January, August & Final Reports

REPORTS

Part 1 - Summary Details

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

CRDC Project Number: ANU6C

January Report:	☐	Due 29-Jan-01
August Report:	☐	Due 03-Aug-01
Final Report:	☒	Due within 3 months of project completion

Project Title: Testing the tomato I-2 gene for its ability to confer Fusarium resistance in cotton

Project Commencement Date: 1/1/00 **Project Completion Date:** 30/9/02

Research Program: Diseases Weeds

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Signature of Research Provider Representative: _____

Part 3 – Final Report Format

The points below are to be used as a guideline when completing your final report.

1. Outline the background to the project.

Fusarium wilt is caused by pathogenic fungi in the genus *Fusarium*, which infect the roots of susceptible plants and are able to spread throughout the vascular system, stimulating the production of gums and gels that block metabolite and water transport (Beckman, 1987). In resistant plants, infection is restricted to vascular tissue in the lower part of the root system, coincident with the production of antifungal metabolites and the formation of structural barriers to infection (Beckman, 1987). The *I-2* resistance gene has been introgressed from *Lycopersicon pimpinellifolium* to cultivated tomato (*L. esculentum*) and protects plants from race 2 (Alexander, 1945) of *Fusarium oxysporum* f. sp. *lycopersici* (Stall and Walter, 1965; Cirulli and Alexander, 1966). *I-2* as been isolated by map-based cloning and encodes a cytosolic protein with an N-terminal leucine zipper, a putative nucleotide binding site, and seventeen C-terminal leucine-rich repeats (Simons *et al.*, 1998). A cytological study involving tomato plants containing an *I-2* promoter-*gusA* construct showed that the pattern of *I-2* expression was coincident with the site of pathogen containment in resistant roots (Mes *et al.*, 2000).

Fusarium oxysporum f. sp. *vasinfectum* is found in most countries where cotton (*Gossypium spp.*) is grown. The pathogenicity of seven Australian isolates of this fungus has been analysed and no significant resistance was seen in 11 *G. hirsutum* cultivars, although *G. arboreum* cv. Roseum was resistant (Davis *et al.*, 1996). These results are consistent with the observation that most commercial cotton cultivars show relatively poor resistance to Australian races of *F. oxysporum* f. sp. *vasinfectum*.

Although *F. oxysporum* is probably a species complex with different vegetative incompatibility groups (Fernandez *et al.* 1994; Davis *et al.* 1996) and polyphyletic origins (Skovgaard *et al.* 2001),

it is possible that elicitors may be shared between different formae speciales. Hence, the avirulence factor of *F. oxysporum* f. sp. *lycopersici* race 2 that is recognised by *I-2* in tomato might also be present in races of *F. oxysporum* f. sp. *vasinfectum*. The availability of genetic, molecular and cytological data relating to *I-2*-mediated resistance make *I-2* a good candidate (indeed the only candidate) for expression in plants lacking genetic resistance to *Fusarium*. Many resistance genes function in plants from the same taxonomic family e.g. pepper *Bs-2* functions in other Solanaceaous plants such as tomato (Tai *et al.* 1999), and some are also able to recognise their cognate elicitors and trigger a defence response in plants from other taxonomic families e.g. *Cf-4* in lettuce (van der Hoorn *et al.* 2000) and *Cf-9* in oilseed rape (Hennin *et al.* 2001, 2002) or initiate a ligand independent response e.g. *RPW8* in native tobacco (Peart *et al.* 2002). Hence, it is possible that *I-2* could trigger defence responses in plants outside the Solanaceae, such as cotton. The overall aim of the project was therefore to test the tomato *I-2* gene for its ability to confer *Fusarium* resistance in cotton (*Gossypium hirsutum*).

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

As indicated in previous reports, we have PCR amplified, cloned, sequence checked and engineered the *I-2* gene for expression in cotton under the control of the CaMV 35S promoter. Two attempts to transform cotton with 35S:*I-2* failed indicating that over-expression of *I-2* in cotton may have prevented the formation of transformed callus.

Subsequent objectives included:

• Investigating the possible causes for the failure of the cotton 35S:*I-2* transformation

1. Plasmid DNA was reisolated from the *Agrobacterium tumefaciens* AGL1 culture used in the failed cotton transformations. This DNA was used to transform *E. coli* and yielded tetracycline-resistant colonies indicating the presence of the binary vector. This was further confirmed by the restriction analysis of plasmid minipreps. The presence of the *I-2* gene in the binary vector was also confirmed by PCR-analysis of whole *A. tumefaciens* cells using gene-specific primers.

2. A control transformation was initiated using the same binary vector carrying the CaMV 35S promoter but lacking the *I-2* coding region. This transformation produced transformed callus, supporting the idea that over-expression of *I-2* prevented the formation of transformed callus, rather than a trivial explanation such as an unsuitable vector.

3. A third attempt to transform cotton with the 35S:*I-2* construct undertaken by a person experienced in cotton transformation also failed, further supporting the idea that *I-2* prevented the formation of transformed callus, rather than a trivial explanation such as technical error.

• Investigating the biological activity of the 35S:*I-2* construct in a transient, leaf expression assay in cotton

Three agroinfiltration experiments, involving the 35S:*I-2* binary vector construct in *A. tumefaciens* AGL1, were attempted in cotton plants at 2 weeks after germination and between 6 and 7 weeks after germination. In the first experiment, no specific chlorotic or necrotic responses arose as a consequence of *I-2* over-expression in cotton, but in the two subsequent experiments a necrotic response was observed. Subject to further confirmation, these results indicate that over-expression of *I-2* in cotton does indeed trigger plant defences and further supports the idea that over-expression of *I-2* prevented the formation of transformed callus in cotton transformation experiments.

