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ORIGINAL PAPER

The effect of *Gossypium* C-genome chromosomes on resistance to fusarium wilt in allotetraploid cotton

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Abstract *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*) has the potential to become the most economically significant pathogen of cotton in Australia. Although the levels of resistance present in the new commercial cultivars have improved significantly, they are still not immune and 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. Forty-six BC_3 *G. hirsutum* × G. *sturtianum* multiple alien-chromosome-addition-line (MACAL) families were challenged with a field-derived *Fov* isolate (VCG-01111). The *G. hirsutum* parent of the hexaploid MACAL is highly susceptible to fusarium wilt; the *G. sturtianum* parent is strongly resistant. Twenty-two of the BC_3 families showed enhanced fusarium wilt resistance relative to the susceptible *G. hirsutum* parent. Logistic regression identified four *G. sturtianum* linkage groups with a significant effect on fusarium wilt resistance: two linkage groups were associated with improved fusarium wilt resistance, while two linkage groups were associated with increased fusarium wilt susceptibility.

Introduction

Fusarium wilt of cotton in Australia is caused by *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*). Fusarium wilt was first recorded in 1993 in the Cecil Plains/Brookstead region of Queensland (Kochman 1995). Since then, the incidence of fusarium wilt in Australian cotton crops has increased drastically, spreading rapidly to most cotton growing regions in New South Wales and Queensland (Reid et al. 2002). Despite robust improvements in the fusarium wilt resistance (FWR) of Australian cotton cultivars, cotton breeders continue to look for new sources of resistance. Genetic variability for resistance to fusarium wilt seems to be limited in the cultivated cotton germplasm, and consequently cotton breeders are looking beyond the cultivated gene pool. Moreover, little is known about the genetics of resistance to fusarium wilt in cotton, particularly in Australia (Wang and Roberts 2006).

Most genetic studies of FWR in cotton have been conducted outside Australia (Famhy 1927; Smith and Dick 1960; Mohamed 1963; Kappelman 1979, 1980; Netzer 1982; Hillocks 1984; Megahed et al. 1984), so the applicability of these data in the Australian pathosystem is questionable. Firstly, root knot nematodes are frequently associated with fusarium wilt infection outside Australia and it is likely that some genetic studies from these areas may confound resistance to fusarium wilt with resistance to the nematodes (Smith and Dick 1960). Nematodes are not involved with fusarium wilt of cotton in Australia and this difference may well explain why overseas cultivars reported to be fusarium wilt resistant fail to perform well in Australia. Secondly, the *Fov* that causes fusarium wilt in Australia is genetically distinct from all overseas races of *Fov* (Davis et al. 1996; Kim et al. 2005). Genetic and evolutionary studies conducted by Wang et al. (2004) established that the Australian *Fov* is closely related to a lineage of indigenous *Fusarium oxysporum* found in several areas of Australia, including the Darling Downs. Thus, the mechanisms of pathogenicity of Australian *Fov* and the genetics

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of FWR against the Australian *Fov* could differ significantly from that of overseas *Fov*.

The genus *Gossypium*, to which the cultivated tetraploid cottons, *G. hirsutum* L. and *G. barbadense* L. belong, contains 44 diploid and 5 disomic tetraploid species (Fryxell 1979, 1992; Fryxell et al. 1992; Percival et al. 1999). Cytogenetically, they have been subdivided into eight diploid genomes, designated A to G and K (Beasley 1940; Edwards and Mirza 1979; Endrizzi et al. 1985; Phillips and Strickland 1966; Stewart 1995), and one tetraploid genome, designated AD (Endrizzi et al. 1985). Seventeen of the 44 diploid species in this genus, the C (2), G (3) and K (12) genomes, are indigenous to the Australian continent. These 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). In addition, because they have a simpler genome than the cultivated cotton, they could also serve as useful genetic models for elucidating the genetics of FWR. Recently, McFadden et al. (2004), studying the levels of FWR both in cultivated and wild Australian *Gossypium* accessions, observed that one Australian cotton relative, *G. sturtianum* J.H.Willis (Gos-5275; C-genome) has shown high levels of FWR relative to three cultivated cottons (*G. hirsutum* cv. Sicot189, DeltaEMERALD and Siokra 1–4) and that this FWR is expressed in a synthetic hexaploid (Gos-5271) that combines *G. sturtianum* (Gos-5275) and *G. hirsutum*. The Gos-5271 hexaploid was significantly more resistant than the commercial cultivars DeltaEMERALD and Sicot 189 and its *G. hirsutum* parent (CPI-138969). Given that CPI-138969 and Siokra 1–4 were clearly identified as susceptible, it suggests that the C-genome gene(s), when present in the *G. hirsutum* background, can have a positive cumulative effect towards FWR.

Transfer of alien genes from *Gossypium* species other than the A- or D-genomes involves the production of hybrids through "tetraploid" (trispecific) and/or "hexaploid" (bispecific) bridging populations followed by successive backcrossing (Brubaker et al. 1999; Stewart 1995). The tetraploid bridging pathway should maximise recombination because the A and D chromosomes have no autosyndetic partners and theoretically should pair with the chromosome of the wild Australian species of the C-, G- and K-genomes (Vroh Bi et al. 1999; Ahoton et al. 2003; Brubaker and Brown 2003). However, extremely low levels of fertility limit the recovery of recombinant individuals in early generations and therefore restrict their use in genetic studies (e.g., Fusarium wilt resistance) where availability to a large number of progeny is required. On the other hand, the hexaploid bridging pathway offers the possibility of generating large number of progeny because successive generations of aneuploids are increasingly fertile. Although, autosyndesis in hexaploid bridging populations reduces the level of homoeologous chromosome recombination, it is hoped the larger number of individuals recovered offset this limitation (Brubaker et al. 1999).

