Cotton Leaf Curl Virus Epidemic in Pakistan: Virus Characterization, Diagnosis and Development of Virus Resistant Cotton through Genetic Engineering

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The cotton leaf curl virus (CLCuV), a whitefly-transmitted geminivirus (Mansoor et al, 1993) has caused heavy losses to the cotton crop and still remains the most important constraint for the development of the cotton sector in the country. The symptoms produced on the cotton plant are leaf curling, thickening of veins, enations and stunted plant growth. Cotton leaf curl disease was recorded as early as 1967 from Pakistan (Hussain et al, 1991). Since 1991/92, cotton leaf curl disease

that was only curiosity previously is now a major threat to the sustainability of this crop. The epidemic of CLCuV in Pakistan is one of the best examples of the dramatic shift in importance of an unimportant endemic disease in the past. It is estimated that the disease has resulted in a loss of 4.98 million bales of cotton with an estimated value of US$7.4 billion.

Recent advances in molecular biology and genetic engineering have opened new avenues in understanding and controlling the disease epidemics. Genetic engineering of crop species such as cotton allows introduction of a specific character such as disease resistance to be incorporated in existing varieties without compromising other agronomic characters. This technology is superior to conventional plant breeding as breeding for disease resistance using resistant germplasm may result in some undesirable characters contributed by resistant germplasm.

The National Institute for Biotechnology and Genetic Engineering (NIBGE) is a federal research institute with a mandate to apply modern and innovative techniques in agriculture, health, environment and energy. Realizing the potential of molecular biology and genetic engineering in solving the CLCuV problem in the country, NIBGE initiated the research program with the following objectives.

- Biological and molecular characterization of CLCuV which includes virus purification, cloning and sequencing of the genomic components and generation of infectious clones.
- Development of PCR/DNA probe-based diagnostic test for the detection of virus in insects and plants and use of this diagnostic test for the identification of alternate hosts of CLCuV.
- Development of virus-resistant cotton through genetic engineering.
- Molecular diversity and distribution of virus in cotton growing areas of Pakistan.

The Cotton Group of NIBGE extensively studied leaf curl disease and made vital contributions to solve this problem.

Biological and Molecular Properties

A basic understanding of the causative agent is essential for devising control strategies. Some of the biological properties such as insect transmission, transmission of virus to indicator host plants, detection of natural alternate hosts, virus particle morphology and detection of virus coat protein by Western and ELISA was carried out. The molecular characterization of virus is essential for genetically engineered resistance as well as development of molecular diagnostic methods. The work on molecular characterization of CLCuV includes cloning of full length genomic components, determination of complete nucleotide sequence, generation of infectious clones and evaluation of molecular diversity of CLCuV.

Biological Properties

Insect Transmission Studies

Our research has shown that CLCuV can be transmitted from cotton to cotton and tobacco by whiteflies. Factors affecting insect transmission efficiency have been studied. The efficiency of virus transmission was greatly affected by temperature. At higher temperatures (40-45C^{o}) the virus efficiency was increased ten fold and CLCuV could be transmitted by a single whitefly. Aphids and jassids were also tried but were unable to transmit the virus, thus whitefly is the only insect vector of CLCuV.

Experimental Host Range

Traditionally plant viruses are experimentally transmitted to some indicator host plants for the observation of symptoms produced. Tobacco plants which were infected by whitefly transmission were used in graft transmission. Infected tobacco leaves were grafted onto *Nicotiana benthamiana*, *N. tabacum* var. Samsun, datura and tomato var. moneymaker. Grafting from cotton to cotton was done to maintain virus infected plants. For sap inoculation a young infected leaf was ground in 0.06 M phosphate buffer pH 6.5, centrifuged in an Eppendrof tube for 3 minutes and supernatant was collected. Sap mixed with carborundum was rubbed on young leaves of tobacco, *N. benthamiana* and tomato plants. CLCuV was successfully transmitted by grafting from tobacco to *N. benthamiana*, tomato and datura and all these plants showed identical symptoms. Mechanical inoculation did not show symptoms on indicator plants suggesting that CLCuV could not be transmitted by sap.

