A Fusarium Wilt Resistance Gene in *Gossypium barbadense* and Its Effect on Root-Knot Nematode-Wilt Disease Complex

C. Wang and P. A. Roberts

Department of Nematology, University of California, Riverside 92521.
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ABSTRACT

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Fusarium wilt, caused by the soilborne pathogen *Fusarium oxysporum* f. sp. *vasinfectum* race 1, is a vascular disease in cotton (*Gossypium* spp.), and is a component of a disease complex with root-knot nematodes (*Meloidogyne incognita*). Genetic analysis of two interspecific crosses (*G. barbadense* Pima S-7 × *G. hirsutum* Acala NemX and Pima S-7 × Acala SJ-2) showed that one major gene (designated *Fov1*) with allele dosage effect conferred resistance to *F. oxysporum* f. sp. *vasinfectum* race 1 in Pima S-7. Two amplified fragment length polymorphism (AFLP) markers were linked to *Fov1* in Pima S-7, with genetic distance from the gene of 9.3 and 14.6 centimorgans. Less severe wilt symptoms in Acala NemX than Acala SJ-2 indicated that Acala NemX possesses one or more

minor genes contributing to delay of wilt symptoms. Highly resistant plants in F₂ and F₃ (Pima S-7 × NemX) families indicated transgressive segregation effects of minor genes in Acala NemX combined with *Fov1* from Pima S-7. The effects of wilt and nematode resistance on the nematode-wilt disease complex were assayed with two inoculation methods. In the presence of both pathogens, wilt damage measured as shoot and root weight reductions was greatest on wilt- and nematode-susceptible Acala SJ-2 and least in root-knot nematode-resistant and wilt-susceptible Acala NemX. Intermediate damage occurred in wilt-resistant and root-knot nematode-susceptible Pima S-7. The results indicated that nematode resistance was more effective than wilt resistance in suppressing wilt symptoms when either resistance was present alone. Nematode resistance combined with intermediate wilt resistance, as in the F₁ (Pima S-7 × NemX), was highly effective in protecting plants from root-knot nematodes and race 1 of Fusarium wilt as a disease complex.

Fusarium wilt of cotton (*Gossypium* spp.) is a vascular disease caused by the soilborne pathogen *Fusarium oxysporum* f. sp. *vasinfectum*. It is widespread and causes substantial crop losses in most of the major cotton-producing areas of the world. The incidence, rate of development, and severity of Fusarium wilt in cotton can increase in the presence of root-knot nematodes (*Meloidogyne incognita*) as a disease complex (1,10). Garber (11) found that Fusarium wilt symptoms occurred with ≥77,000 *F. oxysporum* f. sp. *vasinfectum* propagules per gram of soil without root-knot nematode on root-knot nematode-susceptible Acala SJ-2, whereas as few as 650 *F. oxysporum* f. sp. *vasinfectum* propagules caused Fusarium wilt on plants co-inoculated with 50 root-knot nematode infective juveniles under controlled greenhouse conditions. Fusarium wilt disease symptoms of cotton appeared sooner with an increase of population levels of either or both *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* (11). In California, Fusarium wilt symptoms typically are associated with the presence of *M. incognita* in fields with coarse-textured sandy soils (10,17). In the San Joaquin Valley of California, root-knot nematode-Fusarium wilt complex infection occurs in up to 20% of the cotton-planting area (2,12). During the last 50 years, the nematode and wilt disease complex has been controlled effectively with fumigant nematicides (19). More restrictive uses of nematicides, due to environmental and human health concerns and economic considerations, have resulted in greater emphasis on the development of resistant cultivars for the management of nematodes and wilt disease. Resistance to *M. incognita* has proved to be an effective control strategy for the root-knot nematode-Fusarium wilt disease complex (24,27).

In California, four races (races 1, 3, 4, and 8) of *F. oxysporum* f. sp. *vasinfectum* were reported by Kim et al. (20). Races 1, 3, and 8 are mildly virulent and cause little or no wilt symptoms in the absence of root-knot nematodes. Race 1, the focus of this study, is widely distributed in the San Joaquin Valley and has been the predominant form of *F. oxysporum* f. sp. *vasinfectum*. Race 3 and race 8 were identified recently in a limited number of fields in Tulare and Fresno counties (20). However, race 4 of *F. oxysporum* f. sp. *vasinfectum*, which also was identified recently in California (20), caused severe wilt symptoms and economic loss in Pima (*G. barbadense* L.) cotton fields in the San Joaquin Valley, even in the absence of nematodes. The occurrence and threat of new races or virulent strains of *F. oxysporum* f. sp. *vasinfectum* emphasizes the need to develop cultivars with multiple resistance to different races or strains and multiple pathogens. For example, California imports tons of seed for cattle feed from Australia (20), which has extremely virulent strains of *F. oxysporum* f. sp. *vasinfectum* that cause extensive damage to Australian cotton in the absence of root-knot nematodes (9). An improved understanding of genetic resistance to each race or strain is an important step towards incorporating resistance genes into elite cultivars. Previous reports about the genetic resistance to Fusarium wilt mostly focused on nematode-wilt disease complex in field experiments, revealing one to several genes involved in Fusarium wilt resistance (13,15,18,28,29). Netzer et al. (23) reported one dominant gene involved in determining resistance to race 3 of *F. oxysporum* f. sp. *vasinfectum* in Acala SJ-2 in Israel under greenhouse conditions.