Becerra Lopez-Lavalle and Brubaker (2007) assessed the extent of homoeologous recombination in *G. hirsutum* × *G. sturtianum* and *G. hirsutum* × *G. australe* alien chromosome derivatives using species-specific molecular markers. Their results documented the loss of large cohorts of markers from *G. sturtianum* and *G. australe* specific linkage groups, suggesting that introgression had occurred. However, the expectation was that once introgression had occurred the markers would then segregate and this was not observed. Even if one assumes that there will be significant recombination blocks between the *G. hirsutum* and *G. australe* or *G. sturtianum* chromatin, some subsequent breakdown of the linkage block was expected. In contrast, the observed patterns were the loss of blocks of markers followed by the stable transmission of the modified *G. sturtianum* and *G. australe* linkage group. It is quite clear from Becerra Lopez-Lavalle and Brubaker's results that while the hexaploid bridging pathway may maximize the recovery of fertile progeny it may not increase the likelihood of recovering recombinant individuals. Despite this limitation, the hexaploid bridging approach has made possible the construction of a number of multiple alien chromosome additions lines (MACALs) that partition the C-, G- and K-genomes. This provides the opportunity to examine the genetic contribution of individual *G. australe* and *G. sturtianum* chromosomes in genetic analyses and evolutionary studies (Ahoton et al. 2003; Brown and Brubaker 2003).

These MACALs can be characterized using molecular markers, such as amplified fragment length polymorphisms (AFLPs). Brubaker and Brown (2003) successfully developed and used a suite of *G. australe* chromosome-specific AFLP markers to determine the frequency at which *Gossypium* G-genome chromosomes were transmitted and whether they had been transmitted intact to the *G. hirsutum* × *G. australe* MACALs. In addition, the distribution of these G-genome specific markers among 18 *G. hirsutum* × *G. australe* MACALs aided in the identification of incorrectly mapped AFLPs onto a *G. australe* × *G. nelsonii* F_2 map by independently confirming linkage and establishing linkage relationships where intervening loci were absent. Similarly, a suite of co-segregating *G. sturtianum* chromosome-specific AFLP markers has also been developed and used to characterize the transmission of C-genome chromosome among 11 *G. hirsutum* × *G. sturtianum* MACALs. This aided in the generation of linkage assemblages in a *G. sturtianum* F_2 genetic map (Becerra Lopez-Lavalle and Brubaker 2007).

Although the primary purpose for developing a suite of co-segregating markers for *G. australe* and *G. sturtianum* was to track the frequency and fidelity of alien chromosome

transmission in hexaploid bridging families, the assemblage of alien-genome specific markers into linkage groups has made it possible to identify chromosomes carrying genes for traits of interest. For instance, among 18 *G. hirsutum* × *G. australe* MACALs all BC_1 individuals carrying linkage group Aust-A had brown lint, whereas those that retained the pink (mauve) flower colour of *G. australe* were the individuals that retained linkage group Aust-F (Becerra Lopez-Lavalle and Brubaker 2007). Similar findings have been confirmed in *Raphanus sativus* × *Brassica rapa* alien chromosome derivatives, where morphological traits for each alien chromosome addition type were characterized in the BC_2 individuals, and their inheritance tracked for three successive generations (Kaneko et al. 2003). In this study we set out to elucidate the genetics of FWR in *G. hirsutum* × *G. sturtianum* alien chromosome derivatives. We therefore, assessed 46 *G. hirsutum* × *G. sturtianum* BC_3 MACALs [$ADC_{x\,(0–13)}$] to determine if FWR could be correlated with the retention of specific *G. sturtianum* chromosome or chromosome segments in the *G. hirsutum* background.

Materials and methods

Plant materials

Plants were selected from the BC_3 generation derived from nine BC_1 C-genome MACALs, by successive backcrossing with the *G. hirsutum* (Sicala V-2) (Fig. 1). In total 46 BC_3 MACAL families carrying 0–4 C-genome chromosome additions were challenged with *Fov* in a series of three trials performed in 2001, 2002 and 2003 at CSIRO Plant Industry Black Mountain Laboratories (Canberra-ACT) (Fig. 1). Accession CPI-138969 was included in every experiment as a susceptible control. For this study, seeds from all accessions were sown directly into seedling trays containing stem-sterilised compost: perlite (70:30) mixture amended with 1 g osmocote®/L.

Inoculation method

Fov isolate 24595, obtained from the Cecil Plains area of the Darling Downs of Queensland-Australia, was used to challenge the cotton seedlings (provided by N. Moore; Indooroopilly, Queensland Department of Primary Industries). The fungus was maintained on sterile filter paper at 4°C (Correll et al. 1986) and recovered on 25% (w/v) potato dextrose agar (Difco; PDA) plates at 25°C for 4–7 days before the preparation of inocula.

Fov conidia, for inocula preparation, were obtained by flooding fungal colonies grown on 25% PDA with sterile water and rubbing with a glassrod. Approximately 0.5 ml of this conidial suspension was used to inoculate 500 mL of potato dextrose broth (Difco; PDB) in 2L Erlenmeyer flasks that were incubated at 25°C on an orbital shaker at 100 rpm for 3–7 days. The PDB cultures were filtered through three layers of Kleenex tissue to remove hyphal fragments and the conidial concentration was determined using a haemocytometer. Conidial concentration was adjusted to 10^7 conidia mL^{-1} with sterile water. The inocula were maintained at room temperature (15–25°C) and used within 4 h of preparation.

For inoculation, 2-week-old MACAL and *G. hirsutum* seedlings were carefully removed from the soil; the roots were washed and then dipped into the inoculum (20 plants/200 mL inoculum) for 20 min before being re-potted singly in fresh potting mixture in 10 cm plastic pots (Wang et al. 1999; McFadden et al. 2004).

Seedlings were arranged in a randomized complete block design in a bio-safe glasshouse (temperature range 18–27°C) across three trials (Fig. 1). *Fov* disease symptoms were assessed 23–42 days after inoculation.

Fov disease assessment

Disease assessments for all plants in each trial were conducted at a time (23–42 days after inoculation) determined by the progression of disease symptoms in each trial. Fusarium wilt disease symptoms were visually assessed using a categorical scoring system (vascular browning index = VBI) inferred by the extent of the vascular browning, where: 0 = no vascular discoloration, 1 = discoloration restricted to base of stem only, 2 = discoloration of the hypocotyl, 3 = discoloration of the epicotyl, 4 = complete vascular discoloration of stem and 5 = plant dead. The mean VBI score of CPI-138969 (susceptible control) was used to assess the level of *Fov* disease response in *G. hirsutum* for comparison with accessions carrying C-genome chromosomes. Plants with VBI scores of "0" or "1" were were designated "resistant", while those with VBI scores of "4" or "5" were designated "susceptible". Asymptomatic plants (VBI = 0) may also represent escapes from infection owing to an unequal penetration of the *Fov* conidia into the wounded roots, but escapes should occur in all tested lines, the resistant parent, susceptible parent, and the MACAL BC_3 progeny, with equal frequency. However, the inoculation conditions used in this study have been previously assayed and the percentage of escapes is much lower than for other methods, particularly when compared to field trials (Wang et al. 1999).