Preparative Scale Purification of Geminivirus Particles from Infected Plants

Cotton is a woody plant and upon homogenization of tissues produces a lot of phenolic compounds which interfere with virus purification. Conditions were optimized for the purification of intact geminivirus particles from infected cotton plants. One to three months old healthy and infected cotton plants were used in this study. The geminivirus particles isolation was performed according to Czosnek et al. (1988) with some modifications. The samples were treated with 2% aqueous uranyl acetate (negative staining) and observed on JEOL transmission electron microscope at a power of 40 Kv. Intact geminivirus particles were observed in one of the fractions. The optimization of isolation procedures of CLCuV from the cotton plant itself is the first report of its kind in the world. The purification of intact virus particles has allowed development of antisera which can be used for the detection of virus in infected plants by ELISA.

Molecular Properties of CLCuV

DNA Isolation, PCR Amplification and Cloning of PCR Amplified DNA

Total DNA was isolated from tobacco leaves by the Kirby

method. For the isolation of total DNA from cotton a method modified from Doyle (J.K.Brown, personnel communication) was used. Universal primers for dicot-infecting geminiviruses were used in PCR for the amplification of CLCuV DNA. African Cassava Mosaic Virus (ACMV) and Indian Cassava Mosaic Virus (ICMV) were used as a positive control where as DNA isolated from healthy *N. benthamiana* was used as a negative control. The PCR product of the expected size was obtained both from infected cotton and tobacco plants. The amplification of viral DNA was confirmed by using ACMV DNA A as a probe. PCR amplified DNA was digested with SaII and EcoRI and a fragment of about 1.2kb was cloned in SaII-EcoRI site in Bluescript vector. For the amplification of DNA B degenerate primers reported by (Rojas et al, 1993) were used in PCR. ACMV which is known to be amplified by these primers was used as a positive control. However, no amplification was obtained with primers for DNA B.

Cloning of Components DNA-1 and DNA-2

Double stranded replicative form of CLCuV was purified from infected cotton plants. Clones of DNA 1 of CLCuV were selected by southern hybridization. Full length clones of genome A were obtained either by cloning at unique restriction site or by combining two cloned fragments in such a way that desired open reading frames (ORF) remained intact. Sequencing of several clones was carried out and two variable clones of DNA 1 were identified named as CLCuV Pak-1 and CLCuV Pak-2. Analysis of clones identified another clone which did not hybridize to DNA-1clone but hybridized to viral DNA. The sequence analysis of this clone did not show homology to know geminiviruses. The clone did not hybridize to intergenic probe. This clone has been named as CLCuV DNA-2. PCR primer designed on the basis of this clone suggest that this genomic component is associated with both whitefly-transmitted geminiviruses associated with the disease.

Complete Nucleotide Sequence of CLCuV Pak-1

CLCuV Pak-1 was completely sequenced by dideoxy chain termination method using radioactive 35S or 33P dATP. The sequence data were assembled and open reading frames were identified. The two variable clones have genome organization typical of old world geminiviruses. There were two open reading frames AV1 and AV2 in the virus sense where as five open reading frames in complementary sense namely AC1, AC2, AC3, AC4 and AC5 were identified. The two strains (Pak-1 and Pak-2) differed in their genome size and predicted amino acid sequence of complementary sense genes. However, the most striking differences were in the intergenic or common region. The sequence data has been submitted to EMBL data bank and this is the first sequence of CLCuV in databank.

Total No. of nucleotides = 2749
Length of common region = 278

Table 1. The Genome Organization of CLCuV Pak-1. EMBL Accession No. X98995 (Mansoor et al, 1996)

ORF	Reading frame	Start	Stop	No. of a.a
AV1	+1	124	432	103
AV2	+2	284	1051	256
AC1	-3	2594	1506	363
AC2	-1	1606	1155	150
AC3	-3	1461	1060	135
AC4	-1	2437	2137	100
AC5	-1	592	70	174

Sequence of several clones identified two geminivirus species. The comparison of these viruses is given in Table 2. The two viruses are named CLCuV PK-1 and CLCuV-PK2.