In contrast to phenotypic selection, which is time-consuming, expensive, and sensitive to screening conditions, marker-assisted selection, based on identification of DNA markers linked to Fusarium wilt resistance genes in cotton, will allow wide selection of wilt resistance in cotton breeding. Genetic markers also will enable unique resistance genes to be pyramided in one genotype or to be deployed individually.

Corresponding author: P. A. Roberts; E-mail address: philip.roberts@ucr.edu

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The objectives of this study were to (i) determine the inheritance of resistance to *F. oxysporum* f. sp. *vasinfectum* race 1 in *G. barbadense* cv. Pima S-7, (ii) screen for amplified fragment length polymorphism (AFLP) markers linked to Fusarium wilt resistance, and (iii) determine the effects of the wilt resistance on the interaction between *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* race 1.

MATERIALS AND METHODS

Plant materials and crosses. Plant genotypes used in this study were *G. hirsutum* cv. Acala SJ-2, *G. hirsutum* cv. Acala NemX, and *G. barbadense* cv. Pima S-7. One intraspecific cross was made between Acala NemX and Acala SJ-2, and two interspecific crosses were made between Pima S-7 and Acala NemX, and between Pima S-7 and Acala SJ-2. The F₁ plants were selfed to produce F₂ and also backcrossed to both parents to produce BC₁F₁ populations. Sixty-eight F₂ (Pima S-7 × NemX) plants were selfed to obtain F_{2:3} families for Fusarium wilt screening. All plants for crossing and producing seed were planted in 15-cm-diameter pots filled with steam-sterilized U.C. Mix #2 (3) soil and grown in the greenhouse. Air temperatures in the greenhouse were maintained between 28 and 35°C during the day and 24°C at night. Plants were fertilized with 17-6-10 controlled release fertilizer (Scotts-Sierra Horticultural Products Co.) and were drip irrigated.

Fungus inoculum. Four isolates of *F. oxysporum* f. sp. *vasinfectum* from two Fresno County fields (2F and 3F) in California were kindly provided by Dr. Shirley N. Smith, Plant Pathology Department, University of California, Davis (Table 1). Isolates I and III belong to the high-sporulating culture type (FOV_{HS}), which produces conidia rapidly, a red-purple pigment in potato dextrose agar (PDA) and other culture media, and an unusual, fragrant odor. Isolates II and IV are low-sporulating culture types (FOV_{LS}), with slow conidia production, no pigmentation, and no fragrance (30,31). Isolates I, III, and IV were identified as race 1 and isolate II as race 8 by Kim et al. (20). Cultures from single spores were stored on filter paper at -20°C. To produce inoculum, the isolates were grown in 9-cm-diameter petri dishes containing 20 ml of PDA with 3 mM streptomycin per liter at room temperature for 1 week. The spore suspensions were prepared by adding distilled water onto the culture grown in petri dishes, scraping the agar surface with a glass slide, and filtering the suspension through four layers of cheesecloth. *Fusarium* spore suspensions were adjusted to desired concentrations for each experiment using a hemacytometer.

Nematode inoculum. A culture of *M. incognita* race 3 (isolate Project 77), originating from a San Joaquin Valley, California cotton field, was maintained and multiplied on tomato cv. Tropic. The species and race identity of the culture were confirmed by

TABLE 1. Pathogenicity of four *Fusarium oxysporum* f. sp. *vasinfectum* isolates from the San Joaquin Valley, California, on three cotton cultivars (Pima S-7, Acala NemX, and Acala SJ-2)^a

Isolates	Race	Disease index		
		SJ-2	NemX	Pima S-7
I	1	5.00 a	4.08 a	1.33 a
II	8	4.53 b	4.29 a	0.87 a
III	1	4.93 a	4.11 a	1.67 a
IV	1	4.87 ab	4.23 a	1.75 a
<i>P</i> > <i>F</i>	...	0.0418	0.9441	0.4098
LSD	...	0.3424	0.7877	1.0697

^a Data are the means of 20 replicate plants per treatment. Observations were made 3 weeks after inoculation using the root-cut dip method. *P* = probability level of analysis of variance; LSD = least significance difference at the 0.05 level. Means within a column with the same letter are not different. Disease index, 0-to-5 scale, where 0 = no symptoms and 5 = plant death. Noninoculated control plants remained healthy (data not shown).

isozyme phenotyping and a host differential test as described previously (26). Inoculum was prepared by extracting eggs from tomato roots with NaOCl (14).

