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IDENTIFICATION AND ASSOCIATION OF NBS-LRR ENCODING DISEASE RESISTANCE GENE ANALOGS FAMILY IN DIPLOID COTTON (*GOSSYPIUM ARBOREUM* L.)

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Abstract

Identification of genes at molecular level and association as linked markers to resistance in diploid cotton with emphasis on major diseases of cotton was studied via a conserved domain strategy by polymerase chain reaction (PCR). Three rounds of PCR amplifications by using degenerate oligonucleotide primers corresponding to the conserved motifs, nucleotide binding sites (NBS) and leucine rich repeats (LRRs) were performed. According to the primer wise expected PCR products size, forty nine required size products amplified were eluted and some were directly precipitated from PCR reactions. The identified eluted products were cloned and sequenced. Through this approach resistance genes analogs (RGA) like sequences were determined that are may be candidates for known disease resistance loci. All of the eluted identified candidate resistance genes loci's (RGLs) are not RGAs. However some of the common types of equal size RGLs may be used as markers to identify the resistance genes. These results demonstrates that mapping of RGA sequences can be useful for landing markers linked to known resistance genes analogs and possibly in identifying candidate resistance loci in different species of cotton. Genome characterization for cotton leaf curl virus (CLCuV) study suggested that 800 and 1200 base pair size (bps) fragments could be used as markers for resistance to CLCuV. We named these markers as *MCLCuV-1* and *MCLCuV-2*.

Key words: Identification, Cloning, Characterization, Genes, Cotton, species, PCR.

Introduction

Cotton is an important fiber crop around the world and its production has a big impact on the economy of cotton producing countries. In Pakistan tetraploid cotton *Gossypium hirsutum* L. is sown on about 95% of total area. It is generally uses in world's textile production. In Pakistan, the cropped area of cotton is 2.079 million hectares, with annual production of 7.064 million bales (Anon., 2020-2021). In Pakistan we required 25 million bales of lint by 2025 (Haidar *et al*., 2007).

Various diseases attack this upland cotton. Therefore, lot of efforts has been done for improvement in cotton varieties with resistance to diseases. Consequently, due to these efforts Pakistan become one of the top five cotton producing countries of the word in 1991-92 (Zafar *et al*., 1997). But the seed cotton yield could not sustain due to wide spread prevalence cotton leaf curl virus disease (CLCuD). This disease continuously causing heavy losses for many years and is still prevailing despite efficient management practices adopted by breeders, agronomists and farmers. Besides CLCuD, cotton crop is also amenable to many fungal and bacterial diseases, which cause heavy yield losses. Limited genetic variability is available in the locally developed germplasm (Haidar *et al*., 2012). Selection of suitable germpalsm keeping in view of different characters and disease resistance has been carried out to improve yield and disease resistance in cotton (Haidar & Aslam, 2016; Haidar *et al*., 2016, Aslam *et al*., 2018 a.b).

However, it is important and imperative to look for possible genetic solution of this ever-threatening problem. Almost all of the local cultivars are tetraploid cotton *G. hirsutum* L. and are susceptible to most of diseases. The only source of resistance to most of diseases is diploid species (*G. arboreum* L. and *G. herbaceum* L.). The diploid cotton *G. arboreum* L. is indigenous to the Indus civilization, which constitutes present day Pakistan. Although this short staple and low yielding cotton is not as important as the long staple tetraploid cotton but it has some importance i.e. it is less complex (diploid), natural source of resistance against the pests and diseases, and source of genetic diversity in tetraploid cotton.

Therefore, *Gossypium arboreum* L., natural source of resistance genes can be used to amplify the disease resistance genes or resistance genes like sequences. These sequences then used to find the relationship of resistance genes with other disease resistance genes by different analysis and other molecular techniques.

