MOLECULAR TECHNIQUES CORROBORATE RESISTANCE TO WHITEFLY TRANSMITTED COTTON LEAF CRUMPLE DISEASE

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Abstract

Ten cotton, *Gossypium hirsutum* L., cultivars or breeding-lines were evaluated in the field for resistance to the cotton leaf crumple (CLCr) disease caused by (Genus *Begomovirus*, Family *Geminiviridae*) *Cotton leaf crumple virus* (CLCrV) transmitted by silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring. The cultivars Texas 121, AP 4103, AP 6101 and Stoneville 474 and the breeding-lines were NK 2165C, NK 2108SS, NK 2387C, NKX C429-93-2ct, NKX 2907, and NKX 2207 were in Imperial Valley, CA. Cotton entries were rated for severity of CLCr disease symptoms and the presence of CLCrV in leaves of selected plants of each cultivar/breeding-line was determined by dot blot hybridization with a CLCrV DNA probe and PCR analysis with degenerate geminivirus primers. DNA sequencing of geminivirus DNA-A and DNA-B fragments, amplified from symptomatic cotton plants, was used to confirm geminivirus infection and partial characterize CLCrV. Results showed differences in whitefly infestation levels and virus disease symptoms among cotton entries. The cultivar AP 4103 had a higher CLCr disease rating than other entries except AP 6101. The breeding-line NK 2387C, with Cedix parentage, had a lower CLCr disease rating than other entries except Stoneville 474 and NK X2207. There were visible CLCr disease symptoms in Stoneville 474 and NKX 2207, but KN 2387C did not display visible CLCr disease symptoms nor was viral DNA detected in this line.

Introduction

Whitefly-induced economic losses to cotton occur as a result of reduced cotton yield (Mound 1965) and contamination of lint with honeydew and sooty molds (Davidson et al. 1994). The whitefly-transmitted cotton leaf crumple (CLCr) disease, caused by the begomovirus *Cotton leaf crumple virus* (CLCrV), can also cause extensive reduction in yield (Dickson et al. 1954, Duffus and Flock 1982). Cotton plant height and yield are significantly reduced by CLCrV infections at early and mid-growth stages (Brown et al. 1987). In 1991, silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring became a pest of cotton in the southwestern United States (Bellows et al. 1994). Insecticides provide temporary control of silverleaf whitefly (Chu et al. 1993 and Natwick 1993), but CLCr disease is still common. A long term solution that offers economical and environmental advantages is needed. Development of pest-resistant cultivars is a goal that warrants increased attention (Natwick 1995 and Natwick et al. 2000). The objectives of this study were to: 1) Use a viral rating system to evaluate cotton lines for resistance to CLCr disease; 2) Confirm that the virus associated with CLCr symptoms in the field plots in Imperial County is, in fact, a begomovirus and determine the relationship of this virus with previously characterized geminiviruses; and 3) Use molecular tools to evaluate resistance to CLCrV in upland cotton.

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Material and Methods

Ten cotton, *Gossypium hirsutum* L., cultivars or breeding-lines were evaluated in the field for resistance to the silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring, transmitted cotton leaf crumple (CLCr) disease caused by the begomovirus *Cotton leaf crumple virus* (CLCrV) in 2000 at the University of California Desert Research and Extension Center in Imperial Valley, CA. The experimental design was randomized complete block with four replicates. Each plot was 15 m long and 4 m wide. Rows were 1 m apart. Seeds of ten cotton genotypes were sown and irrigated on 28 March 2000. The cultivars were Texas 121, AP 4103, AP 6101 and Stoneville 474 and the breeding-lines were NK 2165C, NK 2108SS, NK 2387C, NKX C429-93-2ct, NKX 2907, and NKX 2207.

Whitefly adults were sampled weekly, starting 25 May through 28 August, from ten plants in each plot using the leaf turn method (Naranjo & Flint 1995). Ten 5th node from the terminal leaves per plot were taken weekly for sampling whitefly eggs and nymphs, starting 7 June through 28 August. Whitefly eggs and nymphs were counted on single leaf disks of 1.65 cm^2 from each of the ten leaves.

