# IDENTIFICATION OF MOLECULAR MARKERS LINKED TO LEAF CURL VIRUS DISEASE RESISTANCE IN COTTON

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## Abstract

The identification of molecular markers linked to leaf curl virus (CLCuV) disease resistance in cotton has the potential to improve both the efficiency and the efficacy of selection in cotton breeding programs. Genetic analysis suggested that CLCuV resistance is controlled by a single dominant gene. In this study, an interspecific $F_2$ population derived from a cross of *Gossypium barbadense* and *Gossypium hirsutum* was phenotypically classified into CLCuV susceptible and resistant plants. A subset of these $F_2$ plants was evaluated by selective genotyping, with restriction fragment length polymorphism (RFLP) to identify DNA markers linked to the CLCuV resistance gene. Sixty seven $F_2$ derived $F_3$ families were evaluated for segregation at 137 RFLP loci. Three DNA marker loci, linked to each other, also showed significant association with CLCuV resistance. Sequencing of linked markers will permit locus-specific DNA primers for use in PCR-based identification of CLCuV-resistant plants in breeding populations.

## Introduction

Cotton (*Gossypium hirsutum* L.) is the most important cash crop of Pakistan and is grown over about 2.8 million hectares; 10% of the arable land in the country. It provides more than 90% of the raw material to the 350 textile mills and 1100 ginning factories and constitutes 60% to domestic edible oil production. Recently cotton leaf curl virus (CLCuV) disease has emerged as the most important disease of cotton in Pakistan. CLCuV is largely responsible for the drop in cotton production from 12.8 million bales in 1991-92 to about 8.0 million bales in 1993-94; equivalent to a decrease in gross domestic product (GDP) of 3-4%. The impact on Pakistan's farm and household income, downstream manufacturing sector and foreign exchange earnings has been severe.

**Keywords:** Cotton; RFLP; Molecular markers; CLCuV disease resistance; Linked; Identification

CLCuV disease was first observed near Multan in 1967 [1] and has been noted consistently since then. The disease reached economic importance in 1987-88 and became epidemic in 1991-92. CLCuV disease is characterized by the upward curling of leaves and the thickening of leaf veins (more pronounced on the underside). In extreme cases, the formation of a cup-shaped or leaf laminar out-growth called "enations" appears on the underside of the leaf. The CLCuV disease is transmitted by the feeding of the whitefly,

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*Bemisa tabaci* (Genn) and it has many alternate hosts among cultivated and wild *Malvaceae* (mallows, including cotton). It was confirmed that CLCuV belonged to the *Gemini* group, whose vector is whitefly [7].

Measures such as the control of insect vectors and crop rotation help to control the disease, but resistant cotton varieties must be developed to overcome this epidemic. The objectives of the present study were to identify the DNA marker(s) linked to CLCuV disease resistance and to develop a methodology to accurately identify CLCuV-resistant genotypes in early segregating generations.

## Materials and Methods

### Population Development and Phenotypic Analysis

Crosses were made between *G. barbadense* L. (Giza-45) susceptible to CLCuV and *G. hirsutum* L. (Reba P-288) resistant to CLCuV. An $F_2$ population of 285 individuals was grown from self-pollinated progeny of $F_1$ plants. Both the $F_1$ and $F_2$ populations were exposed to CLCuV disease under natural infestation during 1994 and 1995, respectively, using spreader rows of highly susceptible cultivar S-12 to encourage uniform inoculation. Disease intensity was measured as described by Siddig [11]. Plants without any symptoms were scored as resistant, while plants with vein thickening and severe curling, or with "enations" were scored as susceptible.

### DNA Marker Analysis

DNA was extracted from bulked $F_3$ tissue samples [9]. DNA was digested with each of four restriction endonucleases (EcoRI, EcoRV, Hind III and XbaI), according to the manufacturers instructions (Promega, Madison, WI). Restriction fragments were separated by 0.8% agarose gel electrophoresis for 22-26 h at 16-22 Volts. Southern blotting and autoradiography were performed [10]. Linkage map and related statistics were determined using Mapmaker 1.0 [6].

## Results

### Inheritance of CLCuV Resistance

Resistance to CLCuV, derived from *G. hirsutum* line Reba P-288 appears to behave as a single dominant gene in this cross. $F_1$ plants were exposed to CLCuV and none showed symptoms of the disease. Among 285 $F_2$ plants, 223 were resistant; 62 were susceptible. These data are not significantly different from a 3:1 ratio ($\chi^2 = 1.606$, $p > 0.2$).

### Genetics Mapping of CLCuV Resistance

Based on the inference that a single locus controlled CLCuV resistance in this cross, our strategy for genetic mapping used the method of "selective genotyping" [5]. From the $F_2$ population, 35 susceptible plants and 32 resistance plants with unequivocal phenotypes were selected, and selfed to provide $F_3$ families which could be pooled to represent the DNA marker genotype of the individual $F_2$ plants (cf. Paterson *et al.*, 1991). A total of 137 DNA marker loci detected by 112 probes were evaluated. These DNA probes were chosen based on a previously-constructed map of a *G. hirsutum* × *G. barbadense* cross [10] and were found to be discernibly linked to about 80% of the tetraploid cotton genome.