Molecular analysis

Six AFLP primer combinations, which comprised a suite of 172 *G. sturtianum* chromosome-specific molecular markers, were used to identify the *G. sturtianum* chromosomes present in the BC_3 individuals following Becerra Lopez-

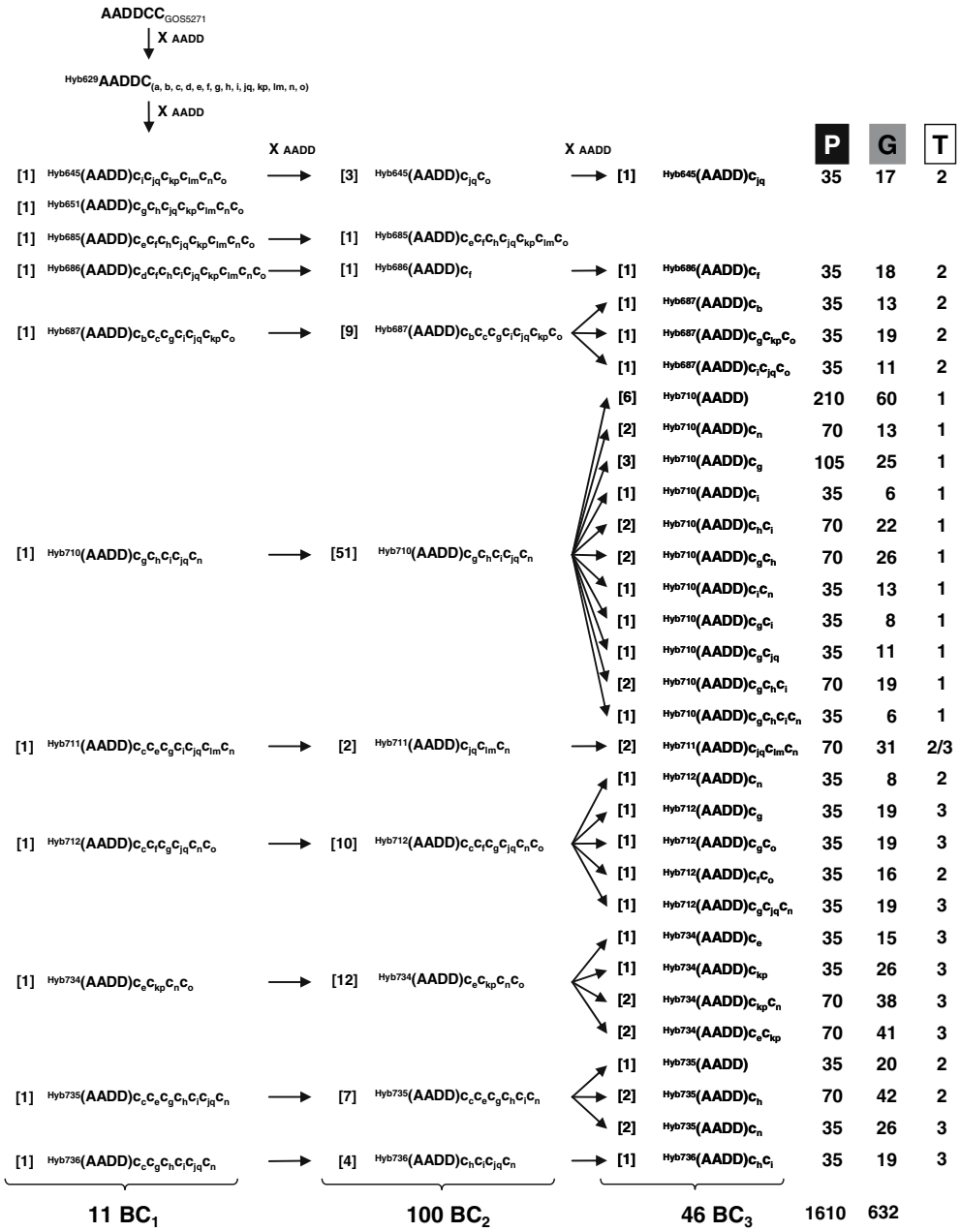

Fig. 1 Pedigree and inferred genomic constitution of 11 BC_1, 100 BC_2 and 46 BC_3 *G. hirsutum* X *G. sturtianum* aneuploids generated by crossing a synthetic *G. hirsutum* (genomic designation AADD; CPI 138969) × *G. sturtianum* (genomic designation CC; Gos-5275) allohexaploid (genomic designation AADDCC; Gos-5271) to *G. hirsutum* (CPI 138969) to generate an AADDC pentaploid F1 (Hyb-629) that contained a haploid complement of the *G. sturtianum* chromosomes. This pentaploid was subsequently backcrossed as female to *G. hirsutum* (Sicala V2; *Fov* susceptible) to generate the second and third generation 4N+ aneuploid plants that contain the full *G. hirsutum* tetraploid genome complement accompanied by subsets of *G. sturtianum* haploid genome. *Uppercase letters A, D* and *C* indicate haploid genomes; *lower case letters with subscripts* designate individual chromosomes. *Column P* indicates the number of BC_3 individuals phenotyped for vascular browning index (VBI); *column G* indicates the number of BC_3 individual genotyped; *column T* indicates the glasshouse trial in which they were tested

Lavalle et al. (2002) (Table 2). Leaf tissue was collected for all plants in each trial, immediately frozen in liquid nitrogen and stored at −80°C until DNA was extracted. Total genomic DNA was extracted using the DNeasy® Plant Kit (QIAGEN GmbH-Hilden-Germany) as indicated by the manufacturer. The AFLP method was performed as described by Vos et al. (1995), with minor modifications for cotton. The AFLP templates were prepared by digesting 1 μg of genomic DNA with the restriction enzymes *Eco*RI and *Mse*I for 2 h at 37°C. *Eco*RI and *Mse*I adapters were ligated to the restriction fragments. Ligation reactions were incubated at 37°C for 3 h and then held at 4°C overnight.