Fusarium wilt assays. A root-cut dip method described by Smith et al. (31) with modifications was performed for the *F. oxysporum* f. sp. *vasinfectum* pathogenicity tests. One-week-old seedlings grown in seedling trays were uprooted, and roots were rinsed with tap water, trimmed to 2 to 3 cm in length, and immediately dipped for 3 min into an aqueous suspension of *Fusarium* macro- and microconidia (1×10^6 conidia/ml). Seedlings then were transplanted into steam-sterilized U.C. Mix #2 (3) soil in new pots with five plants in each pot. Disease severity was measured weekly for 3 weeks after inoculation. To study disease progress in three parents (Pima S-7, Acala SJ-2, and Acala NemX) and two F₁ cotton genotypes (F₁ [Pima S-7 × SJ-2] and F₁ [Pima S-7 × NemX]), symptoms were indexed starting at 7 days and continuing every 1 to 2 days until 4 weeks after inoculation. This experiment was conducted twice. Individual plants were rated for disease severity based on the following index: 0 = no symptoms, 1 = epinasty and slight dwarfing, 2 = 1 to 30% chlorotic leaves, 3 = 31 to 80% chlorotic leaves and severe stunting, 4 = 81 to 100% chlorotic leaves, and 5 = plant death. The stems were cut longitudinally and evaluated for vascular discoloration. Two to four 1-cm segments were cut and plated on PDA agar for re-isolation of *F. oxysporum* f. sp. *vasinfectum*.

Plants with a disease index ≤ 2 were classified as resistant and those with an index > 2 as susceptible, based on comparisons with the indices of the resistant Pima S-7 and susceptible Acala SJ-2 and Acala NemX parents (Fig. 1). In the segregating F₂ (Pima S-7 × NemX) population, plants were identified as homozygous resistant when all individual plants in the F_{2:3} family were resistant, as homozygous susceptible when all individual F_{2:3} plants were susceptible, and as heterozygous when plants in an F_{2:3} family segregated susceptible and resistant. Each experiment for genetic analysis of *F. oxysporum* f. sp. *vasinfectum* included the parents and F₁.

Disease interaction assays. To evaluate the effects of wilt and nematode resistance on the interaction of *F. oxysporum* f. sp.

Fig. 1. Progress of wilt disease symptoms of *Fusarium oxysporum* f. sp. *vasinfectum* race 1 on three cotton cultivars and two F₁ after inoculation with the root-cut dip inoculation method. Data are the means of 20 replicate plants per treatment. Bars show ± one standard error. Disease index: 0-to-5 scale (0 = no symptom and 5 = plant death).

vasinfectum and *M. incognita*, two inoculation methods and four treatments were used (plants with *M. incognita* alone, with *F. oxysporum* f. sp. *vasinfectum* alone, with *M. incognita* and *F. oxysporum* f. sp. *vasinfectum*, and with neither pathogen). In the first method (seedling method), 10-day-old seedlings grown in 10-cm-diameter pots filled with 0.45 kg of steam-sterilized sandy soil (93% sand, 3% clay, and 4% silt) were inoculated with 20,000 *M. incognita* eggs in 5 ml of water and with 30×10^6 *F. oxysporum* f. sp. *vasinfectum* spores in 5 ml of water via two holes made around each plant. The control plants were inoculated with only distilled water at equivalent volumes. In the second inoculation method (soil method), which was used to simulate field conditions, *F. oxysporum* f. sp. *vasinfectum* spores were mixed into soil before planting. This method was modified from that described by Garber et al. (11). Each 10-cm-diameter pot contained 0.45 kg of sandy soil with or without 30×10^6 *F. oxysporum* f. sp. *vasinfectum* spores. The inoculum was mixed into soil before soil was placed in pots and planted with one seed per pot. In treatments including nematodes, the soil was infested by pouring a 5-ml suspension containing 20,000 *M. incognita* eggs over the seed before it was covered. The control plants were inoculated with only distilled water. Treatments were arranged in a randomized complete block design with nine replications. The two inoculation methods were used in separate experiments, and each of the experiments was conducted twice (trials 1 and 2). Data on plant height, fresh shoot weight, fresh root weight, and nematode eggs per gram of root were collected 60 days after inoculation. The height of each plant was measured from the soil surface to the growing tip. The stems were cut longitudinally and evaluated for vascular discoloration. Two to four 1-cm segments were cut and plated on PDA agar for re-isolation of *F. oxysporum* f. sp. *vasinfectum*.

To provide optimal conditions for plant growth and for infection by both pathogens, heat mats under pots maintained soil temperature at 28°C in all experiments. Air temperatures in the greenhouse were maintained between 28 and 32°C during the day and 24°C at night. Plants were fertilized with 17-6-10 controlled release fertilizer (Scotts-Sierra Horticultural Products Co.) and pots were drip irrigated.

Data were analyzed using analysis of variance procedure of the SAS statistical software (SAS Institute, Cary, NC). Nematode egg data were $\log_{10}(x + 1)$ transformed before analysis.

DNA extraction and AFLP screening. Fresh or frozen (–80°C) young cotton leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle, and genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Valencia, CA).

Screening of AFLP markers linked to *Fusarium* wilt resistance was based on the bulked segregant analysis (BSA) method (22). Two DNA pools were made, one with seven homozygous resistant plants and the second with seven homozygous susceptible plants from the F_2 (Pima S-7 $\times$ NemX) population based on phenotype screening of $F_{2:3}$ (Pima S-7 $\times$ NemX) families. The bulks together with the two parents were screened for markers showing polymorphisms between the four samples. The AFLP markers with putative linkage to the *F. oxysporum* f. sp. *vasinfectum* resistance gene (*Fov1*) in Pima S-7 were confirmed by screening the individuals within the bulks. The confirmed markers were analyzed further and mapped using an additional 36 F_2 individual plants segregating for *Fov1* based on $F_{2:3}$ family tests. AFLPs were generated using the protocol of IRDye Fluorescent AFLP Kit for large plant genome analysis (LI-COR, Lincoln, NE) with modifications. The half volumes of the protocol described were used for restriction and ligation of genomic DNA and pre-amplification of the diluted template DNA. In all, 80 primer combinations (*EcoRI/MseI*), including 8 IRDye-labeled *EcoRI* primers plus three nucleotides (AAC, AAG, ACA, ACT, ACC, ACG, AGC, and AGG) and 10 unlabeled *MseI* primer plus three

