Sequence characterized amplified regions for the *Cotton Leaf Curl Virus* resistance in the cotton genome (*G. hirsutum*)

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ABSTRACT

Cotton Leaf Curl Virus (CLCuV) is a devastating pest in the North India and in small pockets in Southern states. Recent molecular biology technique like RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment length Polymorphism) approaches enhance the breeding program. The insect and pest resistant traits are governed by the single/double dominant/recessive genes and by the use of molecular markers the characters can be linked to the cotton genome. The genotypes can be identified with the molecular markers resistant to CLCuV and genetically distinct lines can be exploited for crop improvement program. The molecular marker will help the breeding program in screening the F_2 mapping population. The Cotton Leaf Curl Virus resistant and susceptible genotypes CNH 123 and CNH 1020 were characterized using 80 decamer primers by amplification in a polymerase chain reaction. The primer OPC 02 amplified a unique polymorphic fragment in the CLCuV resistant lines CNH 123 and CNH 1012 designated as OPC 02 (1700 bp). Ten resistant and susceptible F2 DNA were pooled for bulk segregant analysis and amplified with the same primer OPC 02, which also produced the 1700 bp fragment and confirmed it repeatedly. This fragment has been converted into SCAR marker and the primer pair designed was 5' GTGAGGCGTCAGAGGGAT-3' (forward) and 5'-GTTGCCGTGCACTAGGCT-3' (reverse). The F_2 segregating RAPD loci were mapped using Mapmaker program into ten groups. The SCAR marker segregated in a Mendelian fashion n the F2 population.

Introduction

Cotton is the most important source of natural fiber and is the second most important oilseed crop. There are four cultivated cotton species, two diploid (*G. arboreum* and *G. herbaceum* 2n=26) and two tetraploids (*G. hirsutum* and *G. barbadence*, 2n =52). Molecular biology technique like RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment length Polymorphism) approaches enhance the breeding program and help in targeting the gene responsible for leaf curl virus. Extensive gene pool exists in the germplasm and wild species, which is potentially available to cotton breeders and is yet to be exploited. Rates of unimproved germplasm carry genes that are linked to the desired traits on the chromosome segment from the germplasm. The disease was first recorded in *G. barbadence* in Nigeria in the year 1912. In 1926, it was established that the disease was caused by viral infection. In 1930, it was confirmed that the leaf curl-causing virus was a Gemini virus transmitted by white-fly *Bemisia tabaci*. The number of bolls reduces by 15% - 87%, boll weight reduces by 0 - 39%, boll weight reduces by 0 – 39% and yield loss recorded up to 58% - 69%.

CLCuV is a serious problem and identifying the true resistant line is a must for the breeders, which is possible by molecular biology techniques. The pest resistant traits can be linked to the cotton genome as they are governed by the single/double dominant/recessive genes and by the use of molecular markers. Genetically distinct lines can be exploited for crop improvement program. The genotypes can be identified with the molecular markers resistant to leaf curl virus. The molecular marker will help the breeding program in screening the F_2 mapping population. Additionally it will establish the database on molecular markers that can be utilized for Marker Assisted Selection (MAS) in the development of leaf curl virus resistant cultivars. MAS is a new molecular tool with the potential to enhance selection efficiency in cotton (Meredith, 1994). MAS enables to select accurately for rapid return to parental type and for minimal linkage drag associated with target gene/s (Young, 1998; Tanksley, 1989). So far, attempts have been made to map the cotton genome with molecular markers for yield component and fiber quality genes (Wang, 1993; Stelly, 1993; Cantrell, 1993; Paterson, 1993; Wing, 1993; Reinisch, 1994; Shappley, 1996). Several reports are available for insect and pest resistant gene linkage with the molecular markers in crops like *Oryza* (Wang, 1994), tomato (Sandbrink, 1995), sunflower (Mouzeyar, 1995), wheat (Schachermayr, 1994), barley (Chen, 1994) etc. The objective of the present research evolves tagging the molecular marker for CLCuV in cotton through molecular biology techniques.

