Genetic Approach to Characterise Fusarium Wilt Resistance in Australian Native and Cultivated Cotton

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Summary

Fusarium oxysporum f. sp. *vasinfectum* (*Fov*) is an economically significant pathogen of cotton in Australia. Although the levels of resistance present in the new commercial cultivars have significantly improved, cotton breeders continue to look for additional sources of resistance. The native Australian *Gossypium* species represent an alternative source of resistance because they could have co-evolved with the indigenous *Fov* pathogens. However, they belong to the tertiary germplasm pool, which is the most difficult group of species from which to introgress genes into cultivated cottons. Interspecific triploid hybrids can be generated but they are sterile. The sterility barrier can be overcome using synthetic polyploids as introgression bridges, but it now clear that there is insufficient homoeologous chromosome interaction at meiosis to make these good breeding lines. A careful analysis of these lines, however, provides, an opportunity to begin to understand the genetics of fusarium wilt resistance in cotton. *Fov* disease bioassays on *G. hirsutum* X *G. sturtianum* BC_3 multiple alien chromosome addition lines revealed that two *G. sturtianum* linkage groups were associated with improved *Fov* resistance, while two *G. sturtianum* linkage groups were associated with increased *Fov* susceptibility. This result suggests many genes are interacting in the cotton plant to determine the level of fusarium wilt resistance. To complete our understanding of the complex inheritance of resistance to the fusarium wilt disease in cotton, quantitative trait loci analyses of the segregation of fusarium wilt resistance in an elite cotton family have been undertaken. We aim to map quantitative disease resistance loci, which act additively to confer disease resistance against Fusarium wilt. By identifying QTLs for fusarium wilt resistance, we intend to identify the genes involved and mark them with molecular marker surrogates that can be used in marker assisted breeding projects to develop a new generation of better fusarium wilt resistant cultivars.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*), is a serious disease of cotton in Australia responsible for significant yield reductions (Reid et al., 2002). Improved farm management strategies can reduce yield losses, but developing resistant cultivars is by far the most effective long-term means of combating fungal diseases of agricultural plants (Keller et al., 2000). Australian cotton breeders have significantly improved the fusarium wilt resistance of their cultivars, and a new cultivar Sicot-F1 with greater resistance was released in 2004. Despite robust improvements in the fusarium wilt resistance of Australian cotton cultivars, cotton breeders continue to look for new sources of resistance. The distant Australian cotton relatives of cultivated cotton may be a valuable pool of germplasm that can be used as a source of genetic resistance to fusarium wilt (McFadden et al., 2004). However, introgressing gene(s) from diploid *Gossypium* species is not straightforward. Differences in

chromosome number and structure restrict homoeologous chromosome recombination, limiting introgression across genomes.

Transfer of alien genes from *Gossypium* species other than the A- or D-genomes involves the production of hybrids through 'tetraploid' and/or 'hexaploid' bridging approaches followed by successive backcrossing (Brubaker et al., 1999; Stewart, 1995). The flexibility of the hexaploid bridging approach has made possible the construction of a number of multiple alien chromosome additions lines (MACALs) dissecting the C-, G- and K-genomes. This provides a powerful tool for genetic analysis and evolutionary studies. Recently, McFadden et al. (2004) studying the levels of fusarium wilt resistance both in cultivated and wild Australian *Gossypium* accessions, observed that one Australian cotton relative, *G. sturtianum* (Gos-5275; C-genome) has shown improved levels of fusarium wilt resistance when hybridized with cultivated cotton (*G. hirsutum* L.) in a synthetic hexaploid (Gos-5271). The hexaploid was significantly more resistant than the commercial cultivars DeltaEMERALD and Sicot 189 and its *G. hirsutum* parent (CPI-138969).

Relatively little is known about the genetic basis of this fusarium wilt resistance or the mechanism of action of the genes controlling it. However, the bulk of the evidence, most notably, the lack of clear discrimination between resistant and susceptible cultivars and the incremental increases in resistance breeders are achieving through selection, suggests that fusarium wilt resistance in cotton is controlled by multiple genes. Here, the effect of *G. sturtianum* chromosomes on fusarium wilt resistance in cultivated cotton was ascertained to develop an understanding of the minimum number of genes involved. This work was designed as a prelude to more extensive genetic analyses of fusarium wilt resistance in cotton pre-breeding lines.