nucleotides (CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT, CGA, and CCG) (LI-COR) were used for selective amplification. Amplification was performed in a MasterCycler Gradient (Eppendorf, Hamburg, Germany). Electrophoresis and detection were performed on a two-dye, model 4000 LI-COR IR² automated sequencer (Global edition, LI-COR). The gel images were analyzed with Saga automated analysis software (LI-COR).

Segregation and linkage analysis. The data for *F. oxysporum* f. sp. *vasinfectum* resistance phenotype and marker segregation ratios were tested for goodness-of-fit to predicted Mendelian inheritance ratios by the χ^2 test ($P = 0.05$). The linkage analysis between the AFLP markers and *F. oxysporum* f. sp. *vasinfectum* resistance locus was performed with the software JoinMap 3.0. The linkage groups were intact up to a log of the likelihood ratio score of 6. The recombination fractions were converted into centimorgans (cM) based on Kosambi's mapping function (21).

RESULTS

Pathogenicity of four *F. oxysporum* f. sp. *vasinfectum* isolates. This experiment was conducted twice (trial 1 and trial 2). Analysis of variance showed no difference between the duplicate trials 1 and 2, and only data from trial 2 are presented (Table 1). All control plants not inoculated with the *Fusarium* sp. remained healthy (data not shown). There was no difference in pathogenicity among the four isolates within one cotton genotype except that isolate II was less virulent than the other three isolates on Acala SJ-2 (Table 1). At the end of the experiment, almost all inoculated plants of Acala SJ-2 and Acala NemX had died, whereas Pima S-7 plants were growing well even though the plants had mild wilt symptoms with stunting. Wilt disease symptoms in Acala SJ-2 and Acala NemX included cotyledon drop, leaf chlorosis and necrosis, plant wilt, and plant death starting at 10 days after inoculation. The growth of Acala SJ-2 and Acala NemX plant stems was limited to the first node, and plants remained stunted until death. Pima S-7 plants grew slowly for a few days after transplanting and then recovered and grew to the second stem node and above. Vascular discoloration occurred along the length of the stem in all plants of Acala NemX and Acala SJ-2, whereas vascular discoloration was limited to the lower part of the stem just above the soil level in Pima S-7. These data showed that Acala SJ-2 and Acala NemX were susceptible and Pima S-7 was resistant but not immune to *F. oxysporum* f. sp. *vasinfectum*. To analyze the inheritance of resistance to *F. oxysporum* f. sp. *vasinfectum* in Pima S-7 and to determine the effects of the wilt resistance on the nematode-wilt disease complex, isolate I was used for the following experiments.

Disease progress of *F. oxysporum* f. sp. *vasinfectum* in parents and F_1 . Wilt disease symptoms caused by *F. oxysporum* f. sp. *vasinfectum* race 1 on the three cotton parents (Pima S-7, Acala NemX, and Acala SJ-2) and two F_1 (Pima S-7 $\times$ NemX and Pima S-7 $\times$ SJ-2) increased with time after inoculation in all genotypes (Fig. 1). The symptoms started to appear 6 days after inoculation in Acala SJ-2, Acala NemX, and the two F_1 . Some plants started to die 10 days after inoculation in Acala NemX and Acala SJ-2 and 14 days after inoculation in the two F_1 . Acala NemX had fewer wilt symptoms than Acala SJ-2. The progress of wilt disease in the two F_1 was intermediate between Pima S-7 and Acala NemX or Acala SJ-2 11 to 17 days after inoculation (Fig. 1). The severity of disease in the two F_1 was less than on both susceptible parents from the onset of symptoms (day 6 to 7) until day 18, from which time on the F_1 showed severe symptoms similar to the susceptible parents. Conversely, the severity of disease in Pima S-7 was less than in Acala SJ-2 and Acala NemX on all assay dates, and less than in the F_1 after day 10 (Fig. 1). The mean disease index in Pima S-7 remained low during the experiment (Fig. 1). Based on these results, plants were harvested 21 days after inoculation in the genetic analysis experiments.