The following rating scale for CLCr disease symptom was used on 14, 23 and 28 August: 1 = leaf smooth, few if any bumps or blisters; 2 = some obvious blisters and crumpling, but less than 50% leaf with symptoms; 3 = Obvious crumpling, blisters, vein clearing from more than 50% to close to 100%, leaf not rolled; 4 = severe crumpling, blisters, leaves noticeably rolled and distorted.

To confirm that a geminivirus was associated with CLCr disease, DNA extracts were prepared from cotton leaves showing characteristic symptoms with a modified CTAB method (Gawe land Jarret, 1991). These extracts were used in the PCR with degenerate primers for the geminivirus DNA-A (pAL1v1978 and pAR1c496) and DNA-B (pBL1v2040 and pBV1c970) (Rojas et al. 1993). PCR-amplified DNA fragments were cloned and sequenced. Sequences were analyzed using the software of the Wisconsin Genetics Computer Group (GCG) and the Blast program was used to compare sequence to those in the DNA sequence database.

To evaluate resistance to CLCrV, ten representative leaves from each variety were collected 6 September, visually rated for CLCr disease symptoms according to the rating described above and then used in a dot blot hybridization analysis. Total genomic DNA was extracted from each leaf with the modified CTAB method (10 leaves/cultivar or line) and DNA from each leaf was dotted onto a nylon membrane. The membranes were hybridized with a cloned CLCrV DNA-A component probe under high stringency conditions (Gilbertson et al. 1991), which should provide CLCrV-specific detection. The intensity of the resulting hybridization signals was determined with a phospoimaging system and ImageQuant software (Molecular Dynamics).

Seed cotton was hand picked from 4-m sections of row in each plot on 5 October. Seed cotton weights were recorded and samples were ginned. Lint weights were recorded, and lint turnout percentages were calculated. Seasonal whitefly density and CLCr disease ratings were analyzed using ANOVA (MSTAT-C 1989). Student-Neuman-Keul's Multiple Range Test (SNKMRT) was employed for means separations.

Results and Discussion

The breeding-line NKX C429-93-2ct had more silverleaf whitefly adults per leaf (30.7) than any of the other entries; $P \leq 0.05$ (Table 1). The silverleaf whitefly adults per leaf for all other entries ranged from 16.6 for Stoneville 474 to 8.7 for NK 2387C. There were more silverleaf whitefly nymphs per cm^2 of leaf on NKX C429-93-2ct (25.5) than any of the other entries and There were more nymphs per cm^2 of cotton leaf (19.4) on Stoneville 474,

which has hirsute leaves, than on any of the other entries except NKX C429-93-2ct. The silverleaf whitefly nymphs per cm^2 for all other entries ranged from 8.8 (NK 2108SS) to 5.2 (AP 6101).

With a CLCr rating mean of 3.53, the cultivar AP 4103 had more severe CLCr disease symptoms than all other entries with the exception of AP 6101; $P \leq 0.05$ (Table 2). The cultivar AP 6101 had a CLCr disease rating of 3.10 which was a more severe CLCr disease rating mean than all remaining entries except NKX C429-93-2ct and NK 2108SS. The breeding-line NK 2387C, with Cedix parentage, had a lower CLCr disease rating mean (1.00) than all other entries except Stoneville 474 and NKX 2207 with a rating mean of 1.58. Stoneville 474 and NKX 2207 had visible CLCr disease symptoms, but NK 2387C did not display visible CLCr disease symptoms.

To confirm that a begomovirus was associated with CLCr, DNA was extracted from leaves with typical symptoms and subjected to PCR with degenerate geminivirus primers. From extracts prepared from all symptomatic leaves, the DNA-A primers directed the amplification of an approximately 1.1 kilobase (kb) DNA fragment, whereas with the DNA-B primers directed the amplification of an approximately 1.5 kb fragment. No fragments were amplified from extracts prepared from leaves with no disease symptoms. These results indicated: (i) the DNA obtained by the CTAB method was suitable for PCR amplification, (ii) that a geminivirus was associated with the CLCr disease symptoms and (iii) that the geminivirus is a bipartite begomovirus. The complete nucleotide sequences of the DNA-A (1226 nucleotides) and DNA-B (1526 nucleotides) fragments were determined and used in BLAST searches of the sequence databank. The results confirmed that these were begomovirus DNA-A and DNA-B sequences. The BLAST search revealed that the closest sequence in the database to the DNA-A sequence was that of Cabbage leaf curl virus (85% identity), whereas to the DNA-B sequence the closest sequence was Tomato leaf crumple virus (88%). We can conclude that our DNA-A and DNA-B represent those of a distinct geminivirus and, as there is no CLCrV sequence in the database, that our sequence represent those of a CLCrV isolate from the Imperial Valley of California. Consistent with this conclusion was the finding that the DNA-A sequence determined in this study was 98% identical to a DNA-A sequences amplified from cotton with CLCr symptoms in 1997 and 1999. These results also indicate the virus has not changed dramatically over the period of 4 years.