Experimental procedure

Plant material

Seeds of the cotton germplasm accessions CNH 123(RCLCuV), CNH 1012(RCLCuV), CNH 1020(SCLCuV) and CNH 120(SCLCuV) were obtained from the gene bank. The plants were grown in pots in a glasshouse. Crossing between susceptible line and resistant line were carried out to obtain the F_1 and it was forwarded to F_2. The resistant and susceptible parents were scored for the symptoms as zero for complete absence of the disease and 'E' as the enation due to severe leaf curling and stunting of the plant. The following crosses made were:

1. CNH 120(SCLCuV) X CNH 123(RCLCuV)
2. CNH 1020 (SCLCuV) X CNH 123(RCLCuV).

DNA isolation

DNA isolation chemicals were obtained from (Hi media Ltd., India), DNA amplification kit (Bangalore Genei Pvt. Ltd., Bangalore, India) and oligonucleotide primer (Operon technology, Alameda, CA, USA). The seeds were imbibed in water overnight and the outer coat was removed. Seed tissue (20 mg) were crushed with the extraction buffer (0.1M Tris HCl, 0.05 M EDTA (pH 8.0), 0.5 M NaCl, sodium dodecyl sulphate (5 ml) and transferred to an Eppendorf tube. To the extract 7.5 M ammonium acetate was added and incubated at 65 ^{0} C in water bath for 30 min. The extract was cooled and centrifuged at 15,000 rpm in a refrigerated centrifuge. The supernatant was transferred to fresh tube and equal volume of isopropanol was added and incubated at -20 ^{0}C for 10-min .The content was centrifuged at 4 ^{0}C for 15 min at 14,000 rpm. The pellet obtained was rinsed with 70% ethanol, air-dried and dissolved in TE buffer (0.01M Tris-HCl; 0.0001 M EDTA, pH 7.4).

Polymerase chain reaction (PCR)

The DNA was used as template for PCR amplification. The PCR reaction mixture contains 2 μl of DNA (10 ng conc.), 0.02 M $MgCl_2$, 0.05 M KCl, 0.2 μM primer, 100 μM dNTPs, 0.5 U of Taq polymerase and amplified in a Bio Metra Thermal cycler for 35 cycles of 1 min at 94 ^{0}C, 1 min at 35 ^{0}C and 2 min at 72 ^{0}C by initial denaturation step of 4 min at 94 ^{0}C and a final synthesis step of 10 min at 72 ^{0}C. The amplified products were resolved on 1.5% (W/V) agarose gel along with lambda marker (double digest) in 1X TBE buffer and the bands were visualized by staining with ethidium bromide. The genomic DNA from the susceptible and the resistant plant were isolated and quantified in UV-Spectrophotometer. The quality of the DNA was checked by agarose gel electrophoresis.

Data analysis

Linkage analysis was conducted as per Lander *et al*. (1987) using the software Mapmaker / Exp. 3.1b. Markers were ordered with a minimum of the odds ratio (LOD) score (3.0) and maximum distance (50.0). Recombination fractions were converted into centiMorgans (cM) by applying the Haldane function.