Over the past few years, we have witnessed a breakthrough in cloning of natural resistance genes. It includes N gene in tobacco (Witham *et al*., 1994), HM-1 in Maize (Johal & Brigges, 1992), Pto and Cf-9 in tomato (Martin *et al*., 1993; Jones *et al*., 1994), L-6 in Flax (Lawrence *et al*., 1995), RPS-2 (Bent *et al*., 1994; Mindrinos *et al*., 1994) and RPM-1 (Grant *et al*., 1995) in Arabidopsis, and Xa-21 (Song *et al*., 1995) in rice and so many others. These were identified by PCR approach, transposon tagging, and map-based cloning. The resistance genes were characterized and categorized on the basis of similarities in function and or amino acid sequence of protein they rncode. The class containing majority of cloned gene, even though resistant to a very different pathogen including virus, bacteria, or fungi are characterized by the presence of an N-terminal NBS and a C-terminal stretch LRRs. This conserved domain provides the opportunity to identify and sequence more resistance genes in different species.

Identification of resistance genes at molecular level and characterization in diploid cotton was the main objective of this study with main emphasis on CLCuD and bacterial blight (BB) diseases. Identification and genome characterization in this regard is important to understand clues about the mechanism and interaction of pathogens and evolution of resistance genes. Importantly these identified resistance genes or resistance genes like sequences can be transferred to other species/varieties and will be used as markers for selection criteria in conventional breeding programs.

Materials and Methods

The experiments were conducted jointly in collaboration of Plant Breeding and Genetics department (PBGD), University of Agriculture, Faisalabad (UAF) and National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

Plant material and screening: Plants of species *G. arboreum* L. diploid (variety FDH-170) with A genome, were grown in pots placed in glasshouse at NIBGE, Faisalabad. Viruliferous white flies were raised in cages on diseased plants of cotton with leaf curl virus. These were transferred in glass house to inoculate the plants with cotton leaf curl virus and inoculum of *Xanthomonas compestris* PV *malvecerum* was applied by tooth pick scratching (Bird, 1986) method on the leaves of plants for the infestation of bacterial blight. Diploid cotton (*G. arboreum* L.) is a natural source of resistance to most of cotton diseases therefore all the plants showed resistance and no symptoms of cotton leaf curl virus and bacterial blight diseases were observed on the plants. The population of this specie was also raised at PARS (Postgraduate Agriculture Research Station, University of Agriculture, Faisalabad) for field screening to BB and CLCuD. In field conditions, there were no symptoms of CLCuD and BB diseases. The plant leave samples of this specie were used for DNA isolation.

Total genomic DNA isolation: The procedure of Doyl & Doyl (1987) for DNA isolation from cotton plant was adopted because it is quick and good quality DNA can be isolated. RNase treatment was done and then DNA concentration was estimated by flouremeter DyNA Quant^{TM} 200. Various dilutions were made to optimize the PCR conditions. The DNA dilutions 2µl, 5µl of DNA in 500 µl of d.d^2 H_2O were made to check the PCR optimization conditions. The dilution of 2µl of DNA in 500 µl of d.d^2 H_2O was adopted for amplification of resistance gene analogs (RGAs).

Dot-blot hybridization: CLCuD is caused by two groups of viruses i.e. Geminivirus and Nanovirus. These viruses are sub-groups of Begomoviruses. Geminiviruses DNA-A and Nanoviruses DNA-1 are collectively responsible for causing the disease (Mansoor *et al.*, 1999). Recently another component, which is responsible for causing the disease i. e. β particle, has been discovered. DNA-1 is responsible for causing symptoms in plants while DNA-A is involved for replication of viruses. DNA-1, DNA-A and β particle have been sequenced (Mansoor *et al.*, 1999; Briddon *et al.*, 2000). The probed clones of DNA-1, DNA-A and β component were used for dot-blot hybridization to detect causing virus component in the genomic DNA of *G. arboreum* L. diploid cotton (variety FDH-170). This was for screening of these species at DNA level.

