Screening Cotton Germplasm with Molecular Markers for Enhanced Tolerance to Fungal Wilt Disease

Karin Jacobsen Lyon, Alison E. Cook, Melissa K. Hill and Bruce R. Lyon
Cooperative Research Centre for Sustainable Cotton Production, School of Biological Sciences A12,
The University of Sydney, Sydney NSW 2006.

1. Introduction

In a cool, wet season when conditions are favourable for the *Verticillium dahliae* fungal pathogen, Verticillium wilt disease can cost the Australian cotton industry millions of dollars in lost production. The susceptibility of cotton plants to Verticillium wilt does however vary between cotton species and cultivars (Ramsay *et al.*, 1996), and breeders have attempted to select cotton germplasm with increased resistance to the disease. True resistance can be found in cultivars of *Gossypium barbadense*, such as the American cultivar Pima S-7, whereas cultivars of *Gossypium hirsutum* at their best only display tolerance to the disease, restricting the development of disease symptoms and allowing the production of a reasonable crop. Plant breeders from the CSIRO have now developed several *G. hirsutum* cultivars with increased tolerance to Verticillium wilt such as Sicala V-1, Sicala V-2 and Siokra V-15.

The genetics of the different resistance levels displayed by cotton plants to Verticillium wilt remains unclear. Cross breeding of resistant and susceptible cultivars suggests that two or more genes determine resistance in *G. barbadense*, while tolerance in *G. hirsutum* is considered to result from the activity of many genes. The involvement of several resistance genes complicates traditional breeding techniques, which may not be entirely successful in maximising the selection of Verticillium wilt resistance. The identification of genes linked with improved disease resistance would enable breeders to utilise molecular markers in the selection of breeding lines with particular combinations of genes, and thereby allow the possible pyramiding of resistance mechanisms. This would result in new elite cultivars with superior and potentially more robust resistance to Verticillium wilt.

Cotton plants that are more resistant to Verticillium wilt have also been shown to be more resistant to Fusarium wilt, a fungal disease new to cotton production regions in Australia which is increasingly causing concern within the industry. The development of molecular markers for resistance to Verticillium wilt, and the use of these markers in the breeding of elite disease resistant cultivars, could thus have broad benefits for the control of both Verticillium wilt and Fusarium wilt in cotton.

In our laboratory, we have undertaken to isolate and characterise genes that are activated in the *Verticillium*-tolerant *G. hirsutum* cultivar Sicala V-1 upon infection with the *Verticillium* pathogen. Such genes are potentially involved in the defence response of the host plant against *Verticillium* infection, and thus may represent candidate molecular markers for enhanced Verticillium wilt resistance in cotton (Hill, 1998, Hill & Lyon).

2. Resistance mechanisms in cotton to *Verticillium* infection

2.1 Isolation of cotton defence response genes

Two separate approaches were taken to identify genes involved in the defence response of the cotton cultivar Sicala V-1 towards Verticillium wilt. A targeted approach was chosen for the Chitinase and 3-hydroxy-3-methylglutaryl reductase (HMGR) genes which are known to be involved in the defence response of other plant species and therefore could be isolated from the cotton plant based on known DNA sequence.

To identify other, potentially unknown genes that contribute to defence response mechanisms in cotton, a cDNA gene library was constructed from the roots of two-week-old wilt-tolerant Sicala V-1 plants that had been inoculated with *Verticillium* 24 hours earlier. A large number of cloned genes from the library were screened for their activity in *Verticillium*-infected and non-infected root tissue and categorised according to their activity profile. Candidates for involvement in the plant's defence response are found among those with infection-associated activity and those showing an increase in gene activity upon *Verticillium* infection. We therefore embarked on the characterisation of genes from these categories by DNA sequencing, followed by comparison of the sequences with known genes which may aid in the identification and provide clues about potential function in the plant.