The CLCr disease ratings from ten representative leaves from each variety and the CLCrV-specific dot blot rating and PCR amplification results are presented in Table 3. The rating results indicated that AP 4103 had the highest disease rating (3.6), followed by AP 6101 (3.0); these materials are highly susceptible to CLCr. The breeding line NK 2387C had the lowest rating (1.0) and is highly resistant to CLCr, whereas Stoneville 474 is also resistant (1.2). These results are consistent with those of the visual rating taken in August (Table 2). The dot blot results were consistent with the visual ratings in regard showing that breeding line NK 2387C was highly resistant (dot blot rating of 1.8, which is essential virus-free) and Stoneville 474 was highly resistant (dot blot rating 6.2) (Figure 1). The varieties AP 4103 and AP 6101, with the highest CLCr disease ratings, also had the high dot blot ratings (36.5 and 24.2, respectively. The other materials can be considered to be moderately susceptible (Table 3; Figure 1). It is interesting to note that NK 210855 and NKX 2207, which both had visual ratings of 1.8, had considerably different dot blot ratings (28.1 and 14.9, respectively). This suggests that some degree of disease tolerance may exist in NK 210855. These results suggest that dot blot hybridization may be an effective method for evaluating cotton lines for CLCr resistance. The dot blot method also did not have the high levels of background hybridization that were previously experienced with squash blot hybridization analyses.

The results of the PCR detection experiments revealed high levels of CLCrV infection is all plants except those of NK 2387C, which appears immune to CLCrV based on these results (Table 3). The PCR results are consistent with the detection of some degree of CLCrV infection in all the other breeding lines/cultivars based on the dot blot results. However, the PCR data is not particularly informative in regards to indicating relative levels of resistance and, thus, is not a method of choice for evaluating resistance to CLCrV.

With the exception of Stoneville 474 and NK 2108SS, the breeding-line NK 2387C had the highest percentage turnout of lint (40.4 %) among cotton entries; $P \leq 0.05$, (Table 4). NK 2907 had the lowest percentage turnout (35.3 %). The cultivars AP 6101 and AP 4103 had the greatest yield as pounds of lint per acre with 1069 and 1051 pounds, respectively, which was greater than all other entries except NK 2387C, Stoneville 474 and NK 2108SS, $P \leq 0.05$, (Table 4). The lowest cotton lint yield was 602 pounds per acre for NKX 2907, but was not lower than other entries in the study except AP 6101, AP 4103 and NK 2387C.

We confirmed that the whitefly-transmitted begomovirus CLCrV is associated with CLCr disease symptoms in Imperial Valley cotton. There are differences among the cotton entries for adult whitefly densities, but the differences in severity of CLCr disease symptoms among entries does not appear to be related to whitefly densities. NK 2387C appears to be immune to CLCr disease. Heritable traits for CLCr disease resistance in NK 2387C should be developed and release as a CLCrV-resistant cotton variety.

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Table 1. Silverleaf whitefly seasonal means for adults per leaf and nymphs per cm^2 of leaf for cotton varieties, Holtville, CA, 2000.

Variety	Adults[a]	Nymphs
NKX C429-93-2ct	28.6 a	25.5 a
Stoneville 474	16.1 b	19.4 b
NK 2108SS	12.0 bc	8.8 c
NKX 2207	11.6 bc	8.7 c
AP 6101	11.4 bc	5.2 c
AP 4103	10.8 bc	6.1 c
NKX 2907	9.8 bc	7.7 c
Texas 121	9.8 bc	7.4 c
NK 2165C	9.1 bc	5.3 c
NK 2387C	8.7 c	6.1 c

[a] Log transformed data used for analysis; reverse transformed means reported. Mean separations within columns by SNKMRT, P≤0.05.