Subsequent objectives also included the initiation of an alternative approach using the *I-2* promoter, which involved:

• PCR-amplifying the *I-2* promoter.

The *I-2* promoter was successfully amplified using primers that added either SalI or ClaI restriction sites to the ends of the product to facilitate further cloning steps.

• Constructing an *I-2* promoter:*gusA* reporter gene fusion.

An *I-2* promoter:*gusA* fusion was constructed in a binary vector, transferred to *Agrobacterium rhizogenes* by triparental mating, and used to transform cotton and tomato (control) explants in tissue culture.

Analysis of glucuronidase activity in the resulting hairy roots revealed that the *I-2* promoter is active in cotton cells and that the binary vectors used to prepare the *I-2* constructs should be capable of producing transgenic cotton plants.

• Replacing the 35S promoter of the 35S: *I-2* construct with the *I-2* promoter.

A new construct containing the *I-2* gene under the control of its own promoter has been prepared using the error-free coding region of *I-2* prepared for the 35S: *I-2* construct. The ligation junctions within this construct have been confirmed by sequencing and it has been placed in a binary vector and transferred to the *A. tumefaciens* strains AGL1 and LBA4404 in preparation for cotton and tomato transformation, respectively. These transformations have been initiated and the cotton transformation with *I-2:I-2* has already progressed beyond the point at which the *35S:I-2* transformations failed.

3. How has your research addressed the Corporations three outputs: Sustainability, profitability and international competitiveness, and/or people and community?

Cotton cultivars have poor resistance to Australian races of *Fusarium oxysporum* f. sp. *vasinfectum*, and the possibility of transferring *I-2*-mediated resistance from tomato to cotton would greatly reduce the economic and environmental costs of the disease to cotton growers. This could help the Australian cotton industry to compete with producers overseas, where cotton cultivars have been found that are relatively resistant to the prevalent *Fusarium* races. The benefit of a vigorous and sustainable cotton industry to communities dependent on cotton production are obvious. Fusarium wilt is a major threat to the industry and this research has the potential to provide a solution to the fusarium wilt problem. The engineering of constructs and plant transformation are time-consuming processes, but the generation of transgenic plants for pathogen testing is imminent and the outcome of these tests should make the implications of this research for industry, the environment and the community clearer.

4. Detail the methodology and justify the methodology used.

DNA manipulations and PCR

Unless otherwise stated, DNA manipulations were performed using standard methods (Sambrook et al., 1989). Analytical PCR was performed on purified tomato genomic DNA using REDTaq DNA polymerase and buffer (Sigma-Aldrich Co., St Louis, MO) and a MJ Research (Watertown, MA) PTC-200 thermal cycler. The Expand DNA polymerase system (Roche Diagnostics, Mannheim, Germany) was used for PCR-amplification of the *I-2* promoter, since it contains both Taq DNA polymerase to maximise efficiency of amplification, and a proofreading polymerase to minimise PCR-errors.

Production of a 35S: I-2 construct

The complete *I-2* coding region was amplified from genomic DNA (prepared according to Rogers and Bendich, 1993) of the tomato cultivar Mobox using the Expand kit and the oligonucleotides 5'-TTG ATC GAT GGA GAT TGG CTT AGC AGT TG-3' and 5'-GGA TCC TCA TGC CAG GAG CAC CGC TCC TTC-3'. PCR products were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequence analysis revealed PCR-errors at the 3' end of one clone, termed '113', and at the 5' end of another, termed '114' and this problem was overcome by cloning the region between the ClaI site and the SacI site from '113' and the region between the SacI site and the BamHI site from '114' into ClaI-BamHI-cut binary vector pCBJ10, downstream of the CaMV 35S promoter. The cloned *I-2* gene in the 35S:*I-2* construct contains a ClaI site just before the start codon of *I-2*, SalI and SacI sites at nucleotides 241 and 2614 in the coding region, relative to the start codon, and a BamHI site downstream of the 3' untranslated region. This construct, designated pCBJ240, was verified by restriction analysis.

PCR-amplification of the I-2 promoter

The *I-2* promoter region was amplified as a ClaI fragment with the oligonucleotides 5'-ATCGATGCTAATGTTTGCTCTTTCTATTCTC-3' and 5'-ATCGATCAAATCTGCAAAAGCAAAACAAGGAAAC-3' using the Expand DNA polymerase kit and genomic DNA isolated from the tomato cultivar Mobox. Similarly, the region between the 5' end of the *I-2* promoter and the SalI site within the *I-2* coding region was amplified as a SalI fragment with the oligonucleotides 5'-GTCGACTGCTAATGTTTGCTCTTTCTATTCTC-3' and 5'-GTCGACAGCATCTCGAAGCTC-3'. The PCR products with ClaI and SalI sites were cloned into pCR2.1 using the TA cloning kit and designated pCBJ257 and pCBJ258, respectively. The pCBJ257 plasmid was amplified in dam- dcm- *E. coli* cells to allow the ClaI fragment to be excised without dam methylation of the ClaI site at the 3' end of the *I-2* promoter.

Production of an I-2 promoter-gusA construct

The binary vector pSLJ10621 has a multiple cloning site that allows promoters to be inserted upstream of the *gusA* gene as ClaI fragments. Hence, the ClaI *I-2* promoter fragment was excised from pCBJ257 and cloned into ClaI-cut pSLJ10621 to produce pCBJ259, which contains the *gusA* gene under the control of the *I-2* promoter.

