pathogens *in planta*. Hexima Ltd, who are the joint owners of the NaD1 patents, have indicated to us that they will continue to support this work. If the results presented in this report can be verified then it is likely that transgenic plants expressing NaD1 will be assessed under field conditions. Should this technology be proven in the field the commercial potential of NaD1 would be significant.

b) other information developed from research (eg discoveries in methodology, equipment design, etc)

We have found that an infected soil based bioassay is more reliable than a seedling root dip assay to assess transgenic plants. This method (described in 3.2.2) resulted in high disease pressure for Fov in glasshouse trials.

c) are changes to the Intellectual Property register required?

The NaD1 patent "Plant-derived molecules and genetic sequences encoding same and uses thereof" has recently been granted in Australia (patent number not yet available) and in New Zealand (Patent number 523345).

8. Detail a plan for the activities or other steps that may be taken:

(a) To further develop or exploit the project technology.

This technology is moving closer to a commercial outcome. Hexima is committed to continuing the assessment of transgenic lines and a decision on whether to conduct field trials will be made in 2005.

(b) for the future presentation and dissemination of the project outcomes.

This research will be published when further experiments are completed. The Fov and root rot assays must be repeated to verify the early results. Some of the work planned for the last 8 months of the project will be funded by Hexima, and Dr David Nehl has indicated that he is willing to repeat the black root rot experiments. Once further data is obtained, the research will be published in the appropriate scientific journals.

(c) for future research.

We will continue with our research plan that was proposed for the final year of the project. The major focus will be to:

- assess the existing transgenic lines for resistance to Verticillium wilt
- study the localisation of NaD1 and its interaction with Fov using microscopy
- continue the production of new transgenic lines with different promoters

9. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

James McKenna, Fung Lay, Marilyn Anderson, David Guest and Robyn Heath
The potential of the antifungal protein NaD1 for control of Fusarium wilt and Verticillium wilt. Proceedings of the 12th Australian cotton conference, Gold Coast, 2004

J. McKenna, F. Lay, M. Anderson, D. Guest and R. Heath. Transgenic cotton for the control of Fusarium wilt. Proceedings of the 11th Australian Cotton Conference, Brisbane, 2002.

10. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry or the Australian community.

Although some of the recently released cotton varieties produced from the Australian breeding programs have shown increased resistance to Fov, these varieties are not fully resistant and there is still a need to produce new varieties with higher Fov disease rankings than currently available. This will allow growers in areas such as the Darling Downs to grow cotton in fields heavily infested with the fungus and potentially result in a reduction in the disease load in the soil over time.

If further experiments confirm the results presented in this report, then the next step will be to transfer the NaD1 gene to elite varieties that already have a high Fov disease ranking. As the resistance mechanism for NaD1 is likely to be different to that bred into the elite varieties, the expression of the gene should provide additional resistance to Fov. It will take at least five years before commercial varieties carrying the NaD1 gene would be available. Such varieties could potentially increase cotton yields by up to 50% in regions where Fov is endemic.

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Umbeck, P. F. (1992). Genetic engineering of cotton plants and lines. US patent no. 5,159,135

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

This project was designed to assess the potential of a plant derived antifungal gene (NaD1) for control of cotton pathogens. The NaD1 gene codes for a small protein called a plant defensin. Previous *in vitro* assays have shown that purified NaD1 can inhibit the germination and hyphal growth of the fungus *Fusarium oxysporum* f.sp *vasinfectum* (Fov) which causes Fusarium wilt in cotton.

The major aim of the project was to assess the efficacy of the defensin protein, NaD1, against three cotton pathogens: *Fusarium oxysporum*, *Verticillium dahliae* which causes Verticillium wilt and *Thielaviopsis basicola* which causes black root rot.

Four transgenic cotton lines transformed with the NaD1 gene were supplied by Hexima Ltd for this project. The lines expressed the NaD1 protein at high levels in the leaves and roots of seedlings. At least three of the lines had a single copy of the NaD1 gene.

The lines were assessed in glasshouse bioassays using either a seedling dip infection method or an infected soil based method. In limited trials, one transgenic line showed enhanced resistance to Fov and *T. basicola.* Further bioassays with this line to confirm the project findings is planned.

In the long term it is hoped that this gene can be transferred into elite varieties to enhance the resistance of Fov in the field.

For further information please contact:
Dr Robyn Heath,
School of Botany, the University of Melbourne, Victoria, 3010.
Telephone 03 8344 3323, email: robynlh@unimelb.edu.au