Materials and Methods

Alien Chromosome Addition Lines

A *Gossypium hirsutum* X *G. sturtianum* hexaploid bridging family was derived by crossing a 2X[*G. hirsutum* X *G. sturtianum*] (AADDCC; Gos-5271)) synthetic hexaploid to *G. hirsutum* to generate a AADDC pentaploid that was subsequently backcrossed to various *G. hirsutum* lines (Paymaster784glandless; SiokraV-15; Gregg25Vglandless; SicalaV-2; experimental line 7-92; Zaire landrace G107) to generate BC_1, BC_2 and BC_3 multiple alien chromosome addition lines (MACALs; see Brubaker et al., 1999b for complete pedigrees). Backcrossing the [(*G. hirsutum* X *G. sturtianum*) X *G. hirsutum*] pentaploids to *G. hirsutum* generates aneuploid plants containing the complete *G. hirsutum* chromosome complement accompanied by subsets of the C-genome chromosomes. Total genomic DNA was extracted from 11 $4n+C_x$ BC_1, 100 $4n+C_x$ BC_2 and 46 $4n+C_{x=1-4}$ BC_3 individuals and the parental lines following Brubaker and Brown (2003). The BC_3 MACAL families were challenged with *Fov* in a series of three trails performed in 2001, 2002 and 2003 at CSIRO Plant Industry Black Mountain Laboratories (Canberra-ACT). Accession CPI-138969 was included in every experiment as a susceptible control.

Molecular analysis

Amplified fragment length polymorphism (AFLP) species-specific markers were examined among *Fov*-tested BC_1, BC_2 and BC_3 MACALs following Brubaker and Brown (2003). Total genomic DNA was extracted from leaves using the DNeasy® Plant Kit (QIAGEN) as indicated by the manufacturer.

Results

Segregation of *G. sturtianum*-specific AFLPs in *G. hirsutum* X *G. sturtianum* BC_1 MACALs

Of the 844 *G. sturtianum*-specific AFLPs inherited by the *G. hirsutum* X *G. sturtianum* hexaploid, 22 (2.6%) were not transmitted to the [*G. hirsutum* X *G. sturtianum*] X *G. hirsutum* pentaploid. Of the 822 AFLPs inherited by the pentaploid, 717 segregated among the 11 BC_1 MACALs and could be assigned to 1 of 16 linkage groups, designated Sturt-B to Sturt-N (Table 1). A 17th linkage group, Sturt-A, was inferred by a block of 79 AFLP markers present in the *G. hirsutum* X *G. sturtianum* hexaploid and the [*G. hirsutum* X *G. sturtianum*] X *G. hirsutum* pentaploid but missing from the BC_1 MACALs. That Sturt-A was real linkage group was confirmed in an independent intraspecific *G. sturtianum* F_2 family (discussed below). Of the 796 interpretable AFLPs, 572 (70%) were produced by *Eco*RI/*Mse*I primers and 224 (27%) by *Pst*I/*Mse*I primers. Sixty-two AFLPs (7%) had confounded patterns suggesting the two fragments were comigrating in the gels.

Table 1. Frequency and occurrence of co-segregating suites of *G. sturtianum* -AFLP alleles among 11 *G. hirsutum* X *G. sturtianum* BC_1 MACALs. Boxes denote LGs that have lost AFLP markers during transmission.