Table 2. Putative Open Reading Frames (ORFs) of Cotton Leaf Curl Virus (CLCuV) species

ORFs	Start	Stop	No. of Amino Acids	Protein Mr (kD)	Similarity Index (%)
A	1	2750			71
AV2	139 (124)	495 (435)	118 (103)	13.7 (12.1)	90
AV1	299 (284)	1069 (1054)	256 (256)	29.7 (29.7)	74
AC1	2600 (2594)	1518 (1503)	360 (363)	40.3 (40.8)	87
AC2	1615 (1606)	1211 (1154)	134 (150)	15.3 (17.4)	66
AC3	1470 (1461)	1066 (1058)	134 (134)	15.7 (15.6)	66
AC4	2445 (2680)	2144 (2135)	100 (181)	11.1 (20.6)	72
AC5	814 (592)	290 (68)	174 (174)	19.9 (19.3)	68

Values outside parenthesis represent CLCuV-Pk2/Fsd/1.
Values inside parenthesis represent CLCuV-Pk1/Fsd/3.
V in an ORF represents virion sense strand and C denotes the complementary strand.

Biological Variability in Symptom Expression

Biological and molecular variability of CLCuV was also studied. The important variation observed in the field is the upward or downward curling of the leaves. It is not known whether the curling is determined by different viruses or is a plant response to virus infection. To study this phenomenon, cotton plants showing upward or downward curling were passaged by grafting to healthy cotton plants and the symptoms on the plants were recorded. It was found that plants showing downward curling during passage produced plants with upward curling. Similarly, one of the plants where graft was showing upward curling produced downward curling symptoms. The results show that symptom phenotype was not maintained and that curling is either a plant response or may be due to the dominance of one strain.

Molecular Diversity: Evidence of Presence of Two New Geminivirus Species in CLCuV Pak-1

For the evaluation of molecular diversity several clones were obtained either by ccc DNA cloning or PCR. As discussed earlier, the common region is the most diverse region among CLCuV isolates. A common region of six clones was completely sequenced. Four of the isolates have a common region sequence of PK-1 strain while 2 of the clones had a sequence of Pak-2. The data suggested that considerable variability exists in CLCuV. Based on the sequence data, Pak-1 and Pak-2 specific primers were designed and the desired product has been confirmed by cloning and sequencing. These primers are being used for the assessment of distribution of two strains of CLCuV.

Detection and Differentiation of Geminiviruses

Previously we have used the polymerase chain reaction (PCR) for the identification of alternate hosts of cotton leaf curl virus. Surveys were conducted for the collection of additional plant species which were not reported previously to be infected with geminivirus. Samples from infected cotton plants were also collected from different cotton growing areas of Pakistan. DNA A of ACMV or CLCuV was used as a probe for the detection of whitefly transmitted geminiviruses. A number of well characterized geminiviruses both from the old and new world were used as positive control while DNA extracted from healthy plants of various species was used as negative control. It was found that use of ACMV or CLCuV as probe was able to detect geminiviruses by dot blot hybridization both from the old and new world viruses. Similarly the use of ACMV or CLCuV as probe detected geminivirus in 30 plants species out of 40 plant species suspected of whitefly-transmitted geminiviruses.

For the differentiation of an alternate rate of CLCuV from other geminiviruses, a probe of CLCuV common region was prepared. The common region of whitefly-transmitted geminiviruses is the most diverse region and may serve as a virus specific probe. It was found that under high stringency level the use of a common region as probe specifically detected CLCuV both in cotton and alternate hosts and further confirms that members of the Malvaceae family are the alternate hosts of CLCuV.