Production of an I-2 promoter-I-2 coding region construct

A construct containing the *I-2* coding region and its own promoter was prepared for cotton transformation because three previous transformation attempts with a 35S:*I-2* construct had failed, most likely because over-expression of *I-2* prevented formation of transformed callus. Preparation of an *I-2*:*I-2* construct was also justified by a report suggesting that *I-2* is unlikely to function properly in tomato unless its expression is controlled by its own promoter (Mes et al., 2000). The *I-2* gene had previously been assembled by a difficult three-way ligation in a large binary vector carrying the CaMV 35S promoter. Owing to the large size of the 35S:*I-2* vector, its low copy

number and the absence of unique restriction sites, the *I-2* gene was reassembled in a smaller, high copy number vector carrying appropriate unique restriction sites. The 7 kb XhoI-NotI fragment containing the 35S:*I-2* construct from pCBJ240 was cloned into pDarkBluescript KS+ (Jones *et al.*, 1992) to generate pCBJ260, which was used as the source of the ClaI-SacI fragment comprising the 5' end of *I-2*. The SacI-BamHI fragment of '114', comprising the 3' end of *I-2,* was cloned into Dark Bluescript KS+ to produce pCBJ261. The ClaI-SacI fragment of pCBJ260 and the SacI-BamHI fragment of pCBJ261 were cloned into pDarkBluescript that had been cut with ClaI and BamHI to produce pCBJ262. The SalI fragment of pCBJ258 containing the *I-2* promoter was added to SalI-cut pCBJ262 to produce pCBJ265, containing the *I-2* coding region under the control of the *I-2* promoter. In addition to gene-specific PCR and restriction analysis, the sequences of the two SalI junctions at the 5' end of the construct as well as the downstream SacI and BamHI junctions were verified by sequencing. The XhoI-SacI (3.9 kb) and SacI-XbaI (2.1 kb) fragments of pCBJ265, containing the *I-2* promoter and coding region, were cloned into the binary vector pSLJ7291 that had been cut with XhoI and XbaI and the resulting construct, pCBJ267, was verified by restriction analysis and PCR, using gene-specific primers.