Phenotypic and genetic analysis of *F. oxysporum* f. sp. *vasinfectum* resistance in segregating populations. Inheritance of resistance to *F. oxysporum* f. sp. *vasinfectum* race 1 was studied in segregating populations derived from two crosses between resistant Pima S-7 and either susceptible Acala NemX or Acala SJ-2. The observed resistant:susceptible (R:S) ratios in different generations or segregating populations fit R:S ratios expected for a model of one gene determining resistance in Pima S-7 (Table 2). Among the susceptible parents, less severe wilt symptoms were observed consistently in Acala NemX compared with Acala SJ-2 in all tests using the root-cut dip method (Tables 1 and 2; Fig. 1), indicating that Acala NemX carried minor resistance genes contributing to delay in the development of wilt symptoms. The progress of disease over 3 weeks in susceptible plants showed a trend of accumulating wilt severity (Fig. 1). All Acala SJ-2 and F₁ (Pima S-7 × SJ-2) plants were highly susceptible to *F. oxysporum* f. sp. *vasinfectum*, but some plants of Acala NemX and the F₁ (Pima S-7 × NemX) were less susceptible. In addition, when both F₁ (Pima S-7 × NemX) and F₁ (NemX × Pima S-7) were backcrossed to Pima S-7, the observed R:S ratios were >1:1, whereas the observed R:S ratios were <1:1 in the backcross populations of Pima S-7 × F₁ (Pima S-7 × SJ-2) and Pima S-7 × F₁ (SJ-2 × Pima S-7) (Table 2). These results indicated a small but consistent difference in wilt susceptibility between Acala SJ-2 and Acala NemX. In all, 12 of 57 BC₁ Acala NemX × F₁ (Pima S-7 × NemX) plants and 2 of 61 BC₁ Acala SJ-2 × F₁ (Pima S-7 × SJ-2) plants were scored as resistant among all expected susceptible plants (Table 2), further indicating lower susceptibility in Acala NemX. Higher resistance than in the resistant parent Pima S-7 also was observed in some F₂ and F₃ (Pima S-7 × NemX) families. In addition, five individuals had susceptible responses in resistant Pima S-7 and some plants showed resistant responses among expected susceptible plants (Table 2).

AFLP markers associated with resistance to *F. oxysporum* f. sp. *vasinfectum* race 1. Of 80 AFLP *Eco*RI/*Mse*I primer combinations, 13 were tested for potential association with Fusarium resistance based on BSA. After individuals within the bulks were screened, 10 potential markers within eight primer combinations were used to screen the F₂ (Pima S-7 × NemX) individual plants. Based on the wilt resistance assays of F_{2,3} families to determine the F₂ phenotype, two coupling-phase dominant markers (M-

CAA_E-AAC-85 and M-CAA_E-AGC-169; Fig. 2A and B, respectively) were found to be linked to the major gene determining resistance in Pima S-7, with distances from the resistance gene of 9.3 and 14.6 cM, respectively (Fig. 2C).

Effect of resistance on the nematode-wilt disease complex. Experiments on the effects of single and combined inoculations with *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* race 1 on the three cotton cultivars with both the seedling and the soil method were conducted twice (trials 1 and 2). Similar results were obtained between trials based on analysis of variance, and the results presented are from one trial for each inoculation method. No wilt symptoms were observed in the noninoculated control or the nematode-only treatment (data not shown). Among plants inoculated with *F. oxysporum* f. sp. *vasinfectum* race 1 alone, only Acala SJ-2 plants inoculated by the soil method showed wilt symptoms (data not shown). Plants inoculated with both pathogens were shorter and weighed less than noninoculated plants and plants inoculated with either *M. incognita* or *F. oxysporum* f. sp. *vasinfectum* alone (Tables 3 and 4). The percent reduction in plant height, shoot weight, and root weight in the presence of both pathogens was greater in all three cultivars compared with the control, *M. incognita*, and *F. oxysporum* f. sp. *vasinfectum* treatments. The greatest reduction in plant growth was in Acala SJ-2, followed by Pima S-7 and then Acala NemX. Growth of Acala SJ-2 inoculated with *M. incognita* was less than growth of noninoculated plants regardless of inoculation method (Tables 3 and 4). Shoot weight of Pima S-7 inoculated with *M. incognita* by the soil method was less than that of the control (Table 4).

In Acala SJ-2 inoculated by the soil method (Table 4) but not by the seedling method (Table 3), plant height, shoot weight, and root weight were less in the *F. oxysporum* f. sp. *vasinfectum* alone treatment than in the *M. incognita* and control treatments.

In the *M. incognita* treatment, numbers of nematode eggs were equivalent in the nematode-susceptible genotypes Pima S-7 and Acala SJ-2, and greater in the susceptible genotypes than in the resistant Acala NemX. Nematode egg production in Acala SJ-2 plants infected with both *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* pathogens was lower than in the *M. incognita* alone treatment (Table 5).

Vascular discoloration was observed in stem tissues of all three cultivars in the presence of both pathogens. Acala SJ-2 plants

TABLE 2. Classification for resistance to *Fusarium oxysporum* f. sp. *vasinfectum* race 1 of parent lines and segregating populations derived from crosses of resistant Pima S-7 with susceptible Acala NemX and susceptible Acala SJ-2, using root-cut dip inoculation method^v