Results and Discussion

The amplification profiles were studied for the genetic polymorphism. Eighty primers amplified a total of 392 scorable DNA fragments out of which 20% were polymorphic. The rest of the bands were found to be monomorphic. The Primer OPC 02 amplified a unique polymorphic fragment in CLCuV resistant lines CNH 123 and CNH 1012 designated as OPC 02 (1700 bp) (Figure 1). The F1 resulted by the cross between CNH 120(SCLCuV) X CNH 123(RCLCuV) and CNH 1020 (SCLCuV) X CNH 123 (RCLCuV) has introgressed the unique 1700 bp fragment. Ten resistant and ten susceptible F_2 DNA were pooled to carry out bulk segregant analysis and they were amplified with the same primer OPC 02 which has also produced the 1700 bp fragment. The PCR product was stable in the same RAPD experiment with three repeats of amplification. The 1700 bp fragment segregated in Mendelian fashion in the F_2 lines. In order to transform the RAPD marker into SCAR marker we eluted the band and purified the DNA. The primer pair was designed based on the sequence. Primer pair designed was 5'-GTGAGGCGTCAGAGGGAT-3' (forward) and 5'-GTTGCCGTGCACTAGGCT-3' (reverse). The PCR products resulted from SCAR primers were the same with the RAPD amplifications in the template DNA of parents and F_2 population. The result shows the resistance was controlled by single dominant gene showing the Mendelian 3:1 ratio in the F_2 population. This marker may be applied in the marker-assisted breeding. The RAPD products were analyzed using the Mapmaker program. The linkage groups with RAPD markers (Figure 1) were numbered after the primer kit and designated as F 174 and so on. RAPD markers were linked into ten linkage groups out of which in linkage group I and VI they were clustered together and in others they were located apart from each other.

The results show the presence of the unique band in the resistant lines, which reveals that the gene expression may be dominant in nature and it will be helpful in tagging CLCuV resistant gene in the mapping population. The unique resistant and susceptible nature of these genotypes makes them suitable parents to generate mapping population. The degree of introgression between *hirsutum* genotypes would generate a high level of polymorphism. Some more tolerant lines are available in the exotic germplasm as reported by Iqbal *et al.* (1997), CIM 1100 is distinct and tolerant to CLCuV disease. Our results supports the earlier reports by Siddiq *et al.* (1970) that the resistance to CLCuV is controlled by a single dominant gene. Also, Ali Mahbub (1999) made crosses between highly susceptible and resistant parents and they came to conclusion that a single dominant gene is involved in the control of resistance to virus attack. The genetic control of their resistance to the disease is not yet certain. The study is in progress in our laboratory.

The potential benefits of a marker-assisted selection strategy have been discussed widely by Paterson *et al.* (1991). In this study, PCR- based marker has been linked to the CLCuV resistant gene, which is as reliable as an RFLP marker. In our study, bulk segregant analysis assumes that markers adjacent to the target gene will be in linkage equilibrium (i.e. recombination will not randomize the markers with respect to the gene) and as the linkage distance increases, more recombinants will be present in the bulk, culminating in 50% recombinants, no linkage disequilibrium and therefore no difference between the bulk as reported by Michelmore *et al.* (1991). By following this in the present study we have identified RAPD markers linked to the CLCuV resistant gene.

To screen markers linked to single traits with in bulks, RAPD markers provide useful alternative to RFLP analysis. Although the RAPD markers identified in the present study are sensitive to minor changes of conditions, the sequence of the RAPD products could be determined and a specific pair of this region could be generated to tag specific regions reliably as reported by Paran and Michelmore (1993). RAPD amplification can likely be initiated from genomic sites that perfectly match primer sequence. An unpredicted decrease in the number of amplification products has been observed due to decrease in the primer length as discussed by Caeteno-Anolles *et al*. (1992). This study reveals that the GC rich primers bring about amplification. RAPD markers were linked into fifteen linkage groups out of which in linkage group I and VI many were clustered together and located apart from each other showing the amplification may be from the same region of the chromosome and from different region proving the existence of polymorphism.

The SCAR marker identified in this study are the part of the resistant gene and will be helpful for the breeders to screen the breeding material in the F_2 generation itself to select the homozygous resistant line and to develop into a cultivar instead of wasting time, land and manpower until F_7/F_8 generation and even then instability occurs due to segregation.