The pre-selective amplification (primers with one selective nucleotide) products were diluted 30-fold. The selective *Eco*RI and *Mse*I amplification primers carried three selective nucleotides; the *Eco*RI primer was end-labelled with γ-[^{33}P]-dATP. Selective amplification products were loaded on 6% denaturing polyacrylamide gels run at 50 watts for 2.5 h using 1× Tris-Taurine-EDTA (TTE) buffer (10.8 g Trizma base; 3.6 g Taurine; 0.2 g $Na_2EDTA{\cdot}2H_2O$). The polyacrylamide gels were fixed in 10% glacial acetic acid (v/v)–20% methanol (v/v) for 30 min, rinsed twice with ddH_2O, dried at 65°C for 4 h to overnight, and exposed to BiomaxMR® film (Eastman Kodak Co., Rochester, NY) for 1–3 days.

Data analyses

Overall statistical differences in resistant (VBI = 0 or 1) intermediate (VBI = 2 or 3) and susceptible (VBI = 4 or 5) fusarium wilt symptoms between BC_3 MACALs and the control (CPI 138969) were ascertained using a standard chi-square test.

The VBI scores were also analysed by univariate analysis of variance under a generalised linear model (GLM) for each separate trial. Significant C-genome chromosome effects were ascertained for all data sets. These C-genome chromosome effect differences were then separated by comparing the means of the BC_3 MACAL families for significant differences using the Fisher's least significant difference (LSD) test ($P \leq 0.05$). The association between FWR and the presence of C-genome chromosomes in the *G. hirsutum* background was evaluated using binomial logistic regression. The binomial logistic regression was used to model the relationship between resistant and susceptible fusarium wilt symptoms ["0" = resistant (VBI 0&1), "1" = susceptible (VBI 4&5)] and a set of explanatory variables (e.g., the *G. sturtianum* chromosomes or part thereof present in each individual). Like any other linear regression, the goal of the logistic regression analysis was to find the best fitting and most parsimonious, yet biologically reasonable model of a given relationship (Hosmer and Lemeshow 2000). What distinguished the logistic regression model from the linear regression model was that the outcome variable in logistic regression was categorical and most usually binary, with an assumed binomial distribution of errors. All statistical computations were performed using SPSS V.10 (Chicago, USA).

Results

Glasshouse trials

Forty-six BC_3 *G. hirsutum* X *G. sturtianum* MACALs (1610 BC_3 individuals) were assayed for FWR in three consecutive trials (Fig. 1). Thirty-five individuals of the *G. hirsutum* parental line (CPI-138969) (with a FWR comparable to the *Fov* susceptible-industry-standard Siokra 1–4) was included as susceptible control in all three trials. Foliar wilt symptoms were visible by approximately 4 weeks after inoculation. The BC_3 MACALs and the *G. hirsutum* and *G. sturtianum* parental lines were susceptible to varying degrees. The mean VBI score for the *G. hirsutum* parental line ranged from 2.7 to 3.6 indicating a high *Fov* disease pressure in all three trials. The VBI frequencies were different between the parental control and the BC_3 MACALs (Fig. 2a). Overall, VBI scores for the MACALs were significantly lower than for CPI-138969 ($\chi^2 = 21.22$, $P = 0.001$) using data pooled from all MACALs in all three trials. The proportion of resistant plants (VBI scores of 0 or 1) was higher among BC_3 MACALs, with twice as many individuals scoring "0" and "1" relative to the proportion of

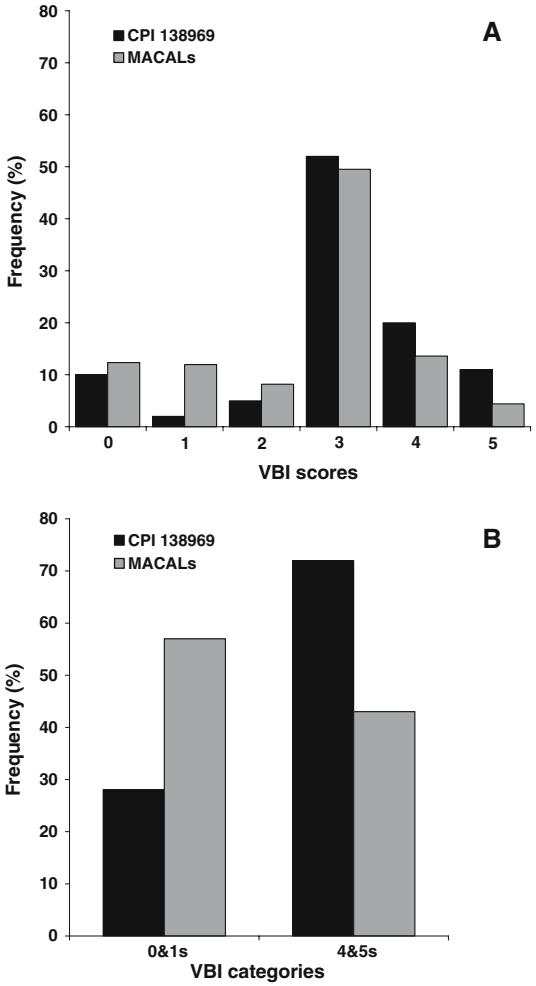

Fig. 2 Pooled frequency distributions of VBI scores of *G. hirsutum* (CPI 138969) and 46 BC_3 *G. sturtianum* × *G. hirsutum* chromosome addition lines (MACALs) (**a**). The proportion of plants displaying extreme *Fov* symptoms in VBI (0 and 1s, resistant) and (4 and 5s, susceptible) categories is given of *G. hirsutum* (CPI 138969) and 46 BC_3 *G. sturtianum* × *G. hirsutum* chromosome addition lines (MACALs) (**b**)

G. hirsutum plants distributed under the same categories. Similarly, the proportion of susceptible plants (VBI scores of 4 or 5) was nearly twofold lower among BC_3 MACALs relative to the distribution of *G. hirsutum* plants under the same categories (Fig. 2b).