Genotype, population	No. of plants	Expected ratio ^w	Observed ratio R:S	χ ²	P value
Pima S-7 (P) × NemX (N) ^x					
Pima S-7	43 ^y	All R	38:5	...	...
NemX	44 ^y	All S	5:39	...	...
F ₁ (P × N)	66 ^y	All S	6:60	...	...
F ₂ (P × N)	133	33.25:99.75 (1:3) R:S	31:102	0.2030	0.6523
F ₃ families ^z	68 ^z	17:34:17 (1:2:1) R:Seg:S	15:34:19	0.4706	0.7903
BC ₁ F ₁					
P × F ₁ (P × N)	85	42.5:42.5 (1:1) R:S	51:34	3.4000	0.0652
P × F ₁ (N × P)	81	40.5:40.5 (1:1) R:S	43:38	0.3086	0.5785
N × F ₁ (P × N)	57	All S	12:45	...	...
Pima S-7 (P) × SJ-2 (S)					
Pima S-7	43 ^y	All R	38:5	...	...
SJ-2	50 ^y	All S	0:50	...	...
F ₁ (P × S)	67 ^y	All S	0:67	...	...
F ₂ (P × S)	143	35.75:107.25 (1:3) R:S	30:113	1.2331	0.2668
BC ₁ F ₁					
P × F ₁ (P × S)	89	44.5:44.5 (1:1) R:S	40:49	0.9101	0.3401
P × F ₁ (S × P)	83	41.5:41.5 (1:1) R:S	39:44	0.3012	0.5831
S × F ₁ (P × S)	66	All S	2:61	...	...

^v These data were collected from two tests. The first test included three parents (Pima S-7, NemX, and SJ-2) and corresponding F₁, F₂, and 34 F₃ (P × N) families. The second test included three parents and two F₁, plus all corresponding BC₁F₁ populations and another 34 F₃ (P × N) families.

^w Expected number of plants for a single gene model of resistance. R = resistant, S = susceptible, and Seg = segregating.

^x P = Pima S-7; N = NemX; S = SJ-2; F₁ (P × N) = F₁ (Pima S-7 × NemX), and so on.

^y The number of plants or families tested are pooled from two tests.

^z Each family included 15 to 25 individual plants.

Fig. 2. Images of amplification products with dominant amplified fragment length polymorphism (AFLP) markers **A**, M-CAA_E-AAC-85 and **B**, M-CAA_E-AGC-169 in the segregating population F_2 (Pima S-7 $\times$ NemX) electrophoresed on polyacrylamide gel with model 4000 LI-COR IR² automated sequencer and **C**, location of *Fusarium oxysporum* f. sp. *vasinfectum* race 1 resistance gene *FovI* in Pima S-7 relative to the two AFLP markers in the F_2 (Pima S-7 $\times$ NemX) population. Linkage shown at log of the likelihood ratio = 6. P_1 = Pima S-7, P_2 = NemX, R = resistant, and S = susceptible, all based on phenotype; M = 700-bp ladder. The arrows point to the marker positions. M-CAA_E-AAC-85 indicates that *MseI* (CAA) and *EcoRI* (AAC) primer combination produced an 85-bp-size marker.

with both pathogens started to die 25 days after inoculation in experiments with both inoculation methods, and all Acala SJ-2 plants had died by the end of the experiments. Vascular discoloration was limited to the lower part of the stem in Acala NemX. Dark vascular discoloration was limited to the first stem node in Pima S-7, with no discoloration between the first and second nodes.

The three F_1 genotypes also were included in the interaction experiments above. In F_1 (Pima S-7 $\times$ SJ-2) and F_1 (SJ-2 $\times$ NemX), shoot weight was less in plants inoculated with both *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* than in non-inoculated plants or in plants inoculated with *M. incognita* or *F. oxysporum* f. sp. *vasinfectum* alone by both the soil method (Fig. 3) and the seedling method (data not shown); similar results were obtained with plant height and root weight (data not shown). However, in F_1 (Pima S-7 $\times$ NemX), shoot weights (and plant height and root weight; data not shown) were similar in all four treatments (Fig. 3). As with susceptible plants inoculated by the seedling method, numbers of nematodes in the *M. incognita* alone treatments were similar in the susceptible genotypes and were greater in the susceptible F_1 (Pima S-7 $\times$ SJ-2) and F_1 (SJ-2 $\times$ NemX) genotypes than in the resistant F_1 (Pima S-7 $\times$ NemX) genotype (data not shown).

DISCUSSION

Genetic analysis of the two crosses Pima S-7 $\times$ SJ-2 and Pima S-7 $\times$ NemX indicated that one major gene with allele dosage effect in Pima S-7 conferred resistance to race 1 of *F. oxysporum* f. sp. *vasinfectum*. This gene is designated here as *FovI*. In addition, less severe wilt symptoms in wilt-susceptible Acala NemX than in Acala SJ-2 indicated that one or more minor genes in Acala NemX contributed to the delay in the development of wilt symptoms and to the small but significant reduction in the severity of wilt symptoms. The presence of some plants in F_2 and F_3 (Pima S-7 $\times$ NemX) families with higher resistance than in resistant parent Pima S-7 suggested that transgressive inheritance occurred and that minor genes in Acala NemX contributed to the resistance in combination with *FovI*. Transgressive inheritance of resistance to *M. incognita* also was observed in $F_{2,7}$ (NemX $\times$ SJ-2) and F_2 , F_3 and backcross populations of Pima S-7 $\times$ NemX

TABLE 3. Effects of single and combined inoculations (using the seedling method) with *Meloidogyne incognita* (RKN) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 1 on plant growth of three cotton cultivars (Acala SJ-2, Acala NemX, and Pima S-7)^x