Development of a Diagnostic Test

Development of PCR Based Diagnostic Test

PCR/DNA probe-based test has been developed for CLCuV by designing two sets of primers. One set of primers is capable of amplifying a portion of viral genome from common region to N-terminal sequence of coat protein. The other set amplifies part of viral genome from replication associated protein (AC1) to common region. Newly developed sets of primers were used to detect presence of CLCuV in cotton plant leaves collected from different cotton growing areas. The amplification of viral genome was confirmed by cloning and sequencing of amplified product. This is a highly sensitive assay for the detection of virus in the plant. Recently, we have developed a multiplex PCR for the detection of two geminiviruses species associated with cotton leaf curl disease in Pakistan. A simplified method is used for the isolation of template suitable for PCR. A rapid profile used PCR primers designed to specifically detect these two virus species. The two species could be found independently or co-infecting the same plants. This is a unique example where two geminivirus species could cause the same disease in a geographical area.

Screening by Southern Hybridization

CLCuV samples were collected from different cotton growing districts of the Punjab and full length cloned viral DNA was used as probe in southern hybridization. The cloned DNA probe hybridized with the samples and thus can be used for screening purposes.

Development of Polyclonal Antisera

Purification of intact geminate particles paved the way for the production of polyclonal antisera against CLCuV. For this purpose rabbits were immunized with viral particles mixed with Freund's complete adjuvant (first dose). Subsequent subcutaneous booster doses were given with Freund's incomplete adjuvant at an interval of 15-20 days. Polyclonal antisera has been distributed to Ayub Agriculture Research Institute — AARI and National Agriculture Research Center — NARC for evaluation by ELISA. We received feed back from both research institutes and they recommended that this material needs further purification for reliable testing for the amplification such that the process is completed in two hours. This method is being used to assess the distribution of two geminivirus species in cotton growing areas of the Punjab, Pakistan.

Development of Monoclonal Antibodies and ELISA Test

As a first step for the development of rapid immunological test for screening, sets of primers based on the sequence to genome A were synthesized for PCR amplification and cloning in expression vectors. These primers amplified 1.1kb fragment from cloned as well as from total DNA isolated from infected cotton leaves. This PCR product has been cloned in bacterial expression vector for large scale protein production and purification. The expressed protein has been used for the generation of monoclonal antibodies. Two clones of hybridoma cell lines producing antibodies against CLCuV have been identified and are being tested for the specificity of monoclonal antibody by ELISA. These studies are being done in collaboration with Department of Pharmacology, John Hopkins University, Baltimore, USA.

Development of Virus-resistant Cotton Through Genetic Engineering

Several approaches have been reported for the development of transgenic resistance against whitefly transmitted geminiviruses.

The approaches that are being used for CLCuV at NIBGE are the following

- Expression of antisense RNA against complete or fragments of AC1 gene
- Over-expression of AC1 in transgenic plants
- Expression of a virus-induced cytotoxin gene in transgenic plants

Tissue Culture of Cotton

The genetic engineering of a plant is heavily dependent on transformation technology whereby a functional foreign gene could be inserted into the genome of the cotton plant. Currently, the two most widely used methods for plant transformation involve agrobacterium-mediated transformation and bombardment of cells with DNA coated particles. These two methods appear to be most important for genetic engineering of cotton both in terms of success and current efforts. Unfortunately, transformation with Agrobacterium requires that the cotton genotype, be regenerable from callus tissues, a feature which so far appears to be limited to some Coker lines and an Australian cultivar Siokra 1-3 among the commercial cultivars. Because of this limitation, researchers have strong interest in alternative transformation processes such as direct transformation of meristems with Agrobacterium.

Tissue Culture of Local/Exotic Varieties of Cotton

Nineteen local/exotic cultivars of cotton were evaluated for in-vitro callus induction and plant regeneration. Varieties S-12, NIAB-78, AEM-1-85, FH-682 and BH-36 produced significantly better calli than other varieties. However, induction was observed to be highly variable not only among different genotypes but also among various explants of the same genotype. Successful attempts were made to control contamination, auto-inhibitory response and decay of calli. Embryoids were observed in some varieties but regeneration was obtained only in Coker-312 and Sikora 1-3 varieties. Further work is in progress.