Contrast analyses were undertaken to determine which BC_3 MACAL genotypes significantly contributed to the enhanced FWR observed (Fig. 3). In the first *Fov* disease trial, there was a significant increase in FWR in 17 of the 22 BC_3 MACALs assayed (mean VBI ranging from 2.4 to 3.1, $P \leq 0.05$). In the second *Fov* trial, the BC_3 MACAL Hyb-687-15 (mean VBI of 1.85, $P \leq 0.05$) ranked as significantly more resistant than the *G. hirsutum* parental cultivar. In the third *Fov* trial, four of the 13 BC_3 MACALs ranked as significantly more resistant than their *G. hirsutum* parent. In total, 22 of the 46 BC_3 MACALs, tested for fusarium wilt symptoms, had significantly higher FWR levels than the *G. hirsutum* parental control (Fig. 3, bars labelled as B, $P \leq 0.05$). The relatively high levels of FWR observed in these 22 *G. hirsutum* × *G. sturtianum* MACALs indicates that the *G. sturtianum* (chromosomes or part thereof) present in these lines contributed to enhanced FWR in the *G. hirsutum* background.

Identifying the *G. sturtianum* chromosomes present in the resistant and susceptible BC_3 MACALs

To assess the *G. sturtianum* linkage groups (Sturt-LGs) present in the resistant and susceptible BC_3 MACALs, 632 BC_3 aneuploid plants from 44 BC_2 MACALs carrying 1–4 *G. sturtianum* chromosomes were analysed using 172 diagnostic *G. sturtianum*-chromosome specific *Eco*RI/*Mse*I AFLPs (Table 1). The 632 plants were selected on the basis of their extreme responses to the *Fov* invasion of the plants' vascular tissues (VBI scores). Thus, the selection pressure was solely driven by the plant's response to the *Fov* attack, and conclusions regarding chromosome recovery from the BC_2 to the BC_3 are subject only to this bias. Of the 632 BC_3 plants assayed, 370 displayed VBI values of 0 or 1 (*Fov*-resistant pool), while 262 displayed VBI values of 4 or 5 (*Fov*-susceptible pool). Of the 172 diagnostic Sturt-LG-specific AFLP markers, 161 were used to characterise the *G. sturtianum* chromosomes present in the 632 BC_3 C-genome MACALs (Fig. 1).

The mean number of Sturt-LGs among the BC_3 progeny ranged from 0.2 for Hyb-710 to 1 for Hyb-711 among the BC_3 *Fov*-resistant pool and from 0 for Hyb-645 and -686 to 1.1 for Hyb-687 among the BC_3 *Fov*-susceptible pool. Overall, the Sturt-LG recovery was 0.5 for both BC_3 *Fov*-resistant and -susceptible pools. The proportion of individual BC_3 MACALs carrying Sturt-LGs was approximately one-third in both pools (35.7% in the resistant pool and 32.1% in the susceptible pool), indicating that the total number of Sturt-LGs recovered in both BC_3 group was comparable and independent from the selection of fusarium wilt-resistant and -susceptible pools (Table 2).

C-genome chromosomes and *Fov* disease resistance

Although the recovery rate of Sturt-LGs among the 632 BC_3 individuals was the same for *Fov*-resistant and -susceptible individuals the representation of individual Sturt-LGs in the two pools was not equal. Broadly, the distribution of Sturt-H suggested a strong association of this Sturt-LG with *Fov*-susceptibility in the *G. hirsutum* background, while LGs Sturt-F, -G, -J&Q, -K&P and –L&M indicated association with *Fov*-resistance. The recovery frequencies of Sturt-LGs Sturt-B, -C, -E, -I, -N and -O appeared equivalent in the two pools.

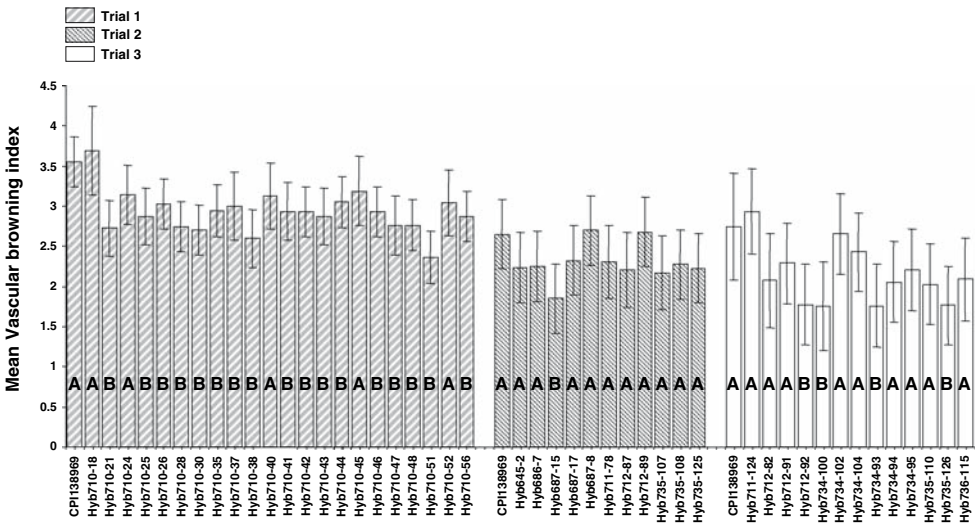

Fig. 3 Results of three *Fov* trials showing means of vascular browning index within 46 BC_3 C-genome alien addition lines and the *G. hirsutum* parent (CPI138969). *Vertical bars* show 95% confidence interval (CI) errors. *Different letters* indicate significant differences based on pair-wise multiple comparisons using the least significant difference test (LSD)

Table 1 *G. sturtianum*-specific AFLP loci used to track the transmission of C-genome chromosomes or part thereof (Sturt-LGs) in the BC_3 MACALs

AFLP-primer combination	No. of secies-specific C-genome AFLP loci per surt-LG[b]																	Total
	A[a]	B	C	D[a]	E	F	G	H	I	[J	Q]	[K	P]	[L	M]	N	O	
EAAC-MCTC	2		6	1	1	1	3	1	1	1		5		2	1	3	1	29
EAGC-MCAT	3		1	1	4			6	3	3		2		1		1	1	26
EAGC-MCTA	2			1		2	3	3	1	3	1	4				2	3	25
EAGC-MCTC	1	1	1	4	1	3	2	1	5	1			4		1	4	2	31
EAGG-MCTC	3		3	2	1	4		2	5	2	4	6	1		1	1	3	38
EAGG-MCTG			1	2	5	1	3	1	2	3		1		1		2	1	23
Total	11	1	12	11	12	11	11	14	17	13	5	18	5	4	3	13	11	172