	BC_1 MACALs												No. of *G. sturtianum* -specific		
Linkage groups	645	651	685	686	687	710	711	712	734	735	736	No. of BC_1 MACALs (%)	EcoRI MseI	PstI MseI	Total (%)
Sturt-A												0	48	31	79 (9.9)
Sturt-*B*					*X*							*1 (9)*	*3*	*7*	*10 (1.3)*
Sturt-C					X		X	X		X	X	5 (45)	28	13	41 (5.2)
Sturt-D				X								1 (9)	27	15	42 (5.3)
Sturt-E			X				X		X	X		4 (36)	51	14	65 (8.2)
Sturt-F			X	X				X				3 (27)	33	8	41 (5.2)
Sturt-G		X			X	X	X	X		X	X	7 (64)	35	19	54 (6.8)
Sturt-H		X	X	X		X				X	X	6 (55)	55	17	72 (9.0)
Sturt-I	X			X	X	X	X			X	X	7 (64)	53	16	69 (8.7)
Sturt-JQ	X	X	X	X	X	X	X	X		X	X	10 (91)	57	27	84 (10.6)
Sturt-KP	X	X	X	X	X				X			6 (55)	55	15	70 (8.8)
Sturt-LM	X	X	X	X			X					5 (45)	36	16	52 (6.5)
Sturt-N	X	X	X	X		X	X	X	X	X	X	10 (91)	44	19	63 (7.9)
Sturt-O	X	X	X	X	X			X	X			7 (64)	47	7	54(6.8)
No. of linkage groups	6	7	8	9	61	5	7	6	4	7	6	71†	572	224	796

†This total does not include LG-B. LG-B was not confirmed as representing a real LG.

Segregation of *G. sturtianum*-specific AFLPs in *G. hirsutum* X *G. sturtianum* BC_2 MACALs

One hundred BC_2 individuals from 10 MACAL families were genotyped using all but 37 of the markers used to genotype the BC_1 (Table 2). All the viable seed from each family were planted; Hyb-651 produced no BC_2 progeny. Of the 548 *G. sturtianum* linkage groups available, 177 (32%) were transmitted to the BC_2 MACALs (Table 2). All the linkage groups present in the BC_1 MACALs were transmitted except for Sturt-D and two of the three altered linkage groups (Sturt-KP_{new} and -LM_{new}). Transmission frequencies were more variable and almost always lower than observed in the BC_1 MACALs. The number of linkage groups per BC_2 MACAL ranged from one to seven ($\overline{X}$ =1.8). Of the 13 linkage groups detected in the BC_1, two (Sturt-B and Sturt-F) were transmitted without further loss of alleles but 32 previously intact linkage groups lost alleles as they were transmitted to the BC_2 MACALs (Table 2).

Table 2: Frequency of *G. sturtianum* LG transmission from ten *G. hirsutum* X *G. sturtianum* BC_1 to the BC_2 MACALs. The numbers of LGs that have lost markers during the transmission are indicated parenthetically. Empty cells indicate the BC_1 parent of the BC_2 family did not contain the LG.

	G. hirsutum X *G. sturtianum* BC_2 MACALs (N)										
Linkage groups	Hyb-645 (3)	Hyb-685 (1)	Hyb-686 (1)	Hyb-687 (9)	Hyb-710 (51)	Hyb-711 (2)	Hyb-712 (10)	Hyb-734 (12)	Hyb-735 (7)	Hyb-736 (4)	No. of BC_1 MACALs (%)
Sturt-A											0
Sturt -B				2							2 (22)
Sturt -C				1		0	2(1)		1	0	4 (13)
Sturt -D			0								0
Sturt -E		1				0		3(1)	1(1)		5 (23)
Sturt -F		1	1				3				5 (42)
Sturt -G				4	25(7)	0	6(1)		1	0	36 (43)
Sturt -H		1	0		18(4)				3(2)	3	25 (39)
Sturt -I	0		0	2	21(4)	0			1	3	27 (35)
Sturt -JQ	1		0	3	17(4)		1		0	1	23 (27)
Sturt -JQ_{new}*		1			17(1)	2(2)					20 (37)
Sturt -KP	0	1		4(1)				9			14 (56)
Sturt -KP_{new}*			0								0
Sturt -LM	0	1				2					3 (50)
Sturt -LM_{new}*			0								0
Sturt -N	0	0	0		9	2(1)	2	3	1	1	18 (20)
Sturt -O	1(1)	1	0	3			4	3			12 (33)
No. of linkage groups	2	7	1	19	90	6	18	18	8	8	177

*These linkage groups represent modified versions of the original linkage group.