Meristem Tip Culture of Local Varieties of Cotton

Meristem shoot tips of ten cultivars of cotton *Gossypium hirstum* were cultured on several media formulated for shoot and root development. The best shoot development was observed on media containing 0.1 mg/liter Kinetin, while rooting was observed on media containing 0.5 mg/l NAA and 0.1 mg/l Kinetin. No inter-varietal variability was observed. A complete protocol was developed from meristem tip culture to field transfer for biolistic gun transformation of cotton.

Development of a Recombinant DNA Construct Based on Sense or Antisense Expression of AC1 Gene

Several technologies have been reported for the development of genetically engineered resistance against cotton leaf curl virus. One of them is the production of antisense RNA against replication associated protein or over-expression of ACI gene in a transgenic plant driven by CaMV S35 promoter.

Open reading frame coding for ACI was identified on clone PS1. Primers (V2417, V2418) were designed such that an NCoI site was incorporated at 5' end of both primers. A product of expected size (1.2 k) was obtained. The PCR product was digested with NCoI enzyme and was cloned in PJIT 166 (a plant expression vector) both in the sense and antisense orientation. The orientation of clones was confirmed by restriction analysis. The clone was also confirmed by sequence analysis. GUS gene from the vector was removed by digestion with SmaI and relegated to give pSJITAC1. The construct was lifted from pSITAC1 by digestion with Sph1 and Sst I and ligated in pBin plus vector digested with the same enzyme and vector pSBinJIT AC1 was obtained. Electro-competent cells of agrobacterium strain C58 (disarmed strain) were used for electro transformation and transformed cells were selected by kanamycin resistance.

Development of Recombinant DNA Construct for Resistance Against CLCuV Based on Virus-induced Expression of a Cytotoxic Gene

During recent years, several techniques have been reported for the development of genetically engineered resistance against geminiviruses. Pioneering work on the use of ribosome-inactivating proteins (RIPs) has been initiated at John Innes Centre. RIPs are naturally occurring plant toxins that are presumed to provide a defense mechanism against pathogens or predator by disrupting protein synthesis in damaged eukaryotic cells. One of these RIP, dianthin has been exploited in these studies. The expression of dianthin is driven by viral coat protein promoter and is activated in-trans by one of the viral gene product (AC2). The activation of transgene expression during virus infection avoids the constitutive expression of the transgene.

A set of primer was designed to amplify the coat protein promoter of CLCuV. Necessary restriction sites were introduced to ensure that the promoter is in frame with the dianthin gene. The primers were successfully used and the desired product was cloned in pGEMT vector and checked by restriction analysis. The cloned fragment was further checked by sequence analysis. The clone was sequenced by use of a T7 sequencing kit using 33P labeled nucleotide. The sequence analysis confirmed that the desired product has been cloned. A plant expression vector pJIT163 was used for the expression of transgenic dianthin. Double 35S promoter in the vector was replaced by coat protein promoter and dianthin was cloned in the correct reading frame. The construct was lifted by digestion with Sst I and ECoRV and cloned in plant transformation vector pBin Plus. The clone was transferred to agrobacterium strain C58 by electroporation.

Development of Constructs Based on Antisense RNA Expression of Parts of Complementary Sense Genes

To express the viral antisense RNA in transgenic plants, an expression cassette was constructed. A ~1.5 kb fragment containing the double CaMV 35S promotor and poly A terminal sequence was isolated from the plasmid pJit 60 (gift from Dr. P. Mullineaux, J11, Norwich, UK) with Xho1 and Sst1 and subcloned into plasmid pBluescript 11 KS to get pSQMW1. The fragments of ~ 460 bp (Start of AC1-end of AC4 gene), ~520 bp (end of AC4- start of AC2 gene) and ~ 540 bp (Start of AC2-end of AC3 gene) were isolated from the pYASF clone using specific primers (SH1 and SH2, and SH3, SH4, SH5 and SH6 respectively) by PCR. The amplified DNA fragments were end filled with T4 DNA polymerase. The individual fragments were then cloned in PSQW1. More than 60 recombinants were screened for sense and antisense insertions of the above mentioned gene sequences. Confirmation was obtained by cutting the plasmid insert with restriction enzymes. PstI (AC1-AC4 and AC1/AC2/AC3 gene) and Sa11 (AC1 middle region)