[a] C-genome LG not represented in the BC_3 families

[b] *G. sturtianum* linkage group

Table 2 Frequency of *G. sturtianum* LG recovery from nine *G.hirsutum* X *G. sturtianum* BC_2 to 632 BC_3 MACALs

BC_2 Family ID	VBI	No. of BC_3s		C genome Sturt-LGs[b]																No.	<Ave>
		(+)[a] C-gen Chr	(−)[a] C-gen Chr	B	C	E	F	G	H	I	[J	Q]	[K	P]	[L	M]	N	O			
Hyb 645	0/1	5	7								4/12	5/12								9	0.4
Hyb 686	0/1	5	8				5/13													5	0.4
Hyb 687	0/1	22	12	5/11				3/17		6/6	3/6	3/6	8/17	8/17				5/23		41	0.9
Hyb 710	0/1	18	53					8/23	4/17	1/14							0/2			13	0.2
Hyb 711	0/1	9	7									1/16			6/16	6/16	3/16			16	1.0
Hyb 712	0/1	26	32				5/11	13/42			2/17	2/17					3/22	6/41		31	0.5
Hyb 734	0/1	24	53			5/33							17/63	17/63			2/28			41	0.5
Hyb 735	0/1	18	58		3/12				4/28	7/12							6/22			20	0.3
Hyb 736	0/1	5	8						3/13	5/13										8	0.6
Total																					
No.		132	238	5	3	5	10	24	11	28	9	11	25	25	6	6	14	11		184	0.5
%		35.7	64.3	45.5	25.0	15.2	41.7	28.6	19.0	62.2	25.7	21.6	31.3	31.3	37.5	37.5	15.6	17.2		11.1	
Hyb 645	4/5	0	5								0/5	0/5								0	0.0
Hyb 686	4/5	0	5				0/5													0	0.0
Hyb 687	4/5	7	2	2/2				1/2		2/5	1/5	3/6	0/2	0/2				4/7		10	1.1
Hyb 710	4/5	32	99					6/41	23/41	18/46							5/23			52	0.4
Hyb 711	4/5	3	12									1/15			1/15	1/15	2/15			5	0.3
Hyb 712	4/5	11	12				3/3	4/15			0/2	0/2					1/5	6/13		14	0.6
Hyb 734	4/5	19	24			8/23							12/42	12/42			2/10			34	0.8
Hyb 735	4/5	8	17		1/1				7/14	0/1							0/4			8	0.3
Hyb 736	4/5	4	2						4/6	1/6										5	0.8
Total																					
No.		84	178	2	1	8	3	11	34	21	1	1	12	12	1	1	10	10		128	0.5
%		32.1	67.9	100	100	34.8	37.5	19.0	55.7	36.2	8.3	3.6	27.3	27.3	6.7	6.7	17.5	50.0		13.3	

Empty cells indicate that the BC2 family did not contain the LG

[a] (+) present and (−) absent

[b] No. of C genome LGs/total no. of BC_3

For accurate association between fusarium wilt symptoms and C-genome chromosome recovery, 168 BC_3 individuals from 13 BC_3 MACAL families with significantly higher resistance levels ($P \leq 0.05$; Fig. 2) were used validate the associations between high fusarium wilt resistance levels in the BC_3 MACALs and the presence of specific

G. sturtianum chromosomes. These individuals contained linkage groups Sturt-E, -G, -H, -I, -J&Q, -K&P, -N and -O. A binary logistic regression based on model *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 Sturt-LGs to resistant and susceptible fusarium wilt symptoms as shown in Table 3. For the resistant fusarium wilt response, the logistic regression analysis indicated that LGs Sturt-E, -J&Q and -N did not contribute to the model; the negative value for B (logit coefficient or effect coefficient) suggested that LGs Sturt-E,

Table 3 C-genome alien addition lines, assigned *Fov* disease response, distribution of *G. sturtianum* chromosomes per *Fov* disease response category, and results of logistic regression analyses showing *G. sturtianum* chromosomes significantly associated with *Fov* wilt symptoms

BC_3 MACALs[a]	Fusarium wilt symptoms	VBI	No. of Ind.	No. of C-genome Sturt-LGs									
				E	G	H	I	[J	Q]	[K	P]	N	O
C genome chromosome distribution													
687-15	[R]	0/1	17		3					8	8		3
	[S]	4/5	2		1					0	0		2
710-21	[R]	0/1	4			1	2						
	[S]	4/5	6			3	2						
710-25	[R]	0/1	3		1	1							
	[S]	4/5	4		1	1							
710-35	[R]	0/1	5		3	1	3						
	[S]	4/5	9		0	6	6						
710-37	[R]	0/1	1			0	0						
	[S]	4/5	4			1	1						
710-41	[R]	0/1	2		1								
	[S]	4/5	6		0								
710-43	[R]	0/1	4		1								
	[S]	4/5	4		0								
710-46	[R]	0/1	3			1	1						
	[S]	4/5	9			5	3						
710-48	[R]	0/1	5		1		3						
	[S]	4/5	3		0		1						
712-92	[R]	0/1	17		2			2	2			2	
	[S]	4/5	2		0			0	0			1	
734-93	[R]	0/1	14							5	5	2	
	[S]	4/5	3							1	1	1	
734-100	[R]	0/1	14	5									
	[S]	4/5	1	1									
735-126	[R]	0/1	22									6	
	[S]	4/5	4									0	
		0/1	111	5	12	4	9	2	2	13	13	10	3
		4/5	57	1	2	16	13	0	0	1	1	2	2
Total			168	6	14	20	22	2	2	14	14	12	5
Logistic regression analysis													
B			−0.5[b]	−1.1	−1.4	2.0	1.0	−5.1		−2.2		−1.0	1.2
SE			0.2[b]	1.1	0.8	0.6	0.5	15.2		1.1		0.8	1.1
Wald			6.9[b]	0.9	3.0	10.0	4.5	0.1		3.9		1.5	1.2
df			1[b]	1	1	1	1	1		1		1	1
Sig.			0.009***	0.335	0.081*	0.002***	0.034**	0.736		0.050**		0.223	0.272
Exp(B)			0.58[b]	0.34	0.24	7.73	2.81	0.01		0.11		0.37	3.36