Primer designing: Eight known conserved amino acid motifs were used to design oligonucleotide primers. Primer designs were based on conserved motifs in and around the NBS of known resistance genes (Table 1). Primers were dissolved in 10µl d. d^2H_2O.Then for PCR amplification 5µl diluted primer was mixed in 200µl d.d^2 H_2O and stored at –20°C.

PCR strategy and amplification conditions: First and second round of PCR was done in 50µl reaction quantity with 1µl for each pair of reverse and forward primers. DNA amplification reactions were performed in a thermal cycler (Hybaid touch down thermal cycling system). The template of DNA was denatured at 94°C for 2 minutes. Followed by 40 cycles, 30 seconds at 94°C, 30 Seconds at 40°C, 2 minutes at 72°C, with a final extension of 10 minutes at 72°C.

First round of PCR reaction of 50 µl contained 5.0 µl of diluted genomic DNA, 5.0 µl dNTPs (100 µM each dATP, dCTP, dGTP, dTTP), 5.0 µl PCR Buffer (50mM KCL), 3.0 µl $MgCl_2$ (3mM), 30.0 µl d.d^2H_2O, 0.5 µl Taq Enzyme and 1.0 µl of each primer and were subjected to thermal cycling.

Second round was same except that it was carried out 50.0 µl reactions containing 5.0 µl of a 20X dilution of bulked first round of PCR mixture as template and subjected to thermal cycling i.e., 94°C for 2 minutes; 10 cycles of 94°C for 30 Sec., 40°C for 30 C and 72°C for 2 minutes; 30 cycles of 94°C for 30 Sec., 45°C for 30°C and 72°C for 2minutes; 10 minutes at 72°C. Taq polymerase, buffer, $MgCl_2$ and dNTPs were acquired from Perkin Elmer, Noorwalk USA. While primers were got manufactured from Gibco BRL.

PCR products elution and purification: PCR products were eluted from an agarose gel slice using glass milk method, freeze squeeze method and TM-Rapid Gel extraction protocol (Life Technologies). Some of the required size PCR products were directly precipitated.

Cloning of PCR products: Purified and precipitated PCR products/DNA fragments of the required size eluted from agarose gel and were than cloned. Cloning of the desired products was done with pGEM-T Easy cloning kits (Promega).

Sequencing of clones: Double stranded plasmid DNA samples were used for sequencing with ABI-310 genetic analyzer (Perkin Elmer) by using ABI-Prism TM Dye Kit with AmpliTaq. Automated DNA sequencing was based on chain termination and utilizes fluorescent labels. The emitted fluorescence is observed by detectors and the information analyzed by the computer and converted to nucleotide sequence.

Table 1. Oligonucleotide primers used to amplify resistance gene analogs (RGAs) Gibco BRL designed primers (Collins *et al*., 1998).