Plant measurement, cultivar ^y	Control ^z	Treatment		
		RKN	FOV	FOV + RKN
Plant height				
SJ-2	100 ab (28.7)	92 bc	102 ab	39 e
NemX	100 ab (27.1)	97 ab	104 ab	82 c
Pima S-7	100 ab (32.0)	100 ab	109 a	62 d
$P > F = 0.0001$				
LSD = 13.50				
Shoot weight				
SJ-2	100 a (16.1)	74 bc	101 a	2 e
NemX	100 a (19.0)	86 abc	95 ab	62 c
Pima S-7	100 a (16.9)	80 abc	95 ab	37 d
$P > F = 0.0001$				
LSD = 24.78				
Root weight				
SJ-2	100 a (11.3)	72 c	102 a	8 e
NemX	100 a (15.4)	99 a	95 ab	68 cd
Pima S-7	100 a (11.1)	77 bc	102 a	50 d
$P > F = 0.0001$				
LSD = 18.13				

^x Observations were made 60 days after inoculation. Ten-day-old seedlings grown in 10-cm-diameter pots filled with sandy soil were inoculated with 20,000 *M. incognita* eggs, 30×10^6 *F. oxysporum* f. sp. *vasinfectum* spores, or both. Data are mean percentages of each plant growth parameter, calculated from the mean value of the control as 100% for each genotype. Means with the same letter are not different. Data were compared among all treatments and all cultivars for each parameter.

^y P = probability level of analysis of variance and LSD = least significant difference at the 0.05 level.

^z Values in parentheses are means of control plant height (cm), shoot weight (g), and root weight (g).

TABLE 4. Effects of single and combined inoculations (using the soil method) with *Meloidogyne incognita* (RKN) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 1 on plant growth of three cotton cultivars (Acala SJ-2, Acala NemX, and Pima S-7)^x

Plant measurement, cultivar ^y	Control ^z	Treatment		
		RKN	FOV	FOV + RKN
Plant weight				
SJ-2	100 ab (32.2)	89 bc	71 d	35 e
NemX	100 ab (27.7)	101 ab	104 a	87 c
Pima S-7	100 ab (38.8)	90 bc	92 abc	62 d
$P > F = 0.0001$				
LSD = 12.57				
Shoot weight				
SJ-2	100 a (18.6)	82 bc	48 d	2 e
NemX	100 a (16.9)	101 ab	101 a	71 c
Pima S-7	100 a (19.7)	80 c	91 abc	35 d
$P > F = 0.0001$				
LSD = 19.45				
Root weight				
SJ-2	100 a (13.3)	84 bc	61 d	4 e
NemX	100 a (12.2)	106 ab	109 a	76 cd
Pima S-7	100 a (9.5)	104 ab	106 ab	54 d
$P > F = 0.0001$				
LSD = 22.42				

^x Observations were made 60 days after inoculation. Each 10-cm-diameter pot contained sandy soil with or without 30×10^6 *F. oxysporum* f. sp. *vasinfectum* spores (mixed into the soil before seeding). In treatments including nematodes, 20,000 *M. incognita* eggs were inoculated over the seed before it was covered. Data are mean percentages of each plant growth parameter, calculated from the mean value of the control as 100% for each genotype. Means with the same letter are not different. Data were compared among all treatments and all cultivars for each parameter.

^y P = probability level of analysis of variance and LSD = least significant difference at the 0.05 level.

^z Values in parentheses are means of control plant height (cm), shoot weight (g), and root weight (g).

TABLE 5. Effects of single and combined inoculations with *Meloidogyne incognita* (RKN) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 1 on nematode egg production on three cotton cultivars (Acala SJ-2, Acala NemX, and Pima S-7) using the seedling and the soil inoculation methods^y

Cultivar	Treatment and inoculation method			
	Seedling method		Soil method	
	RKN	RKN + FOV	RKN	RKN + FOV
SJ-2	4.239 a	3.138 b	3.793 a	2.402 b
NemX	2.040 c	1.514 c	1.315 c	1.375 c
Pima S-7	4.500 a	4.416 a	3.918 a	3.467 a
$P > F$	0.0001		0.0001	
LSD ^z	0.727		0.883	

^y Observations were made 60 days after inoculation. Nematode egg data were transformed to $\log_{10}(\text{eggs per gram root} + 1)$ for analysis and the values are means for nine replicate plants per treatment. P = probability level of analysis of variance and LSD = least significant difference at the 0.05 level within an inoculation method. Means within an inoculation method with the same letter are not different.

^z Data were compared among treatments of RKN alone and RKN + FOV in all three cultivars.

(C. Wang, unpublished data), and to Verticillium wilt in an F_2 (resistant Pima S-7 $\times$ susceptible Acala 44) population (5).

This study identified two AFLP markers that are linked to *FovI* in Pima S-7. For effective marker-assisted selection, markers must be tightly linked to the resistance genes. In our study, we had only 50 F_2 (Pima S-7 $\times$ NemX) plants to map *FovI*, and the two AFLP markers were not tightly linked to *FovI*. Therefore, these markers would not be useful for indirect selection of *FovI*-based wilt resistance, and converting them to PCR-based markers may not be worthwhile. However, the two AFLP markers could be useful for fine-mapping studies and map-based cloning of the *FovI* gene. To map *FovI* at the chromosome level, we will use microsatellite markers with known chromosome positions to screen the F_2 population. In the meantime, we will use more F_2 plants based on phenotype screening of $F_{2:3}$ families for fine localization.