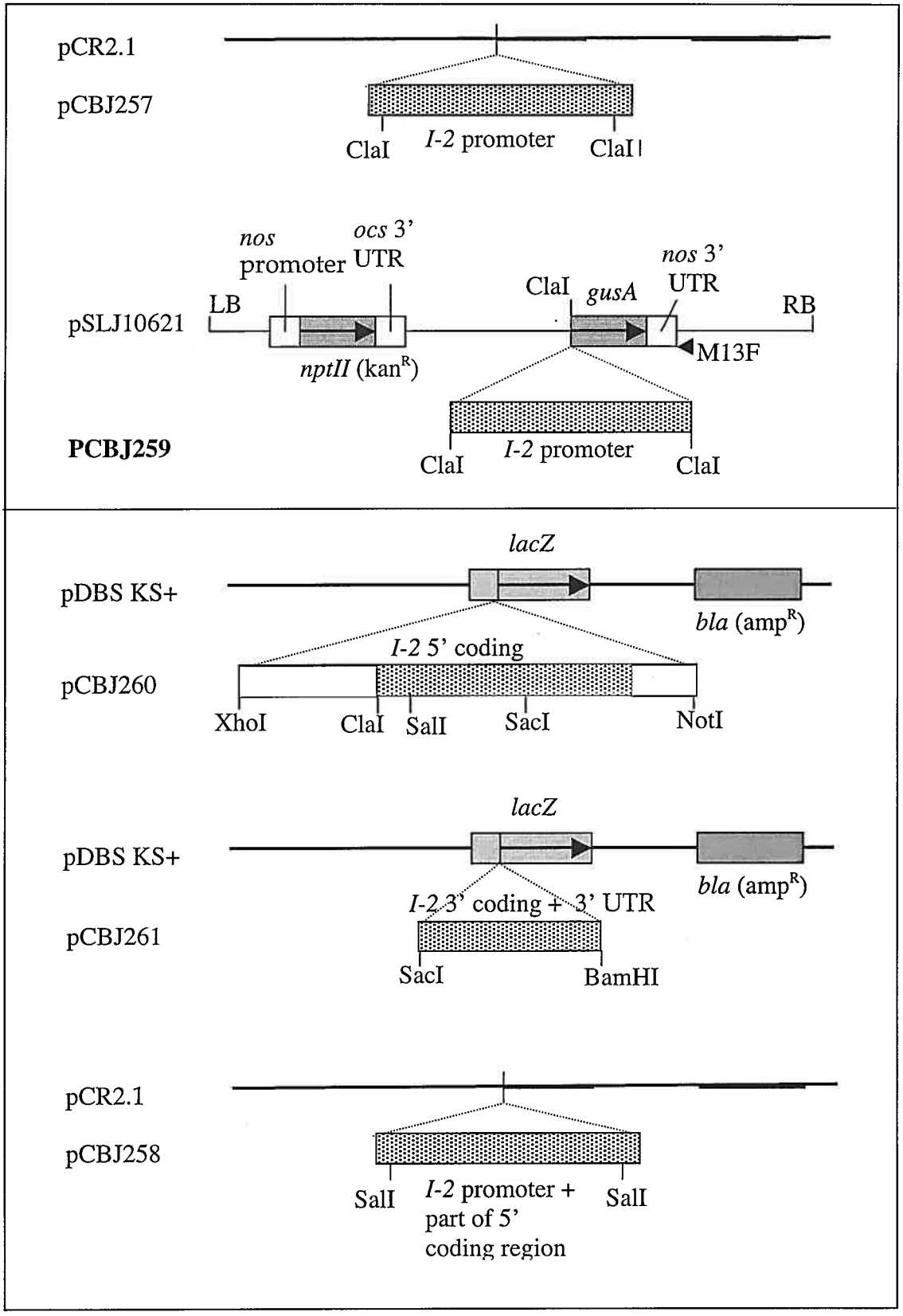

Fig. 1A. DNA constructs

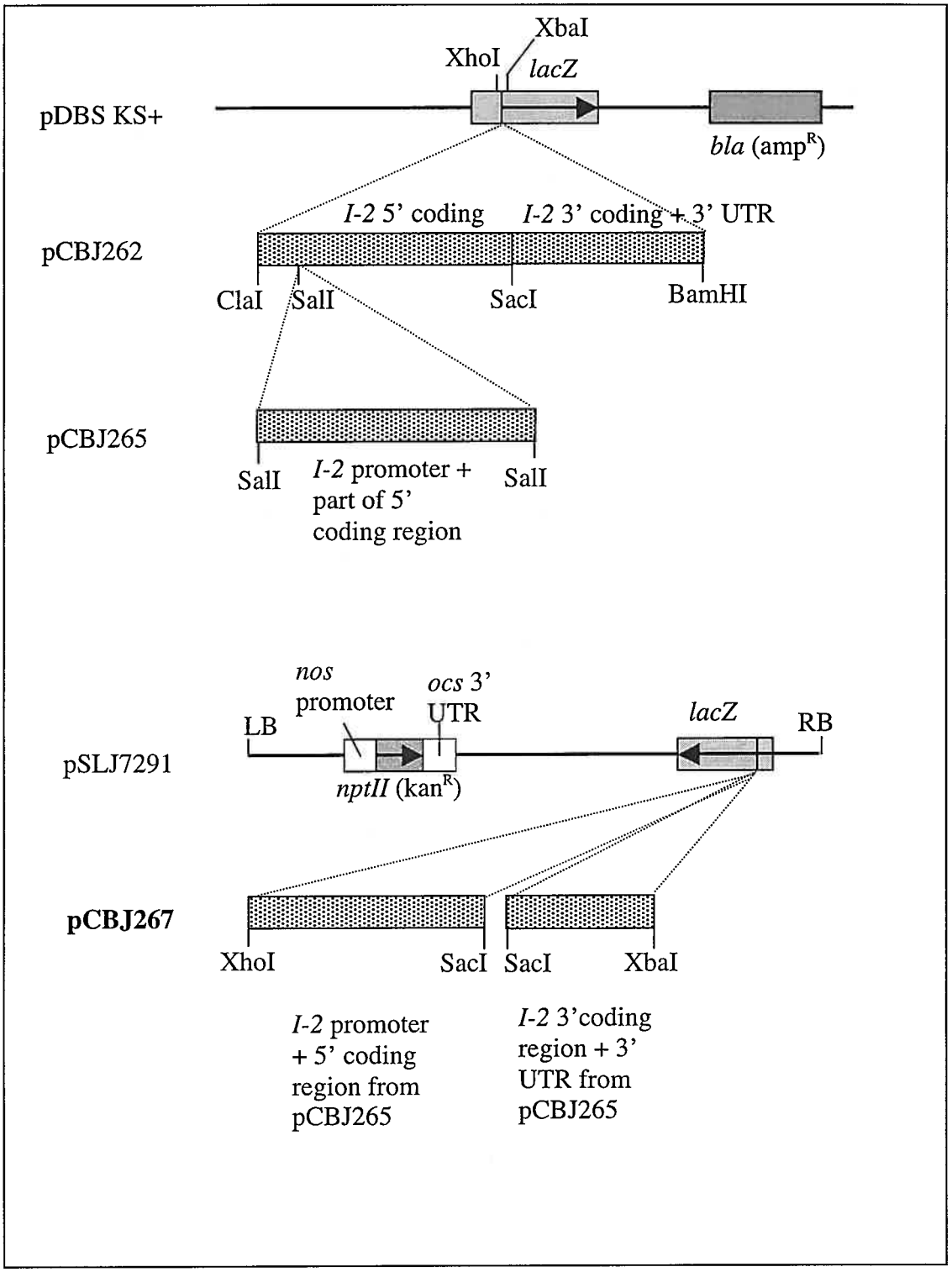

Figure 1B. DNA constructs (continued)

Conjugations

Triparental mating (Jones et al., 1994) was used to transfer pCBJ240 (35S:*I-2*) and pCBJ267 (*I-2:I-2*) to the *Agrobacterium tumefaciens* strains AGL1 and LBA4404, pCBJ10 (35S control construct) to *A. tumefaciens* strain AGL1, and pCBJ259 (*I-2:gusA*) to AGL1 and *A. rhizogenes* strain A4.

Transient expression studies in tobacco and cotton plants

Agroinfiltration is a rapid and sensitive approach for testing resistance gene function in leaves of plants (Van der Hoorn *et al.* 2000; Thomas *et al.* 2000) and was used to test whether cotton transformation with the 35S:*I-2* construct had failed because of constitutive activation of defence signalling resulting in cell death and whether transient expression could be used as a bioassay for *I-2* activity in cotton. The cotyledons of two-week old cotton seedlings and the cotyledons, first or second true leaf and the youngest fully-expanded leaf of seven week-old cotton plants were infiltrated with *Agrobacterium* cell suspensions containing various experimental and control constructs in duplicate trials. Infiltrated cotyledons or leaves of cotton plants were scored for necrotic or chlorotic symptoms 14 days after treatment.