C-genome chromosomes and *Fov* disease resistance in the BC_3 MACALs

A binary logistic regression based on a model of *Fov* disease resistance and susceptibility using C-genome-specific chromosome inheritance as predictor variables was used. This model correctly

classified 72% of the 168 lines by giving the probabilities of contribution of the C-genome LGs to resistant-and susceptible-fusarium wilt resistance. For the resistant-fusarium wilt resistance, the logistic regression analysis indicated that the probability of the Wald statistics was marginally significant (p= 0.081) for LG-G, indicating that its contribution to the model is not conclusive and has to be taken with caution; the negative value of B for presence vs. absence of LG-G indicates that more LG-G linkage groups were present in the resistant BC_3s after controlling for the other predictors. The value of the 'odd ratios' (Exp B) was 0.24, which implies that susceptible BC_3 individuals were 76% less likely to carry the LG-G. Also, the logistic regression indicated that LG-K&P contributed significantly to the model (p=0.05); the negative value of B suggested that LG-K&P tend to be 'present' more often than 'absent' in the resistant BC_3s, after controlling for the other predictors. The value of the 'odd ratios' was 0.11, which implies that susceptible BC_3 individuals were 89% less likely to carry the LG-K&P.

For fusarium wilt susceptibility, the logistic regression analysis indicated that the probability of the Wald statistics for LG-H and -I contributed significantly to the model (p=0.002 and p=0.034, respectively); the positive value of B suggested that LG-H and -I tend to be 'present' more often than 'absent' in the susceptible BC_3s, after controlling for the other predictors. The values of the 'odd ratios' for LG-H and –I were 7.73 and 2.81, respectively. These results indicated that resistant BC_3 individuals were approximately three- to eight-fold less likely to carry the LG-I and -H, respectively.

Overall, binomial logistic regression analyses identified four putative C-genome LGs associated with significant differences in fusarium wilt responses in the BC_3 MACALs. Linkage groups 'G' and 'K&P' were significantly associated with *Fov* resistance, while linkage groups 'H' and 'I' were associated with *Fov* disease susceptibility.

Discussion

The results of the logistic regression analysis suggested that the improved fusarium wilt resistance observed in the BC_3 MACALs is most likely to be associated to the transmission of linkage groups 'G' and 'K&P'. In other words, BC_3 MACALs that were designated as fusarium wilt susceptible had a relatively lower probability of carrying linkage groups G and K&P. In contrast, those BC_3 MACALs identified with susceptible phenotypes had a higher probability of carrying linkage groups 'H' and 'I'. Thus, this method identifies four *G. sturtianum* linkage groups ('G', 'H', 'I', 'K&P') as being associated with fusarium wilt resistance. Furthermore, the identification of four C-genome chromosomal LGs indicates that more than a single gene is affecting fusarium wilt resistance trait suggesting a multilocus control of fusarium wilt resistance in the BC_3 MACALs.

This study has not only confirmed that *G. sturtianum* is a source of resistance to fusarium wilt, but that this resistance is controlled by multilocus interactions. Many of the useful alien genes, contained on the chromosomes affecting fusarium wilt resistance are probably quite different from these of the cultivated species and are therefore potentially useful for providing novel and affective sources of resistance to this economically significant disease. However it is clear that it will not be possible to transfer these genes to cultivated cottons by traditional breeding means, and that we need to adopt more technically complex methods. The next step in this process is to undertake a quantitative trait loci (QTL) analysis using an experimental breeding population derived from a cross between

susceptible and resistant parents. This will identify specific genomic regions in the cotton genome were import genes for fusarium wilt resistance are located and mark their location with molecular markers. With an understanding of the number of genes involved in fusarium wilt resistance, how they interact and where there are located in the genome, cotton breeders can maximise the genetic potential of their breeding lines, insuring that the resistance gains made to date will continue.

Future Research: Developing of 20 cM cotton framework map for QTL mapping of Fov resistance

To undertake a QTL analysis of fusarium wilt resistance, one must develop a suitable experimental genetic family and develop molecular markers. The experimental cotton mapping population that has been developed is based on a pre-breeding line that combines a fusarium wilt resistant parent with good lint characteristics with a fusarium wilt susceptible parent with good yield and plant architecture characteristics. Through multiple years of single seed descent, sub-lines (also called recombinant inbred lines or RILs) are being developed so that this important family of experiment families can be immortalized.

This population is being used to develop a 20 cM framework map of cotton combining a variety of molecular markers, such as simple sequence repeats (SSRs) or sequence tagged sites (STS). This 20 cM framework map is a genetic map of the cotton genome. When an association between a molecular marker and a specific trait is identified (by QTL mapping, see below), the location of the molecular marker on the map indicates where the gene controlling that trait is located.