A total of 3 loci, linked to each other, showed significant association with CLCuV resistance. For three of the markers, none of the 35 susceptible plants showed presence of the *G. hirsutum* (resistance) allele. A genetic map of the region surrounding the CLCuV resistance gene is shown in Figure 1. Two DNA marker loci, detected by probes *A1215* and *A1826*, essentially co-segregate with the locus (one at 0.0 cM and one at 0.1 cM). However, in both cases the Polymorphic restriction fragment linked to CLCuV-R is "dominant", therefore we cannot discern homozygotes from heterozygotes. This has the consequence that our estimates of 0.0 and 0.1 cM have a confidence interval of about ±5 cM, i.e. the makers could be as far as 5 cM from the locus. A third, dominant, restriction fragment detected by the probe *pGH318*, lies about 11.6 cM distal from the locus with a confidence interval of about ±5 cM. Finally a co-dominant locus detected by the probe *pGH286* lies about 29.1 cM proximal to the locus.

The restriction fragments detected by *pGH286* correspond closely in size to a pair which have previously been mapped to cotton chromosome 4 (A.H.P., unpubl.), based on analysis of monosomic substitution stocks [10]. The restriction fragments detected by each of the other three probes have not been previously mapped, however other restriction fragments detected by these probes do not map to chromosome 4, but to other chromosomes (*A1215*, to homoeologous chrs. 6 and 25; *A1826* to putative chr. 22; *pGH318* to homoeologous linkage groups A02 and D03; [10]). Although the *pGH286* restriction fragments segregating in this population appear to be very close to the same size as those previously mapped to chromosome 4, the probe also detected several other restriction fragments in the same size range, therefore we consider the assignment to chromosome 4 to be tentative. Additional evidence from new DNA probes will be sought to

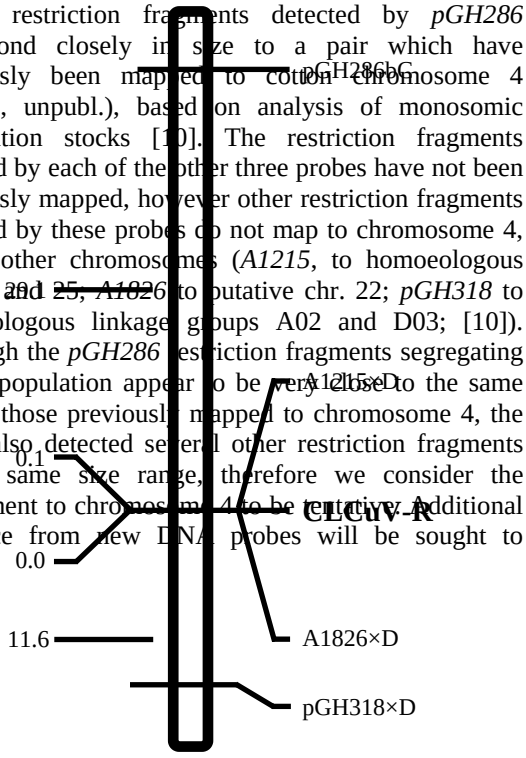

**Figure 1.** Genetic map of CLCuV resistance gene in cotton. The linkage group harboring CLCuV resistance is delineated by four DNA probes (names shown at right), that have been previously mapped [10]. The lower case letter "b" for p*GH286* indicates that multiple loci segregate for this probe, and the segregating DNA restriction fragments correspond to a locus previously designated b. The lower-case "x" following the other three probes, indicates that the specific restriction fragments segregating, have not previously been mapped. Recombinational distances (left) are expressed in centiMorgans [4].

further test this hypothesis.

## Discussion

Establishment of DNA markers diagnosis for CLCuV resistance represent an important step toward accelerated development of cotton cultivars resistance to this relatively new pathogen. With molecular markers, the cotton breeder will be able to select plants resistant to CLCuV on a genotypic basis; an important asset to existing cotton breeding techniques. With this approach, the development of CLCuV-resistant cotton varieties may become more efficient. Sequencing of the CLCuV resistance markers is in progress for the identification of locus-specific DNA primers which can be used for PCR-based identification of CLCuV-resistant plants. In conjunction with efficient techniques for isolating DNA from small amounts of cotton leaves (R. Wright, C. Jiang, A.H.P., unpubl.) or cotton seeds [12], these data will provide the basis for rapid screening of segregating populations for CLCuV resistant plants, using techniques which are well-described [2,3].

Although CLCuV resistance is not presently a significant problem in some cotton producing countries, such as the USA, the identification of DNA markers linked to a resistance gene provides the opportunity for "pre-emptive breeding" and developing resistant cultivars in the absence of a selective pressure. Such actions might reduce the likelihood that CLCuV would become established in locations it has not yet reached. Moreover, the use of a DNA marker-based assay enables one to select for resistance without having to infect plants with the pathogen, thereby reducing the risk that the pathogen might escape into a new environment. CLCuV resistance is the first viral resistance gene to be mapped in cotton. In other *taxa*, "clustering" of resistance genes at specific locations in the genome has often been found suggesting that this region of the cotton genome is a likely place to look for additional genes conferring resistance to other viruses of cotton. Further, linked DNA markers, such as we describe for CLCuV resistance, are a logical starting point for molecular cloning of genes for which there is little information on specific biochemical functions. The cotton genome map presently stands at more than 1300 DNA loci (A.H.P., pers. comm.), and is rapidly approaching a marker density suitable for "chromosome walking". The possibility of cloning the CLCuV resistance gene would open up new opportunities to better understand this plant-virus interaction at the molecular level.

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