Agrobacterium rhizogenes transformations of tomato and cotton roots

A. rhizogenes transformations were performed using a method adapted from that of Hwang *et al.* (2000) and were used as a rapid means of testing the activity of the *I-2* promoter in cotton cells and confirming that the binary vectors used in this work would allow transgene expression in cotton. Disinfected tomato and cotton seeds were sown on sterile germination media, comprising MS salts pH 5.7, 1% w/v glucose and 2 g/l Gelrite (Sigma-Aldrich) in Phytacon tubs (Sigma-Aldrich Co., St Louis, MO), and were grown for 7 days with 16 hours

illumination at a constant temperature of 25°C. Fresh *A. rhizogenes* plate cultures were used to inoculate 2 ml liquid cultures in AR medium (5g/l yeast extract, 0.5 g/l casamino acids, 8 g/l mannitol, 2 g/l ammonium sulphate, 5 g/l sodium chloride, 15 g/l agar) with 50 μg/ml rifampicin. Tetracycline (5 μg/ml) was used where cells contained pCBJ259. The 2 ml cultures were grown for approximately 16 hours at 27°C with shaking and were then used to inoculate 15 ml AR cultures with appropriate antibiotics in 125 ml Erlenmeyer flasks, which were incubated for 3 days at 27°C with shaking. Cotyledons of 7-day old cotton or tomato seedlings were cut into explants of approximately 1 cm x 1 cm, dipped in appropriate *A. rhizogenes* cultures and wiped on sterile filter paper to remove excess liquid. Explants were then incubated for 40 hours, abaxial surface upwards, with 16 hours illumination at a constant temperature of 25°C on feeder media containing MS salts pH 5.7, 2% w/v sucrose, 0.1% w/v glucose and 4.2 g/l Agargel (Sigma-Aldrich). After this, explants were transferred, abaxial side downwards, to feeder media supplemented with 500 μg/ml Augmentin (Smithkline-Beecham, Welwyn Garden City, UK) and were incubated for three to four weeks with 16 hours illumination at a constant temperature of 25°C. Hairy roots from a sample of explants were stained for glucuronidase activity as previously described (Jefferson and Wilson, 1991). Some hairy roots were also cryosectioned to allow the pattern of glucuronidase staining to be examined microscopically in transverse sections.

5. Detail results, including the statistical analysis of results.

Transient expression of a 35S:I-2 construct in cotton

Cotton transformation attempts using a 35S:*I-2* construct could have failed due to inhibition of shoot regeneration arising from resistance protein overexpression and constitutive signalling resulting in the activation of various plant defences including hypersensitive cell death. Therefore, the biological activity of the construct was tested in cotton plants by

transient expression following agroinfiltration (Thomas *et al.* 2000; Van der Hoorn *et al.* 2000). Expression of the 35S:*I-2* construct did not result in a reproducible necrotic or chlorotic response in cotyledons or mature leaves of cotton plants in one experiment, but produced a necrotic response in two subsequent experiments. In one of these experiments, the cotyledons and first or second true leaves responded strongly to agroinfiltration with the 35S:*I-2* construct, developing necrosis and red pigmentation in the zone of injection. but poorly or not at all to control constructs (Fig 2). In the other, the cotyledons and first or second leaves did not respond as strongly to the 35S:*I-2* construct, developing chlorosis rather than necrosis and red pigmentation. In both cases, the youngest fully expanded leaves did not respond as well as cotyledons and the first or second true leaves, but this may reflect the greater difficulty experienced in infiltrating these leaves. These agroinfiltration experiments need to be repeated, but they nevertheless the support the idea that overexpression of *I-2* causes the induction of plant defence responses in cotton.

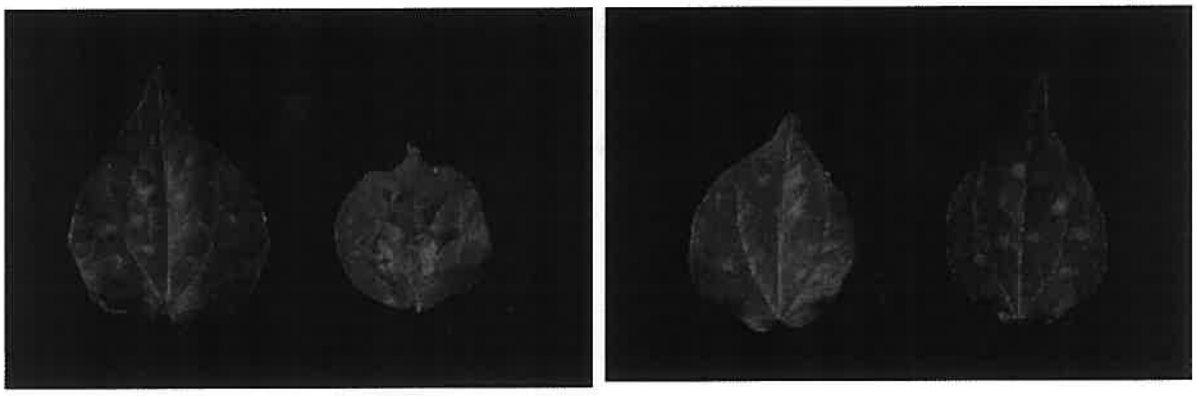

Figure 2. Agroinfiltration of cotton leaves with *Agrobacterium* carrying a 35S:*I-2* T-DNA causes necrosis or chlorosis and red pigment formation suggesting the activation of plant defences. Results of two experiments are shown (left and right panels). In each experiment the control leaf (left) was infiltrated with *Agrobacterium* carrying a 35S T-DNA lacking *I-2* and the experimental leaf (right) was infiltrated with *Agrobacterium* carrying a 35S:*I-2* T-DNA.

Activity of the I-2 promoter in cotton hairy roots

The activity of the *I-2* promoter in cotton cells and the ability of the binary vector backbone used in the 35S:*I-2* and *I-2* promoter: *I-2* constructs to mediate transgene expression in cotton was tested by transforming cotton and tomato (control) explants with *Agrobacterium rhizogenes* containing an *I-2* promoter:*gusA* construct. As expected, hairy tomato roots expressing this construct, but not the roots of explants transformed with *A. rhizogenes* A4 alone, showed b-glucuronidase activity (Fig. 3). Comparable results were seen when cotton explants were transformed with A. rhizogenes A4 with and without the *I-2* promoter*: gusA* construct, suggesting that the *I-2* promoter is active in the cells of cotton plants (Fig. 3).