For the QTL, or quantitative trait loci, analysis of fusarium wilt resistance, representatives of each experimental line will be infected with *Fov* under experimental conditions (fusarium field nurseries or Biosafe facilities). When the subsequent level of disease in each line is compared with its molecular marker fingerprint (using the 20 cM framework markers), markers that are always associated with high levels of resistance or high levels of susceptibility can be identified. The end result will be the identification of the minimum number of genomic regions with genes affecting fusarium wilt resistance, where those genes are located, and how they are expressed and interact.

Once this analysis is complete, the breeders will be able to use the molecular markers to track the inheritance of critical genes more easily and effectively. One of the big advantages is that lines can be screened for optimum fusarium wilt resistance genotypes without exposing them to the disease. At the same time, supplied with the knowledge of the minimum number of genes controlling fusarium wilt resistance and where the can found in the genome, molecular biologists can search for the actual genes involved, and once they have been identified, search for more effective versions in other germplasm, including the wild Australian cotton species.

Progress to date

Rong *et al.* (2004) have genetically anchored a set of STSs composed of 3347 loci (2584 on the tetraploid map and 763 on the diploid map), which provides transferable genetic markers suitable to construct a framework cotton genetic map for studying the inheritance of Fusarium wilt resistance in cultivated cottons. Of the 3347 loci mapped by Rong and co-workers (2004), 457 loci have been

identified as good candidate markers (SSRs and RFLPs) to construct a framework map that equates to an average interval of 20 cM between loci for a *Fov*-QTL mapping strategy in cultivated cottons. Of the 457 loci selected, 371 are STSs resolved by hybridization (RFLPs) and 86 are PCR-based markers (SSRs). RFLP markers are very difficult to integrate into a breeding program for reasons associated with high cost and low efficiency when compare with the PCR-based microsatellites (SSRs). Converting mapped RFLPs loci to a PCR-based assay is an attractive proposition, because it would allow us to translate these cotton mapped markers into a format applicable to a practical breeding approach.

Of the 371 RFLP markers, we have currently tested 245 cotton-STS specific PCR-based markers against the parentals of an experimental family segregating of fusarium wilt resistance (Family '61003'). A total of 239 co-dominant PCR markers had a positive PCR amplification on the parental lines. The results of this screening revealed that of the 239 markers 68 (28%) were polymorphic indicating that more markers will be required to develop the 20 cM cotton framework map. As an adjunct to the Rong & Paterson AD genetic linkage map, a second published cotton map has been investigated. Nguyen *et al.* (2004) developed a cotton genetic map composed of approximately 1200 loci. The genetic linkage information contained in the Nguyen's cotton map will assist in increasing the coverage of our proposed 20cM cotton framework map. Approximately, 330 cotton SSRs have been selected and screened from the Nguyen's map. Of the 330 SSRs, approximately 230 (70%) were polymorphic between the fusarium wilt resistant and susceptible parents. Currently, of 384 PCR-based loci are available for mapping in our fusarium wilt resistance segregating family, 126 have been genotyped and analysed for mapping.

Fov disease screening the '61003' experimental family required an optimization of the bioassay protocol. A series of three glasshouse trials aiming to measure *Fov* disease responses in the fusarium segregating family were conducted between October 2005 and May 2006. A total of 8,100 61003 F_3 individuals has been tested for *Fov* disease responses in the glasshouse. All the data for this bioassay has been collected and collated for final analysis. Similarly, for *Fov* testing under field conditions, a total of 23,000 61003 F_4 individuals were arrange in an unbalanced plot design at the *Fov* Norwin-Fields in Queensland. All data from this bioassay has been collected and collated for final analysis. The results from the F_3 and F_4 progeny will be compared separately against the 20 cM cotton framework map, so markers that are always associated with high levels of resistance or high levels of susceptibility can be identified. Ultimately, we intend to identify the minimum number of genomic regions with genes affecting fusarium wilt resistance, and where those genes are located. The identification of the genomic region containing the genes involved in Fusarium wilt resistance will allow us to identify also markers that would act as surrogates for the selection of resistant genotypes in the absence of *Fov*.

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