The fragments of 2.0 kb harboring the above mentioned genes in both sense and antisense orientation were isolated by cutting the pSQW1 with Sst1 and EcoRV. Finally these fragments were cloned into the plant transformation vector PGA 482 at Sst1/ Hpal site to yield PGS clones. Later these plasmid carrying the respective genes were transformed into agrobacterium tumefacient strain LBA4404 by electroporation.

Transformation of Constructs in an Elite Pakistani Cotton Variety S-12

A protocol was developed for Agrobacterium mediated transformation of an elite cotton variety S-12. The method that uses 3-day-old mature embryos for transformation and selection was made on kanamycin. Analysis of the first batch of plants suggests that the gene has been integrated in these putative transgenic plants. The plants are being tested for the presence of genes and the ability of plants to resist geminiviruses.

Cotton Genome Project

A majority of the present day commercial cotton varieties grown in Pakistan belong to *Gossypium hirsutum* L. (upland cotton) a very few to diploid species *G. arboreum* L. Breeders have evolved these varieties through selection based on morphological and physiological features (yield, fiber quality, resistance against certain pests and diseases, etc.).

Most of the varieties grown in Pakistan originated from intraspecific crosses of *G. hirsutum* L. at various research centers around the country. These hybridization practices resulted in narrow genetic base of the new varieties. Any crop with a narrow genetic base is more prone to natural disasters such as outbreak of a disease.

Morphological features are indicative of the genotype but are represented by only a few loci because there are not enough number of characters available. Moreover, they can also be affected by environmental factors and growth practices. To have an accurate and reliable estimate of genetic relationships and genetic diversity, a large number of polymorphic markers are essentially required.

Random amplified polymorphic DNA (RAPD) technique of Williams et al (1990) provides an unlimited number of markers which can be used for various purposes. In addition to technical simplicity and speed of RAPD methodology, its level of genetic resolution is equivalent to restriction fragment length polymorphism (RFLP) for determining genetic relationships.

RAPD analysis was used to evaluate the genetic diversity of elite commercial cotton varieties in addition to the intravarietal studies. Twenty individual plants of cotton variety S-12 were analyzed with 10 primers for any polymorphisms. No polymorphisms were observed with any of the ten primers indicating that the technique can be used for the analysis of purity of seeds in cotton.

Twenty-two varieties belonging to *Gossypium hirsutum* L. and one to *G. arboreum* L. were analyzed with 50 random decamer primers using polymerase chain reaction (PCR). Forty-nine primers detected polymorphism in all 23 cotton varieties, while one produced monomorphic amplification profiles. A total of 349 bands were amplified and 89.1% of which were polymorphic. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA) showed that 17 varieties can be placed in two groups with a similarity ranging from 81.51% to 93.41%. *G. hirsutum* L. varieties S-12, V3 and MNH-93 showed a similarity of 78.12, 74.46 and 69.56% respectively with rest of the varieties. One variety CIM-1100 showed 57.02% similarity and was quite distinct. The diploid cotton *G. arboreum* L. var. Ravi was also very distinct from rest of its tetraploid counterparts and showed only 55.7% similarity. The analysis revealed that the intervarietal genetic relationships of several varieties is related to their center of origin. The results also showed the genetic relationship of elite commercial cotton varieties with some standard "Coker" and diploid *G. arboreum* L. var. Ravi (old world cotton). As expected, most of the varieties have a narrow genetic base. The genetic similarities obtained can be used for the selection of possible parents to generate a mapping population. The polymorphic profiles can be used for the identification of different varieties and the protection of breeders propriety rights.

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