Figure 3. The *I-2* promoter functions in cotton. Tomato (top row) and cotton (bottom row) hairy roots arising from transformation of explants with *A. rhizogenes* A4 (left column) or A4 (*I-2:gusA*) (right column) were excised and stained for glucuronidase activity.

The distribution of glucuronidase activity in hairy roots with less intense staining of both tomato and cotton (Fig. 3) suggested greater activity in vascular tissues consistent with

previous observations made in tomato (Mes et al., 2000). Samples of hairy roots were cryosectioned to produce transverse sections for microscopic examination (Fig. 4). Glucuronidase activity was found to be localised to cells surrounding the xylem, as previously reported (Mes et al., 2000), but was also found at the root epidermis (Fig. 4). The reason for this difference is not clear, but the data nevertheless show comparable staining patterns in tomato and cotton, although less intense in cotton, suggesting that the *I-2* promoter functions in cotton in much the same way as it does in tomato.

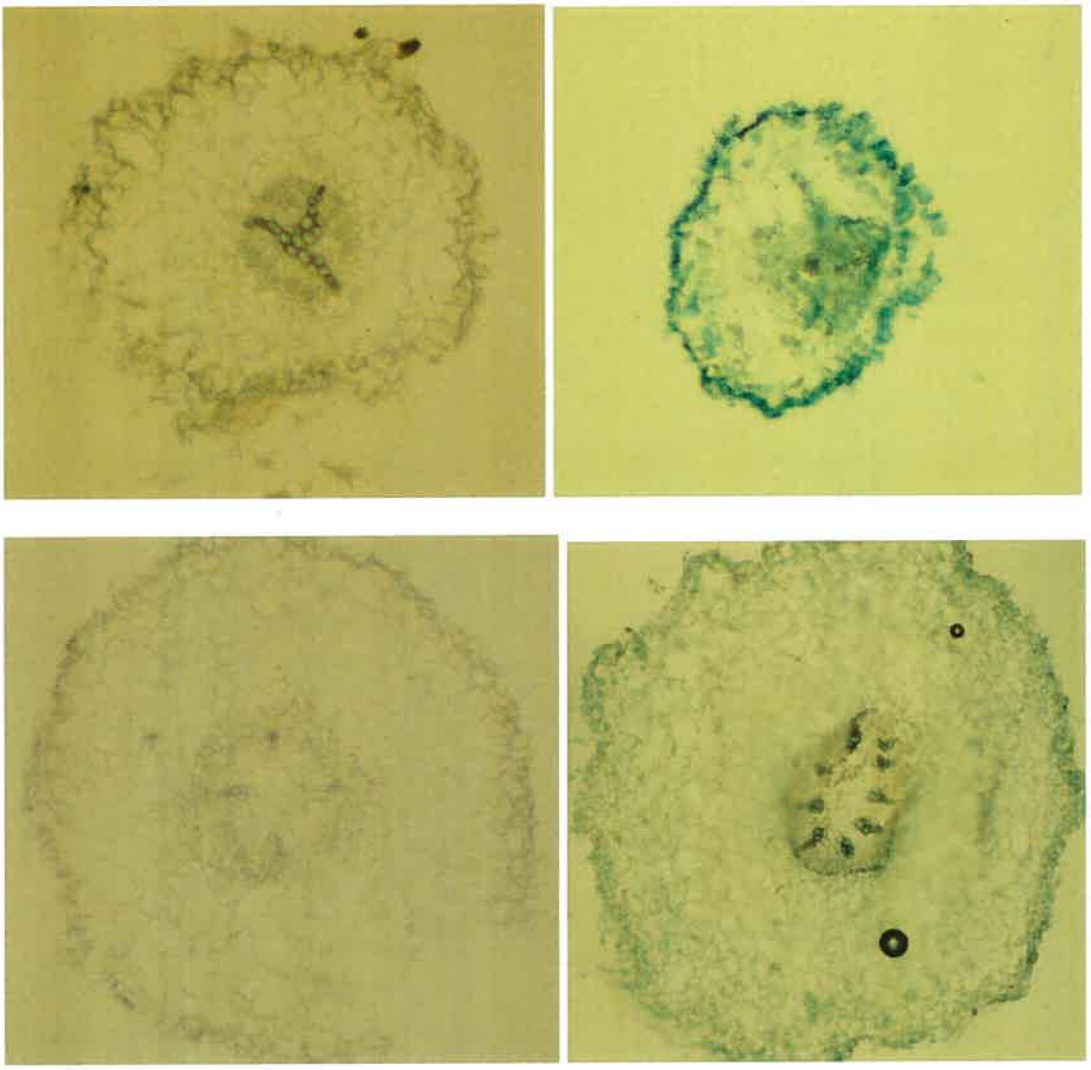

Figure 4. The *I-2* promoter confers a similar spatial pattern of expression in cotton to that conferred in tomato. Tomato (top row) and cotton (bottom row) hairy roots arising from transformation of explants with A4 (left column) or A4 (*I-2:gusA*) (right column) were excised, stained for glucuronidase activity and cryosectioned to produce transverse sections for microscopic examination.