Cotton Gene	**Gene or Protein Similarity**
CHT	Chitinase {cotton}
HMGR	3-hydroxy-3-methylglutaryl reductase (HMGR) {cotton}
VGh126	Metallothionein-like gene {cotton}
VGh136	disease resistance response gene {pea}
VGh141	pathogenesis-related protein (PR-10) {European white birch}
VGh42	phenylalanine ammonia lyase (PAL) {poplar}
VGh110	14-3-3-like protein {tomato}
VGh117	serine/threonine protein kinase {*Arabidopsis*}

Table 1. A small selection of the genes cloned from the cotton cultivar Sicala V-1, followed by the gene or protein cloned from other plants with which they share similarity.

A small selection of putative defence response genes that we have isolated from the cotton cultivar Sicala V-1 are listed in Table 1. Chitinases are hydrolytic enzymes which degrade chitin, a major component of fungal cell walls. Such enzymes are capable of inhibiting fungal growth as well as liberating chiton oligomers which can act as elicitors for further host defensive action. HMGR is a key regulator enzyme in the biosynthesis of phytoalexin antimicrobial compounds, whereas the exact role of the Metallothionein-like gene (VGh126) in plant defence remains speculative. VGh136 is similar to an infection-associated disease-resistance response gene from pea, and VGh141 was identified as a pathogenesis-related protein following DNA sequence comparison. The DNA sequence of VGh42 shows similarity to phenylalanine ammonia lyase (PAL). PAL is an enzyme involved in the production of defence-related compounds, lignin for cell wall strengthening and secondary messengers involved with signalling pathways within the cell. Two genes, VGh110 which is similar to a 14-3-3-like protein from tomato, and VGh117 which is similar to a serine/threonine protein kinase from *Arabidopsis*, both have a putative function in signal transduction pathways which may trigger the onset of the defence response in the host plant. Clearly, our research has identified a number of cotton genes which potentially function in the diverse defence responses of the cotton plant towards *Verticillium* infection.

2.2 Searching for genes that provide superior wilt-tolerance

Our studies have identified genes that display new or increased activity upon *Verticillium* infection of Sicala V-1 and therefore qualify as putative defence response genes directed against the invading fungus. In our search for molecular markers that may help breeders select for *Verticillium*-resistant cotton germplasm, we are however predominantly interested in genes linked with resistance which might help explain the resistance variation observed among some cultivars. For example, Siokra 1-4 is found to be highly susceptible, Sicala V-1 is tolerant whereas Pima S-7 displays resistance towards *Verticillium* infection. It is unclear whether this difference in response to *Verticillium* infection is due to the swiftness and/or intensity of the cultivar's defence response, or rather reflects the possession of one of several possible resistance mechanisms. Being able to discriminate between these possibilities would enable breeders to make more informed choices when deciding on a plant breeding strategy for *Verticillium*-resistant cultivars, either by pyramiding several resistance mechanisms currently found in distinct cultivars or alternatively, by boosting the intensity of a defence response common to all cultivars.

To investigate the mechanism(s) behind wilt resistance in cotton and to identify markers associated with superior wilt tolerance, we compared the relative activity of selected genes between the cultivars Siokra 1-4 (susceptible), Sicala V-1 (tolerant) and Pima S-7 (resistant). Figure 1 shows the activity profile of three genes: Chitinase, HMGR and a Metallothionein-like gene at time points of 0, 6, 12, 24 and 48 hours after *Verticillium* infection (Hill 1998). Chitinase and HMGR display a similar activity profile, with minimal activity in all of the cultivars in the time up to 24 hours post-infection, after which time Sicala V-1 and Pima S-7 both exhibit a significant increase in gene activity that continues at 48 hours. The increase in Chitinase and HMGR activity is not detected in Siokra 1-4, which may suggest a delayed or absent defence response in this disease-susceptible cultivar. On the other hand, the Metallothionein-like gene shows early increased activity (6 hours) in Siokra 1-4 and Sicala V-1, which is not apparent in the resistant Pima S-7 cultivar. This may therefore be an example of a gene involved in a defence mechanism that is present in *G. hirsutum* (Siokra 1-4 and Sicala V-1) but absent from the genetically more distant *G. barbadense* (Pima S-7). The induction of the Metallothionein-like gene persists for 24 hours after infection in the tolerant Sicala V-1, whereas the induction is short-lived in the disease-susceptible Siokra 1-4, which may suggest a failure of this susceptible cultivar to mount a proper and lasting defence against the invading fungus. In conclusion, our data suggest the existence of at least two defence mechanisms involved in *Verticillium* resistance in cotton; one is represented by HMGR and Chitinase, and the other by Metallothionein. In cloning these genes, we have isolated potential molecular markers for each of these mechanisms.