The root-cut dip method of inoculation is useful for screening wilt resistance in cotton and has been used widely for pathogenicity tests of *F. oxysporum* f. sp. *vasinfectum* in cotton (20,30,31). Other inoculation methods, such as the seedling and soil methods

Fig. 3. Effects of single and combined inoculations with *Meloidogyne incognita* (RKN) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 1 (added to soil at planting) on shoot weight of three F_1 cotton genotypes. Observations were made 60 days after inoculation. Data are mean percentages of each plant shoot weight, calculated from the mean value of the control as 100% for each genotype. Bars show $\pm$ one standard error.

used in this study and the stem puncture method (7,16,28), also are informative but give less discriminating wilt disease reactions. When *F. oxysporum* f. sp. *vasinfectum* was added alone by either the seedling or soil method, we observed no differences in the aboveground symptoms exhibited by the three cultivars (Acala SJ-2, Acala NemX, and Pima S-7); these symptoms were always mild. In contrast, when the fungus was added with the nematode, we observed clear differences in symptom expression among the cultivars regardless of the fungus inoculation method.

Previous studies have shown important effects of pathogen resistance in cotton on the nematode-wilt disease complex, including the breakdown of wilt resistance by nematode infection (6,17, 32). Those studies, however, were based on genetically undefined resistance traits. Here, we were able to assess the pathogen and host interactions in the disease complex with cotton genotypes carrying characterized resistance genes, *FovI*-mediated wilt resis-

tance, and *rkn1*-mediated nematode resistance (34). The greatest reduction in plant growth occurred in Acala SJ-2, followed by Pima S-7 and Acala NemX. These genotype differences can be explained by their respective nematode and wilt resistance profiles. Acala SJ-2 is susceptible to both *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* race 1, as shown in the current experiments. Pima S-7, possessing gene *Fov1*, is resistant to *F. oxysporum* f. sp. *vasinfectum* race 1, but is highly susceptible to *M. incognita*. In these experiments, co-infection of Pima S-7 with *F. oxysporum* f. sp. *vasinfectum* race 1 and *M. incognita* caused severe wilt disease symptoms, indicating that nematode infection broke the wilt resistance conferred by *Fov1*. The smaller plant growth reductions and less severe wilt symptoms in Acala NemX in the combined *M. incognita* plus *F. oxysporum* f. sp. *vasinfectum* race 1 treatment were due to *rkn1*, a major gene that confers a high level of resistance to *M. incognita* (34). Furthermore, even though Acala NemX is wilt susceptible, it showed lower wilt disease than the highly susceptible Acala SJ-2 on several assay dates and a lower disease profile overall than Acala SJ-2 during the disease progression study.

The root-knot nematode–Fusarium wilt disease complex experiment among three F₁ (NemX × SJ-2, Pima S-7 × NemX, and Pima S-7 × SJ-2) genotypes provided further support for these findings. The F₁ (NemX × SJ-2) and F₁ (Pima S-7 × SJ-2) genotypes were nematode susceptible and grew poorly in the presence of both pathogens. However, the root-knot nematode-resistant and wilt-intermediate-resistant F₁ (Pima S-7 × NemX) genotype grew better than nematode-resistant and wilt-susceptible parent Acala NemX. Our results indicated that cultivars with resistance to *M. incognita* suffer much less wilt than cultivars with resistance to *F. oxysporum* f. sp. *vasinfectum* race 1 alone. Nematode resistance combined with intermediate wilt resistance was more effective than nematode resistance alone in protecting plants from the wilt disease. Similar interaction affects have been reported for nematode–wilt disease complex in other crop systems (4,8,25).

The *Fusarium* sp. caused much more damage to Acala SJ-2 when the fungal inoculum was added to the soil before planting than to the growing seedling. This result indicated that the early seedling stage of Acala SJ-2 (susceptible to both pathogens) is more sensitive to wilt infection than later seedling stages. More severe wilt infection developed in the 7-day-old seedlings than in the 10-day-old seedlings with the root-cut dip method for *F. oxysporum* f. sp. *vasinfectum* pathogenicity tests (data not shown). Pima S-7 exhibited less severe symptoms when 14-day-old seedlings were inoculated (20) than when 7-day-old seedlings were inoculated in the current study. Thus, how and when plants are inoculated with the pathogens can greatly affect disease expression, and laboratory and greenhouse screens may not represent the true resistance occurring in the field (33).

In the genetic study, severe damage to the roots in Pima S-7 after cutting resulted in five individual plants failing to recover after transplanting. Hillocks (13) also reported that the root-cut dip method may damage the roots and occasionally result in severe wilt even in resistant plants. In addition, we also observed that some plants we expected to be susceptible showed resistance, probably resulting from escaped infection due to large screening tests.

In summary, Pima S-7 was resistant to *F. oxysporum* f. sp. *vasinfectum* race 1, and the resistance was attributed to one gene (*Fov1*), with allele dosage effects based on genetic and AFLP marker analysis. Nematode resistance and wilt resistance of the host genotype affected the severity of wilt disease in the presence of both *M. incognita* and *F. oxysporum* f. sp. *vasinfectum* race 1. In co-inoculations with both pathogens, resistance to root-knot nematodes greatly decreased the wilt symptoms compared with resistance to *Fusarium* wilt alone.

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