Inoculation of Siokra S324 cotton with an incompatible race of *Xanthomonas campestris* pv. *malvacearum* induces chitinase and reduces the severity of *Verticillium* wilt symptoms.

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Introduction

Fungal diseases, in particular *Verticillium* wilt, and, more recently, *Fusarium* wilt, have the potential to cause major losses in Australian cotton production. Research conducted by our group is focussed on genetic approaches to the improvement of cotton resistance to fungal attack.

It has been established for several plant/pathogen systems that interaction of an avirulent race of a pathogen with a resistant plant gives rise to an "incompatible response" by the host plant. This reaction, usually characterised by the development of a local lesion of dead cells around the point of infection or inoculation, results in containment of the pathogen and survival of the plant. The incompatible reaction triggers other responses in the plant including the development of a general tolerance to further pathogen attack known as systemic acquired resistance. One aspect of systemic acquired resistance is the activation of several genes that result in the synthesis of increased amounts of proteins called "pathogen response" (PR) proteins (Ward *et al.*, 1991). Some PR proteins have been shown to have antifungal activity *in vitro* (eg. Mauch *et al.*, 1988) and *in vivo* in transgenic plants (eg. Broglie and Broglie, 1993). The hydrolytic enzymes chitinase and glucanase are frequently found to be among the PR proteins. Induced resistance and elevation of PR protein levels has been described for *Xanthomonas campestris* and turnip (Conrads-Strauch *et al.*, 1990), pepper (O'Garro and Charlemange, 1994) and *Arabidopsis* (Lummerzheim *et al.*, 1995; Buell and Somerville, 1995) but not, as far as we are aware, for cotton and *Xanthomonas campestris* pv. *malvacearum*.

The incompatible reaction of the host is dependent on the presence of an avirulence gene(s) in the pathogen and corresponding resistance gene(s) in the plant. Several avirulence genes from *Xanthomonas campestris* pv. *malvacearum* have been isolated and characterised (De Feyter *et al.*, 1993). It may be possible to express these genes in cotton and, provided that they can be selectively targetted, induce a localised, artificial incompatible response followed by induction of PR proteins and systemic acquired resistance. An avirulence gene from *Cladosporium* *fulvum* constitutively expressed in a resistant host tomato line (Honée *et al.*, 1995) gave plants that became necrotic and died, demonstrating the ability of an avirulence gene to initiate a global incompatible response. The use of suitably targetted pathogen avirulence gene(s) in transgenic plants has a fundamental advantage over the use of genes for individual PR proteins and other putative anti-fungal proteins. It has the potential to activate the whole suite of the plant's defence responses, including induction of PR proteins and activation of the synthesis of antifungal secondary metabolites such as phytoalexins (Górski *et al.*, 1995) as well as other, as yet unknown, responses. The use of avirulence genes could be complementary to the use of single PR genes with inducible promoters that could be activated along with the endogenous plant defences.

The successful deployment of *Xanthomonas* avirulence genes to improve cotton resistance is a long term goal. Several fundamental questions need to be addressed to determine if the approach is viable before we embark on the generation of transgenic cotton. While it is well known that avirulent strains of *Xanthomonas* produce an incompatible response in resistant lines of cotton, it is not known if this causes an increase in the levels of PR proteins or if systemic acquired resistance is induced. Therefore the goal of the preliminary work described here is to establish if *Xanthomonas* infection of cotton has these effects. Chitinase activity was used as an indicator of PR protein induction; the response of chitinase activity to *Xanthomonas* inoculation and various other treatments was monitored. Subsequent infection of inoculated and uninoculated plants with *Verticillium* wilt was used to assess the systemic acquired resistance response. This pathogen was used because of its economic importance, but because the resistance response may be much stronger in leaf tissue than in roots, it might be more appropriate to use a foliar pathogen in future work.

Methods

Xanthomonas inoculation and other pretreatments

Cotton plants (Siokra S324) were sown in potting mix and grown in a glasshouse. They were treated 10-11 days after sowing. Treatments were as follows.

(1) Spray to runoff with water (control).