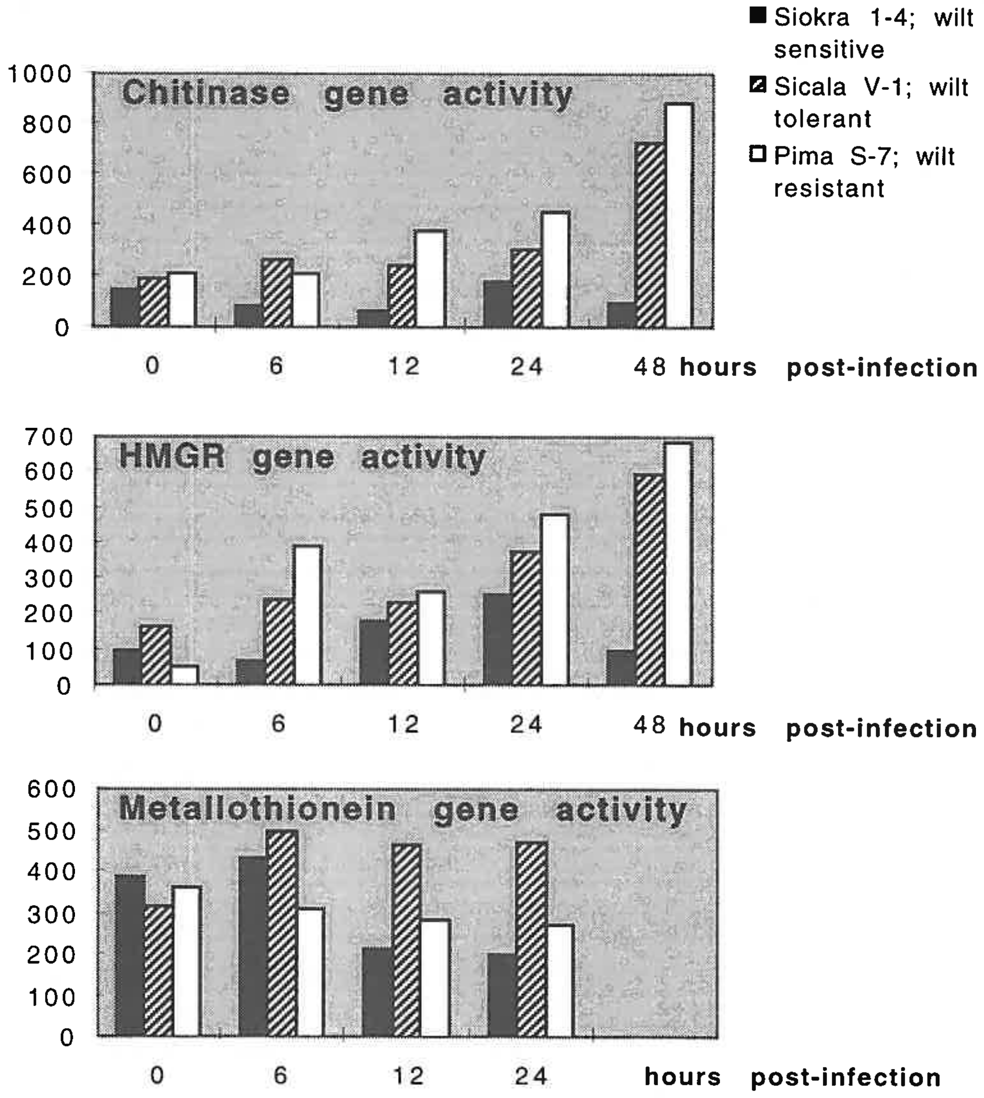

Figure 1. Gene activity of three putative cotton defence response genes, Chitinase, HMGR and Metallothionein, at various time points after infection of the cotton cultivars Siokra 1-4, Sicala V-1 and Pima S-7 with the *Verticillium* pathogen.