6. Discuss the results, and include an analysis of research outcomes compared with objectives.

Transient expression of *I-2* in cotton leaves under the control of the CaMV 35S promoter resulted in chlorosis or necrosis in two experiments, but not a third. Nevertheless, the results of these experiments suggest that the deleterious effects of *I-2* over expression could explain why transformed callus could not be recovered in cotton transformation experiments. These results further suggest the need for a weaker promoter with an appropriate pattern of expression. Following a report showing that gene expression from the endogenous promoter of *I-2* is highly tissue-specific (Mes et al., 2000), the possibility of expressing an *I-2* promoter: *I-2* construct in cotton was explored. Results from hairy root transformation of cotton explants with an *I-2* promoter-*gusA* construct suggested that the *I-2* promoter is active in cotton cells and mediates tissue-specific expression similar to that seen in tomato plants. These results also indicate that the binary vectors used in our work can be applied successfully to cotton transformation. Therefore, a binary vector containing an *I-2* promoter:*I-2* coding construct was engineered and transferred to the *A. tumefaciens* strains AGL1 and LBA4404 for cotton and tomato (control) transformations, respectively. Already, transformation of cotton with this construct has progressed beyond the stage at which the transformations with the 35S:*I*-2 construct failed previously and, if all continues to go well, second generation *I-2:I-2* cotton plants should be available for *Fusarium* testing within the next year or two. If *I-2* is able to protect cotton plants from *Fusarium*, this would demonstrate the potential for transgenic cotton plants to be used in cotton-growing areas of Australia affected by *Fusarium*.

There are also two significant by products of this research. One is the demonstration that cotton is amenable to hairy root transformation, which offers the possibility of rapid testing of transgenes in hairy roots of cotton. If a *Fusarium* infection assay can be developed for hairy roots, then this offers the further possibility of rapid testing of transgenes in hairy roots of cotton for their effectiveness in combating *Fusarium* infection. The other is the demonstration that the *I-2* promoter functions in cotton roots. Even if *I-2* itself doesn't confer *Fusarium* resistance in cotton, the *I-2* promoter could still be used to trial the effectiveness of other transgenes in cotton roots by expressing these transgenes at points where fungus is likely to invade.

7. 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 and future research needs.

This research project will continue beyond the period of CRDC funding and, providing the cotton transformation goes well, the ability of transgenic *I-2:I-2* cotton plants to resist *Fusarium* wilt will be tested in due course. In its favour, we now know that *I-2* is biologically active in cotton and is apparently able to induce a defence response. We also know that the *I-2* promoter is active in cotton roots. If *I-2* does confer *Fusarium* resistance in cotton then the potential benefits will be enormous, although there would be significant costs in further testing and the development and testing of commercial lines. If *I-2* does not confer *Fusarium* resistance in cotton then one potential avenue for the control of *Fusarium* wilt in cotton will be closed. If this is the case, then more research effort will need to be directed towards identification of endogenous sources of *Fusarium* wilt resistance in wild relatives of cotton, particularly the A and D genome relatives. In this regard, I plan to submit a research proposal to CRDC seeking support for an investigation of the A genome relatives *Gossypium*

arboreum and *G. herbaceum* as potential sources of genes for resistance to the two problematic races of *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt in Australian cotton.

8. Describe the project technology (eg. commercially significant developments, patents applied for or granted licenses etc).

No project technology of the kind listed above has been developed thus far by this project. However, should *I-2*:*I-2* cotton plants show Fusarium resistance, then the results will be commercially significant and issues of patents (existing and potential) and licences (to be obtained or granted) will become relevant. Other aspects of the project technology have been described in detail in Section 4 above.

9. Provide a technical summary of any other information developed as part of the research project. Include discoveries in methodology, equipment design, etc.

As indicated in Section 6, two significant methodologies have arisen as by products of this research.

• Development of a hairy root transformation system for cotton. This offers the possibility of rapid testing of transgenes in hairy roots of cotton.

• Demonstration that the *I-2* promoter functions in cotton roots. This offers a promoter suitable for driving the expression of transgenes in cotton roots, particularly in the root epidermis and in cells surrounding the vascular tissue.

10. Detail a plan for the activities or other steps that may be taken;

(a) to further develop or to exploit the project technology

Subject to availability of funds and personnel, future work will/may involve:

• Repeating the cotton agroinfiltration experiments with the 35S:*I-2* construct to show that the necrotic response is reproducible.

• Looking for pathogenesis gene induction following agroinfiltration of cotton with the 35S:*I-2* construct to confirm activation of plant defences

• Identification of the red pigment formed in response to agroinfiltration of cotton with the 35S:*I-2* construct

• Development of a *Fusarium* infection assay for hairy roots and, if successful, testing *I-2:I-2* hairy roots for *Fusarium* resistance.

• Production of *I-2:gusA* transgenic cotton to test the activity of the *I-2* promoter in cotton tissues other than roots

• Molecular analysis of transgenic *I-2:I-2* cotton plants to confirm delivery and expression of the *I-2:I-2* transgene

• Production and harvesting of second generation transgenic *I-2:I-2* cotton seed for *Fusarium* testing

• *Fusarium* testing of second generation transgenic *I-2:I-2* cotton plants

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

I will continue to be involved in meetings and workshops to discuss the *Fusarium* problem in cotton. New results, particularly those arising from the future testing of *I-2:I-2* cotton plants

for *Fusarium* resistance will be reported at these venues. I also intend to publish the results of this research in a peer-reviewed scientific journal (see Section 11 below).

11. List the publications arising from the research project.

There are no publications arising from this work as yet. However, after the outcome of *Fusarium* testing on *I-2:I-2* cotton plants is known, a manuscript will be prepared for submission to a journal such as Functional Plant Biology to report the biological activity of 35S:*I-2* in cotton, the activity of the *I-2* promoter in cotton and the effectiveness or otherwise of the *I-2* gene in cotton against *Fusarium*.

12. Are changes to the Intellectual Property register required?

No.

REFERENCES

Alexander, L. J. (1945). Physiologic specialisation in the tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*. *J. Ag. Res.* **70**, 303-313.

Beckman, C. H., ed. (1987). The Nature of Wilt Diseases of Plants. St Paul, MN: American Phytopathological Society.

Cirulli, M. and Alexander, L. J. (1966). A comparison of pathogenic isolates of *Fusarium oxysporum* f. sp. *lycopersici* and different sources of resistance in tomato. *Phytopathol.* **56**, 1301-1304.