(2) Spray to runoff with a 10^{-4} M aqueous solution (20ppm) of 2,6-dichloro*iso*nicotinic acid (CGA 41396) (a compound shown (Brock *et al.*, 1994) to induce resistance of cotton plants to *Alternaria macrospora* infection).

(3) Inoculation of one cotyledon in two spots with a freshly grown culture of *Xanthomonas campestris* pv. *malvacearum* Race 15 (Brinkerhoff, 1970) infiltrated into the tissue using a

needleless syringe.

(4) Mock inoculation of one cotyledon with sterile growth medium.

Extraction and chitinase determination.

Whole cotyledons were harvested and ground with 0.05M sodium acetate buffer, pH 5.0 (1 ml) and sand plus 50% (w/w) insoluble polyvinylpyrrolidone. The mixture was centrifuged to remove debris, a portion of the supernatant diluted 1 in 5 with water and the diluted supernatant recentrifuged. Chitinase assays were performed on these crude extracts using carboxymethychitin-remazol brilliant violet as substrate according to the method of Wirth and Wolf (1990). Protein levels were determined using a bicinchoninic acid kit (Pierce) with bovine serum albumin as standard.

Verticillium inoculation

Plants were inoculated 5-7 days after *Xanthomonas* inoculation or other pretreatment with a suspension of *Verticillium* conidia in water (10^7 ml^{-1}) prepared from week-old plates. The plants were root-dipped in the conidial suspension or water (control) for 10 min and then repotted singly in fresh potting medium and were grown in a growth chamber with a 16h 25°C light /8hr 24°C dark cycle. Pots were kept constantly moist and plants were examined regularly for wilt symptoms.

Results

Chitinase levels

The chitinase activities observed in extracts obtained four days after pretreatements are presented in Figure 1. A significant increase in chitinase activity was detected after *Xanthomonas* inoculation (4.5-fold increase), after mock inoculation with sterile growth medium (1.9-fold increase), and after spraying with 2,6-dichloro*iso*nicotinic acid (1.6-fold increase). Similar results were obtained seven days after pretreatments. The increased chitinase activity was observed only in the inoculated cotyledon, suggesting that at these time points the response occurs within the inoculated leaf but not the whole plant. Similar results were obtained for two experiments.

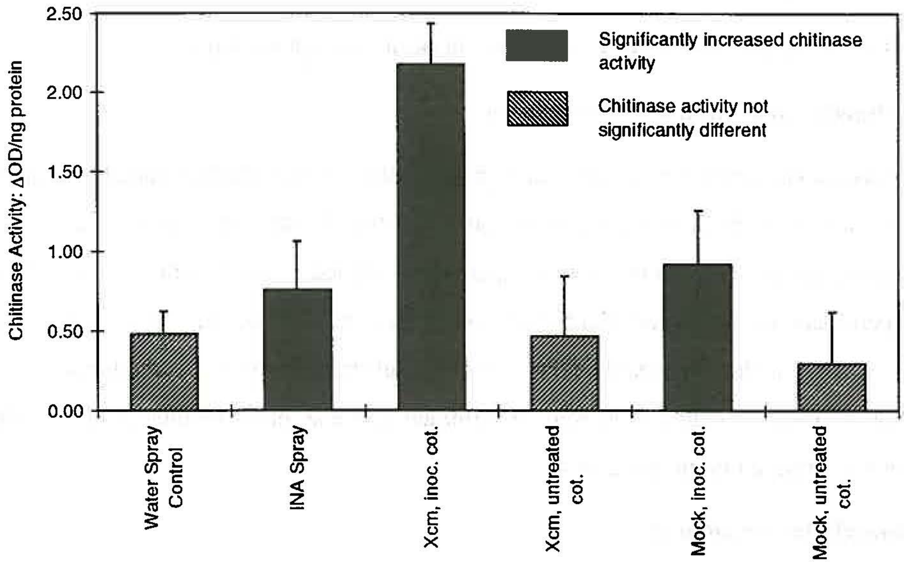

Figure 1. Chitinase activity (expressed as increased absorbance [OD] due to liberation of dye from artificial substrate [carboxymethchitin-remazol brilliant violet] in 30 minutes at 37°C per ng protein) of extracts of cotton cotyledons four days after different pretreatments as detailed in Methods. Abbreviations: INA, 2,6-dichloro*iso*nicotinic acid; Xcm, *Xanthomonas campestris.*, cot., cotyledon. Error bars are the standard deviation of the mean.