***Significant at the 99% confidence level

**Significant at the 95% confidence level

*Significant at the 90% confidence level

[a] BC_3 carrying C-genome chromosomes, which VBI means were significantly different to the VBI mean of CPI 168696

[b] Intercept

-J&Q and -N tend to be "present" more often than "absent" in the resistant BC_3s but not significantly, after controlling for the other predictors. The probability of the Wald statistics was marginally significant ($P = 0.081$) for Sturt-G, indicating that its contribution to the model is suggestive but should only be accepted with caution; the negative value of B for presence versus absence of Sturt-G indicates that Sturt-G was present in the resistant BC_3s more often than could be explained by random occurrence after controlling for the other predictors. The value of the "odds ratios" [Exp (B)] was 0.24, which implies that susceptible BC_3 individuals were 76% less likely to carry the Sturt-G. On the other hand, the logistic regression indicated that Sturt-K&P contributed significantly to the model ($P = 0.05$); the negative value of B suggested that Sturt-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 "odds ratios" was 0.11, which implies that susceptible BC_3 individuals were 89% less likely to carry the Sturt-K&P.

For the plants exhibiting susceptible fusarium wilt symptoms, the logistic regression analysis indicated that Sturt-O did not contribute to the model; the positive value of B suggested that Sturt-O tends to be "present" more often than "absent" in the susceptible BC_3s but not significantly, after controlling for the other predictors. The probability of the Wald statistics indicated that Sturt-H and -I contributed significantly to the model ($P = 0.002$ and $P = 0.034$, respectively); the positive value of B suggested that Sturt-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 Sturt-H and -I were 7.73 and 2.81, respectively. These results indicated that resistant BC_3 individuals were approximately three to eightfold less likely to carry the Sturt-I and -H, respectively. Overall, binomial logistic regression analyses identified four putative Sturt-LGs associated with significant differences in fusarium wilt symptoms in the BC_3 MACALs. Linkage groups Sturt-G and Sturt-KP were associated with *Fov* resistance, while LGs Sturt-H and Sturt-I were associated with *Fov* disease susceptibility.

Discussion

Introgression of agronomically important traits, such as fungal disease resistance, from novel germplasm into cultivated cotton to improve current commercial cultivars represents one of the major aims of cotton breeding (Stewart 1995; Brubaker and Brown 2003). Australian native *Gossypium* species have desirable agronomic traits associated with lint and seed qualities, tolerance/resistance to environmental stresses (i.e., cold and drought), and resistance to fusarium wilt (Stewart 1995; McFadden et al. 2004). Species-specific molecular markers can facilitate such breeding approaches considerably. AFLP analysis (Vos et al. 1995) has proven to be an efficient method for tracking the inheritance of *G. sturtianum* chromosomes in *G. hirsutum* × *G. sturtianum* chromosome addition lines as they are recurrently backcrossed to *G. hirsutum* (Becerra Lopez-Lavalle and Brubaker 2007).

The significant improvement in fusarium wilt resistance observed in a *G. hirsutum* × *G. sturtianum* hexaploid F_1 hybrid (Gos-5271) suggested that *G. sturtianum* (Gos-5275) genes have the potential to contribute good levels of FWR, when present in the *G. hirsutum* background (McFadden et al. 2004). The use of Gos-5271 as female parent in backcrosses with *G. hirsutum* allowed the production of BC_1, BC_2 and BC_3 lines carrying several C-genome alien chromosomes. By using chromosome-specific AFLP markers, it was possible to trace the recovery of individual C-genome alien chromosomes from the BC_1 to the BC_2 progeny (Becerra Lopez-Lavalle and Brubaker 2007). In the assessment of the fusarium wilt symptoms of the 46 BC_3 MACALs, it was found that 22 BC_3 MACALs (48%) outperformed their *G. hirsutum* parent across three trials under high *Fov* disease pressure (Fig. 2). This result suggested that one or more FWR-genes were transferred from *G. sturtianum* (Gos-5275) to the MACALs through the *G. sturtianum* hexaploid bridging hybrid (Gos-5271). It also confirms a previous observation made by McFadden et al. (2004) on the improved levels of FWR of Gos-5271, when compared with CPI-138969. The fact that only a subset (48%) of the C-genome alien chromosome derivatives showed significant levels of fusarium wilt resistance suggested that *Fov* resistance is restricted to a small number of linkage groups. These results and those from McFadden et al. (2004) are consistent with the model that the recovery of specific C-genome chromosomes or part thereof from the diploid species (*G. sturtianum*) to a *G. hirsutum* background improves the resistance of *G. hirsutum* (i.e., CPI-138969; susceptible) to fusarium wilt.

The AFLP markers have not only been very successful in determining the chromosomal status and identity of the different C-genome MACALs generated, but have also been successful in tracking the inheritance of G-genome MACALs generated by Brubaker and Brown (2003). To determine which specific C-genome linkage groups contributed gene(s) involved with the enhanced fusarium wilt symptoms, 172 *G. sturtianum*-specific AFLP markers were screened (Table 2). Overall, selection towards fusarium wilt resistant and susceptible symptoms did not affect our ability to independently estimate the recovery of Sturt-LGs in the third generation of alien chromosome derivatives. The most interesting observation of this analysis was the high frequency of co-occurrence of Sturt-J and -Q, LG-K and P, and LG-L and M.

Even though the selection of BC_3 progeny based on their fusarium wilt symptoms had no impact on the average Sturt-LG recovery, there were indeed differences among the 13 individual *G. sturtianum* chromosomes that were transmitted to the BC_3 progenies between *Fov*-resistant and *Fov* –susceptible pools and among populations (Table 2). Between pools, for example, Sturt-F and -J&Q of *G. sturtianum* were present in the largest number of plants (~40%) in the *Fov*-resistant pool but were not in a single plant in the *Fov*-susceptible pool of Hyb-686 and -645 populations, respectively. At first glance, the Sturt-F and -J&Q transmissions indicated a strong independent association with *Fov*-resistance; however, this trend was reversed in Hyb-712 BC_3 family in which all susceptible plants (three individuals) possessed Sturt-F. Such variation in the recovery of individual alien chromosomes between *Fov*-resistant and –susceptible pools contributes to the level of complexity in estimating the association between FWR and the *G. sturtianum* chromosomes transmitted in the *G. hirsutum* background. The number of C-genome chromosomes transmitted from the BC_2 parents is an important consideration in evaluating the association between FWR and *G. sturtianum* chromosomes in the BC_3 MACALs. If several alien chromosomes are present in an individual plant this gives rise to potential multiple loci interactions between the alien and the AD sub-genomes, complicating the analysis of their association with FWR even further. Consistent with this observation, the overall frequency distribution of C-genome Sturt-H suggested an association with *Fov*-susceptibility in the *G. hirsutum* background, while Sturt- F, -G, -J&Q, -K&P and -L&M were possibly associated with *Fov*-resistance. Based solely on the frequency distribution of Sturt-LGs among the resistant and susceptible BC_3 MACAL pools, the association of C-genome Sturt-B, -C, -E, -I ,-N and -O could not be ascertained.