Methods: Studies of gene activity were conducted by aseptically germinating cotton seedlings in culture pots for 2 weeks, by which time the first true leaves had appeared. The seedlings were carefully removed from their pots and had their roots submerged in a *Verticillium* inoculate for 3 minutes, after which they were returned to their pots. The *Verticillium* inoculate was prepared by scraping 2 week old *Verticillium* strain 1028 (Ramsay *et al.*, 1996) off solid media, followed by homogenisation and filtering through gauze. The *Verticillium* spores were counted and the concentration adjusted to 5 million spores per ml. Root tissues were harvested from the infected seedlings 6, 24 or 48 hours later. RNA was subsequently isolated from the *Verticillium*-infected root samples and gene activity was analysed by the Northern blotting technique using radioactively-labelled Chitinase, HMGR and Metallothionein gene probes.

2.3 Verification of candidate molecular markers

We are attempting to expand on these preliminary findings by screening twenty-five lines of current Australian cotton germplasm, including parental lines, existing varieties and promising new lines to evaluate a number of genes as candidate molecular genetic markers which may signify enhanced resistance to Verticillium wilt. We are focusing on two of the candidate genes described in Table 1, namely HMGR and the Metallothionein-like gene. Based on the small study of three cultivars that we have described above, we are expecting to find a correlation between the gene activity profile of each cultivar and *Verticillium* resistance. Preliminary results reveal a significant variation in activity for the HMGR gene among the twenty-five cultivars, however due to the experimental strategy of blind-testing the cultivars, it is still to be shown whether this trend correlates with the field-determined level of *Verticillium* wilt resistance of each cultivar. The gene activity of the Metallothionein-like gene on the other hand, displays minimal variation among the cultivars, suggesting that it may have less value as a molecular marker for *Verticillium* wilt resistance.

3. Conclusion

We have found that a study of gene activity in the cotton plant in response to *Verticillium* infection provides abundant information that may help the cotton industry combat costly fungal diseases. Firstly, our research may provide molecular markers that can help plant breeders to select superior *Verticillium* wilt-resistant cotton germplasm when selecting progeny from parents of variable *Verticillium* susceptibility. Secondly, we are gaining a better understanding of the mechanisms involved in host plant resistance to *Verticillium* wilt, which could help in the design of the best strategy for breeding elite cultivars with *Verticillium* resistance, for example by pyramiding mechanisms currently found in separate germ lines. Our results suggest the existence of at least two such disease resistance mechanisms. Thirdly, we are collecting a range of potential defence response genes whose activity could be manipulated by transgenic technologies to increase resistance to *Verticillium* wilt in the cotton plant. Improving resistance mechanisms which occur naturally in the cotton plant in this way may have a higher chance of success than the introduction of novel foreign genes, and would result in transgenic plants which may be less controversial when released in the field. Finally, cotton plants that are more resistant to Verticillium wilt are also reported to be more resistant to Fusarium wilt, so the development of new cotton cultivars with superior resistance to Verticillium wilt using the above procedures should have significant benefits for the control of fungal diseases of cotton in general.

4. Acknowledgments

We wish to thank Greg Constable and Peter Reid of the CSIRO Cotton Breeding Unit, Narrabri for providing the twenty-five lines of seed representing current Australian cotton germplasm. This research was funded by the Cooperative Research Centre for Sustainable Cotton Production, including postgraduate scholarships to Melissa Hill and Alison Cook, and by the Cotton Research and Development Corporation.

5. References

Hill M.K. (1998). Gene expression in the cotton plant (*Gossypium hirsutum* L.) following infection with the fungal wilt pathogen *Verticillium dahliae*. Ph.D Thesis, University of Sydney.

Hill M.K. and Lyon B.R. Identification of disease response genes expressed in *Gossypium hirsutum* following infection with the wilt pathogen *Verticillium dahliae*. Plant Molecular Biology (Submitted for publication).

Ramsay J.R., Multani D.S. and Lyon B.R. (1996) RAPD-PCR identification of *Verticillium dahliae* isolates with differential pathogenicity on cotton. Australian Journal of Agricultural Research 47: 681-693.