Table 2. CLCr disease ratings on cotton varieties and breeding-lines, 2000.

Variety	14 Aug	23 Aug	28 Aug	Mean
AP 4103	2.96 a	3.75 a	3.88 a	3.53 a
AP 6101	2.54 ab	3.25 ab	3.50 ab	3.10 ab
NKX C429-93-2ct	2.45 ab	3.12 ab	3.00 bc	2.86 bc
NK 2108SS	2.58 ab	2.63 bc	2.63 cd	2.61 bc
Texas 121	2.35 ab	2.13 cd	2.50 cde	2.33 cd
NK 2165C	1.83 bc	1.88 cde	1.84 def	1.85 d
NKX 2207	1.60 cd	1.63 de	1.94 def	1.72 d
NKX 2907	1.39 cd	1.75 de	1.75 ef	1.63 de
Stoneville 474	1.61 cd	1.50 de	1.63 f	1.58 de
NK 2387C	1.00 d	1.00 e	1.00 g	1.00 e

Mean separations within columns by SNKMRT, P≤0.05.

Table 3. Evaluation of various cotton cultivars for response to cotton leaf crumple virus based upon visual rating of disease symptoms, dot blot detection of viral DNA, and PCR detection of viral DNA.

Variety	Disease Rating	Dot blot Rating	PCR detection No. (+) / Total tested
AP 4103	3.6	36.5	10 / 10
AP 6101	3.0	24.2	10 / 10
NKX C429-93-2ct	2.2	19.1	10 / 10
Texas 121	2.1	40.0 (30.3)[a]	9 / 10
NK 2108SS	1.8	28.1	9 / 10 (1?)[b]
NKX 2907	1.8	22.8	10 / 10
NKX 2207	1.8	14.9	8 / 10 (2?)[b]
NK 2165C	1.8	11.2	5 / 8 (3?)[b]
Stoneville 474	1.2	6.2	9 / 10
NK 2387C	1.0	1.8	0 / 10

[a] Numbers in parenthesis represents mean if one abnormally high sample is discarded.

[b] Numbers of plants which are not clearly determined.

Table 4. Pounds per acre seed cotton and lint, and lint turnout percentages.

Variety	Seed cotton	Lint	Lint % Turnout
AP 6101	2849.5 a	1069.5 a	37.5 bcde
AP 4103	2821.2 a	1051.1 a	37.2 bcde
NK 2387C	2371.2 ab	957.7 ab	40.4 a
Stoneville 474	2025.3 b	797.1 abc	39.3 ab
NK 2108SS	1995.3 b	784.7 abc	39.3 ab
NK 2165C	1881.9 b	705.7 bc	37.2 bcde
Texas 121	1899.5 b	701.3 bc	36.9 bcde
NKX C429-93-2ct	1812.0 b	689.5 bc	38.1 bcd
NKX 2207	1736.9 b	636.7 bc	36.2 de
NKX 2907	1703.7 b	602.0 c	35.3 e

Mean separations within columns by SNKMRT, P≤0.05.

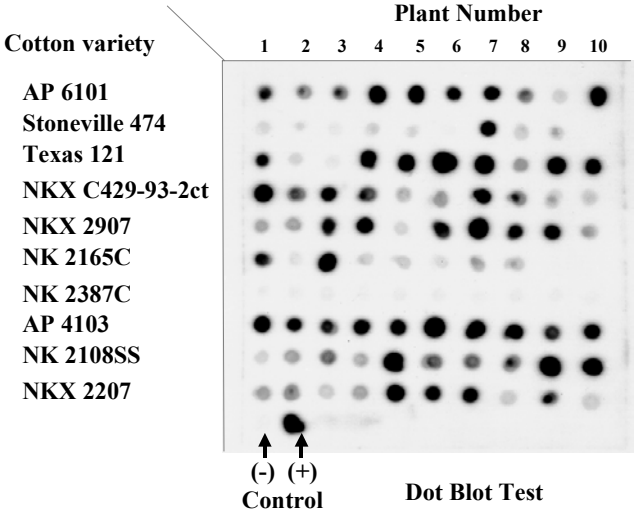

Figure 1. Evaluation of various cotton cultivars/breeding-lines for resistance to cotton leaf crumple virus based upon dot blot hybridization analysis with a CLCrV-specific DNA probe.