Davis, R. D., Moore, N. Y. and Kochman, J. K. (1996). Characterisation of a population of *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt of cotton in Australia. *Aust. J. Agric. Res.* **47**, 1143-1156.

Fernandez, D., Assigbetse, K., Dubois, M.P. and Geiger J.P. (1994). Molecular characterization of races and vegetative compatibility groups in *Fusarium oxysporum* f. sp. *vasinfectum*. *Appl. Envir. Microbiol,* **60**, 4039-4046.

Hennin C. Diederichsen E. and Hofte M. (2001). Local and systemic resistance to fungal pathogens triggered by an AVR9-mediated hypersensitive response in tomato and oilseed rape carrying the *Cf-9* resistance gene. *Physiol. Mol. Plant Path.* **59**, 287-295.

Hennin, C., Hofte, M. and Diederichsen, E. (2002). Functional expression of *Cf-9* and *Avr9* genes in *Brassica napus* induces enhanced resistance to *Leptosphaeria maculans*. *Mol. Plant-Microbe Interact.* **14**, 1075-1085.

Hwang, C. F., Bhakta AV, Truesdell GM, Pudlo WM, Williamson VM. (2000). Evidence for a role of the N terminus and leucine-rich repeat region of the *Mi* gene product in regulation of localised cell death. *Plant Cell* **12**: 1319-1329.

Jefferson, R. A. and Wilson, K. J. (1991). The GUS gene fusion system. In *Plant Molecular Biology Manual*, vol. B (eds. Gelvin, S. B., Schilperoort, R. A. and Verma, D. P. S.), pp. B14/24. Dordrecht: Kluwer Academic Publishers.

Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J. and Jones, J. D. G. (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* **266**, 789-793.

Mes, J. J., Van Doorn, A. A., Wijbrandi, J., Simons, G., Cornelissen, B. J. C. and Haring, M. A. (2000). Expression of the *Fusarium* resistance gene *I-2* colocalises with the site of fungal containment. *Plant J.* **23**, 183-193.

Peart, J. R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D. C., Schauser, L., Jaggard, D. A., Xiao, S., Coleman, M. J., Dow, M., Jones, J. D., Shirasu K. and Baulcombe D. (2002). Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. *Proc. Natl. Acad. Sci. USA* **99**, 10865-10869.

Rogers, S. O. and Bendich, A. J. (1993). Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*, vol. 1 (eds. Gelvin, S. B., Schilperoort, R. A. and Verma, D. P. S.), pp. A6: 1-10. Dordrecht: Kluwer Academic Publishers.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. New York: Cold Spring Harbour Press.

Simons, G., Groenendijk, G., Wijbrandi, J., Reijans, M., Groenen, J., Diergaarde, P., Van der Lee, T., Bleeker, M., Onstenk, J., De Both, M. et al. (1998). Dissection of the *Fusarium I2* gene cluster in tomato reveals six homologs and one active gene copy. *Plant Cell* **10**, 1055-1068.

Skovgaard, K., Nirenberg, H.I., O'Donnell, K. and Rosendahl, S. (2001). Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology. **91**, 1231-1237.

Stall, R. E. and Walter, J. M. (1965). Selection and inheritance of resistance in tomatoes to isolates of races 1 and 2 of the *Fusarium* wilt organism. *Phytopathol.* **55**, 1213-1215.

Tai, T.H., Dahlbeck, D., Clark, E.T., Gajiwala, P., Pasion, R., Whalen, M.C., Stall, R.E. and Staskawicz, B.J. (1999). Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. *Proc. Natl Acad. Sci. USA.* **96**, 4153-14158.

Thomas, C. M., Tang, S., Hammond-Kosack, K. and Jones, J. D. G. (2000). Comparison of the hypersensitive response induced by the tomato *Cf-4* and *Cf-9* genes in *Nicotiana* spp. *Mol. Plant Microbe Interact.* **13**, 465-469.

Van der Hoorn, R. A. L., Laurent, F., Roth, R. and De Wit, P. J. G. M. (2000). Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9/Cf-9*-induced and *Avr4/Cf-4*-induced necrosis. *Mol. Plant Microbe Interact.* **13**, 439-446.

Part 4 – Final Report Plain English Summary

Provide a half to one page Plain English Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web.

The aim of his project was to test the *I-2* gene, which confers resistance to *Fusarium* wilt in tomato, for its ability to confer resistance to *Fusarium* wilt in cotton. The *I-2* gene was engineered for strong constitutive expression under the control of the 35S promoter and used in several attempts to produce transgenic cotton plants. These attempts failed, suggesting that the over-expression of *I-2* may have triggered cotton defence responses resulting in the death of cells receiving the *I-2* transgene. Transient expression of 35S:*I-2* in cotton leaves triggered a chlorotic or necrotic response and the accumulation of a red pigment, consistent with this interpretation. The *I-2* gene's own promoter was then tested as a weaker-expressing replacement for 35S, by engineering a fusion between the *I-2* promoter and a glucuronidase reporter gene, and using *Agrobacterium rhizogenes* to produce transgenic roots carrying the gene fusion. Analysis of glucuronidase expression in these roots indicated that the *I-2* promoter was active in cotton. The *I-2* gene was then engineered for expression under the control of its own promoter and used in a further attempt to produce transgenic cotton expressing *I-2*. This attempt has already progressed beyond the point where transformations with 35S:*I-2* failed and, presuming *I-2*:*I-2* transgenic cotton plants will be produced in due course, second-generation plants will then be tested for *Fusarium* resistance. Given, that the *I-2* promoter is active in cotton and *I-2* appears able to induce a defence response in cotton, the chances that *I-2* may confer Fusarium wilt resistance in cotton are considerably better than they were at the beginning of this project when nothing was known about the way *I-2* would behave in cotton.