The results of the logistic regression analysis on the validation data set, shown in Table 3, suggested that the improved fusarium wilt resistance observed in the BC_3 MACALs is most likely to be associated to the recovery of linkage groups Sturt-G and Sturt-K&P. In other words, BC_3 MACAL individuals that were fusarium wilt susceptible had a relatively lower probability of carrying linkage groups Sturt-G and Sturt-K&P. In contrast, those BC_3 MACAL individuals identified with susceptible phenotypes had a higher probability of carrying linkage groups Sturt-H and Sturt-I. Thus, this method identifies four *G. sturtianum* linkage groups (Sturt-G, Sturt-H, Sturt-I, Sturt-K&P) as being associated with FWR.

The fact that multiple C-genome chromosome segments (Sturt-LGs) were associated with fusarium wilt resistant and susceptible symptoms in the BC_3 MACALs support the conclusion that fusarium wilt resistance in cotton is multigenically controlled (Hillocks 1984; Shi et al. 1991, 1993; McFadden et al. 2004). Remarkably, little is known about the genetics of resistance to fusarium wilt in cotton, particularly in Australia. Reports on the mode of inheritance of resistance to *Fov* have been inconsistent with respect to the number and effect of genes involved. Genetic studies of FWR outside Australia are also confounded by the fact that in most areas increased fusarium wilt severity is associated with root knot nematode (RKN) making it difficult to discriminate between FWR and RKN resistance (Shepherd 1986; Shepherd and Kappelman 1986). Thus, there is a pressing need to understand the genetic control of fusarium wilt resistance so that the resistance in *G. sturtianum* can be fully utilised in improving Australian cultivated cottons. Our results, and those reported by McFadden et al. (2004), indicate that *G. sturtianum* may constitute a good genetic model for unravelling the mode of *Fov* resistance in *G. hirsutum*. This assumption is based on the fact that *G. sturtianum* is diploid, so genetic analysis would not be complicated by the presence of duplicated homoeologs for relevant genes. That there are disease-susceptible *G. sturtianum* accessions available provides the opportunity for developing useful experimental families for genetic analyses. Based on fusarium wilt symptoms and molecular marker diversity we have identified an optimal parental combination for development of FWR segregating families and advanced backcross progenies that are currently being generated to assess whether the basis of resistance in *G. sturtianum* could serve as a model for elucidating the resistance in *G. hirsutum* (McFadden et al. 2004).

This study has not only confirmed that *G. sturtianum* is a potential source of resistance to fusarium wilt, but that this resistance is expressed in the *G. hirsutum* background. Many of the useful alien genes, contained in the chromosome segments transferred to *G. hirsutum*, are quite probably different from these of the cultivated species and are therefore, potentially useful for providing novel and effective sources of resistance to this economically significant disease. Hence, there is a potential for exploiting the Australian wild relatives of cotton with different mechanisms of resistance to increase the level and diversify the basis of resistance to *Fov*.

Alien chromosome addition lines are extremely useful for transferring particular characters from low to high ploidy species, and fusarium wilt resistance of cotton is one practical goal. Successful stable transfer of the *Fov* resistance associated with linkage groups G and K&P is dependent upon the ability of these *G. sturtianum* LGs to recombine with the A&D-sub-genomes. Although the hexaploid bridging strategy, which we undertook to produce the advance *G. hirsutum* × *G. sturtianum* chromosome addition lines, increased our ability to sample more meiotic events than was possible with the tetraploid bridging approach, the distribution of Sturt-LGs in the BC_3

MACALs gave no evidence of recombination between the C-genome and the A and D sub-genome of the *G. hirsutum* (Becerra Lopez-Lavalle and Brubaker 2007). This suggests that one of the potential barriers for introgression of *Fov* resistance genes from *G. sturtianum* into *G. hirsutum* is the apparent limited recombination among the homoeologous chromosomes of *G. hirsutum* and *G. sturtianum*. However, the very low levels of recombination observed in the germplasm material used in this study can potentially be overcome through the development of trispecific allotetraploid hybrids (tetraploid bridging) with a D-genome diploid as the bridging species. Tetraploid bridging derivatives ($A_hD_hD_1C_1$ or $A_hD_hD_5C_1$) such as those used by Vroh Bi et al. (1998, 1999) may offer an opportunity to enhance chromosome pairing between the C-genome with the A chromosomes of *G. hirsutum*. In such crosses, the D_1 or D_5 chromosomes of the tri-species hybrids pair mostly with their homologous D_h chromosomes of *G. hirsutum*, so that the C_1 chromosomes of *G. sturtianum* can pair with the A_h chromosomes of *G. hirsutum*. Considering fusarium wilt resistant in *G. sturtianum* is likely to be controlled by multiple genes, the introgression of these multiple genomic regions into *G. hirsutum* may be facilitated by using the tetraploid bridging strategy, if the rates of real homoeologous recombination are indeed effectively higher. Working with $A_hD_hD_1C_1$ or $A_hD_hD_5C_1$ synthetic tetraploids still require considerable effort to produce enough fertile recombinant progeny to guarantee that all relevant chromosome segments have been introgressed.

Although recombination between *G. sturtianum* and *G. hirsutum* homoeologous genomic regions could not be ascertained in the *G. sturtianum* × *G. hirsutum* aneuploid cytogenetic stocks we developed, the identification of *G. sturtianum* chromosomes associated with economically important traits such as fusarium wilt resistance will facilitate the identification of the homoeologous regions containing these genes in the allotetraploid cultivated species.

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