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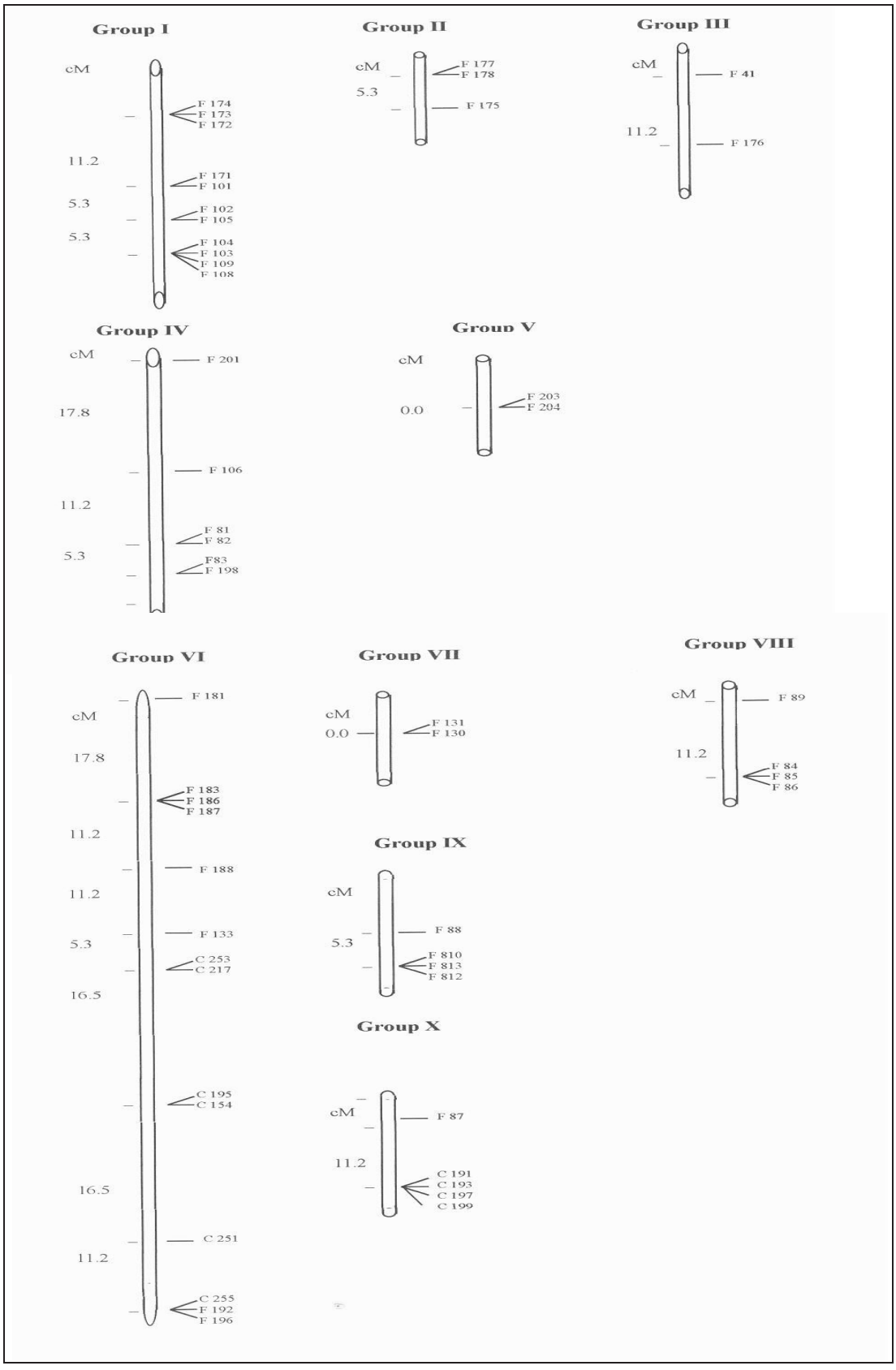

Figure 1.

Linkage groups for RAPD markers. Map Scale is 5.0 cM/cm (Kosambi mapping function. Log-likelihood: -50.00).