Verticillium tolerance

Symptoms of *Verticillium* wilt were observed in inoculated plants about 1 week after inoculation and all inoculated plants were showing some symptoms after two weeks. The severity of the disease was estimated by measuring plant height. The height data obtained 15 days after *Verticillium* inoculation is presented in Figure 2. There is no significant difference between the mean heights of the control (non-*Verticillium*-inoculated) groups, but for *Verticillium*-inoculated plants, the group preinoculated with *Xanthomonas* is significantly taller than the untreated control. Therefore, while *Xanthomonas* inoculation has not given protection against *Verticillium* infection, it appears to have reduced the severity of the disease symptoms. This experiment has only been completed once, so these results should be regarded as preliminary. In this experiment the *Verticillium* inoculum level used was quite high, so it is possible that protection might be observed using lower inoculum levels.

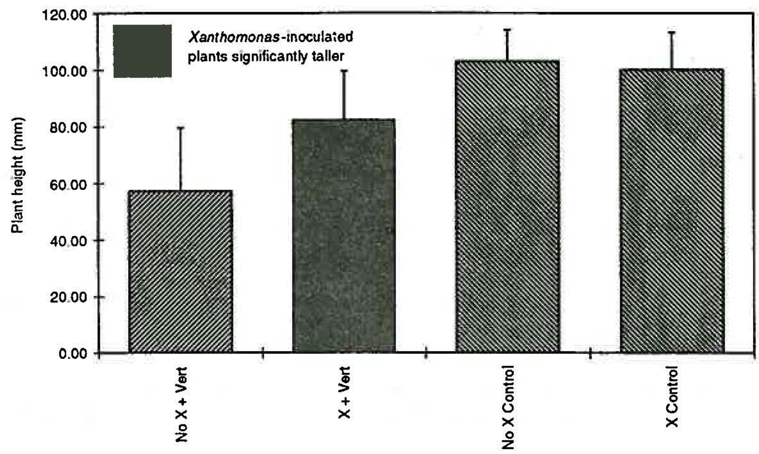

Figure 2. Plant heights 15 days after *Verticillium* inoculation. Plants were pretreated by inoculation with *Xanthomonas* (X) or were untreated (No X) and were then root-dip inoculated with *Verticillium* conidial suspension (+Vert) or water (Control). Error bars are the standard deviation of the mean.

Discussion

The elevation of chitinase activity after inoculation of cotyledons of resistant cotton with an avirulent strain of *Xanthomonas campestris* pv. *malvacearum* was observed. Although some increase was also observed after mock inoculation, probably in response to wounding, the increase was much greater in bacterially inoculated leaves. The increase observed after *Xanthomonas* inoculation was also greater than the increase observed after treatment with 2,6-dichloro*iso*nicotinic acid, a chemical that has been shown in a previous study (Brock *et al.*, 1994) to induce systemic resistance to subsequent inoculation with a leaf pathogen. It is therefore likely that induction of PR proteins is occurring and that systemic acquired resistance is developed. The evidence for induced resistance is tentative at this stage; further work with *Verticillium* and the leaf pathogen *Alternaria macrospora* is planned. Investigation of the levels of chitinase in root tissue after various treatments is also likely to give some indication of the ability of *Xanthomonas* inoculation to induce resistance to *Verticillium* and *Fusarium* infection.

It is not known if *Xanthomonas* avirulence gene products are able to interact directly with cotton tissue to give the incompatible response, as is the case with *Cladosporium fulvum* and tomato. The avirulence gene product may require the presence of other *Xanthomonas* metabolites to produce the actual signalling molecule. If this is the case, expression of the avirulence genes in plant tissue will not have the desired effect. Further experiments are being designed to investigate this problem as the next step towards the goal of using *Xanthomonas* avirulence genes to improve cotton's tolerance to pathogens.

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Acknowledgements

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