Conserved aminoacid motif	Primer's name	Primer's sequence (5' to 3'
GVGKTT (P-Loop)	P-Loop-1	AAG AAT TCG GNG TNG GNA AAA CAA C
	P-Loop-2	AAG AAT TCG GNG TNG GNA AAA CTA C
	P-Loop-3	AAG AAT TCG GNG TNG GNA AAA CCA C
	P-Loop-4	AAG AAT TCG GNG TNG GNA AAA CGA C
	P-Loop-5	AAG AAT TCG GNG TNG GNA AGA CAA C
	P-Loop-6	AAG AAT TCG GNG TNG GNA AGA CTA C
	P-Loop-7	AAG AAT TCG GNG TNG GNA AGA CGA C
	P-Loop-8	AAG AAT TCG GNG TNG GNA AGA CGA C
L (I/V/L) VLDDV	Kinase-2 D	CTA CTG NTN CTN GAC GAG GT
	Kinase-2 E	CTA CTG NTN CTN GAC GAT GT
	Kinase-2 F	CTA CTG NTN CTN GAT GAC GT
	Kinase-2 G	CTA CTG NTN CTN GAT GAT GT
GLPLA	GLPL-1	AAC TCG AGA GNG CNA GNG GNA GGC C
	GLPL-2	AAC TCG AGA GNG CNA GNG GNA GAC C
	GLPL-3	AAC TCG AGA GNG CNA GNG GNA GTC C
	GLPL-4	AAC TCG AGA GNG CNA GNG GNA GCC C
	GLPL-5	AAC TCG AGA GNG CCA ANG GCA ATC C
	GLPL-6	AAC TCG AGA GNG CCA ANG GCA AAC C
KQCFAFCSI	CFA-1	CAR WAI GCR AAR CAY TGT TT
	CFA-2	CAR WAI GCR AAR CAY TGC TT
	CFA-3	ATG GAR CAR WAI GCR AAA CA
	CFA-4	ATG GAR CAR WAI GCR AAG CA
WMAx G (F/I) V	WMA-1	AYR AAN CCN TNT GCC ATC CA
	WMA-3	AYR AAN CCN TNC GCC ATC CA
	WMA-4	AYR AAN CCN TNG GCC ATC CA
MHD	MHD-1	CGA CAG TCN ATC ATG CAT
	MHD-2	CGA CAG TCN ATC GTG CAT
	MHD-3	CGA CAG TCN GTC ATG CAT
	MHD-4	CGA CAG TCN GTC GTG CAT

Sequencing analysis

Search for comparison/identity between the expected sequences of clones was carried out with BLAST SEARCH (Gish & States, 1993).

Results

Diploid cotton *G. arboreum* L. variety FDH-170 showed resistance and no symptoms of cotton leaf curl virus, bacterial blight and other diseases were observed on the plants in the glasshouse and in the field conditions. The probed clones of DNA-1, DNA-A and β component was used for dot-blot hybridization to detect virus causing component in the genomic DNA. No hybridization with either of three components was observed in *Gossypium arboreum* L. DNA that confirmed that this cotton was totally resistant to cotton leaf curl virus. So the DNA of this specie was used to amplify the disease resistance genes via a conserved domain strategy by PCR. Different dilutions of DNA were made for optimization of PCR conditions. DNA 2µl (500ng/2µl) in 500 µl d.d^2 H_2O was found most suitable for PCR amplification.

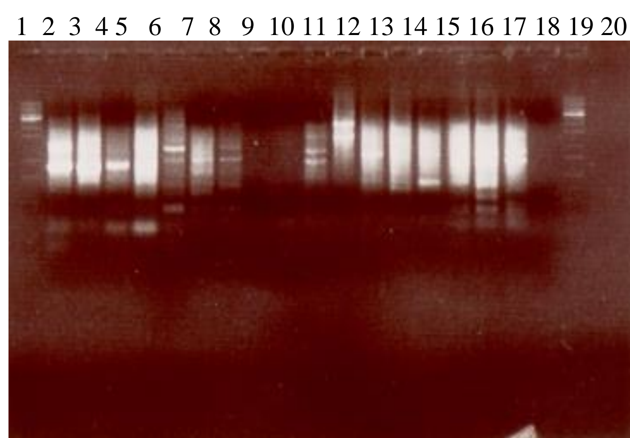
Different primers combinations using forward and reverse primers were used to get required size of PCR amplified products. In some of the PCR nested primer approach combining primers for three or four motifs was used. In this all possible combinations of primers for different motifs were used in different PCR reactions. PCR products were eluted, mixed and used as template in second round of PCRs.

PCR was conducted with possible combinations of 29 primers of six amino acid-conserved motifs, using forward and reverse primers to get required size PCR amplified products. P-loop and Kinase-2 were used as forward primers, while GLPL, CFA, WMA, and MHD were used as reverse primers.

In first round of PCR amplification P-loop primer was used as forward primer with all other reverse primers i. e. MHD, WMA, CFA, and GLPL. In one set, a PCR with 19 combinations including 2 controls was performed. In total, 8 PCR sets were performed in first round of amplification i.e. 152 PCR reactions (Figs. 1, 2).

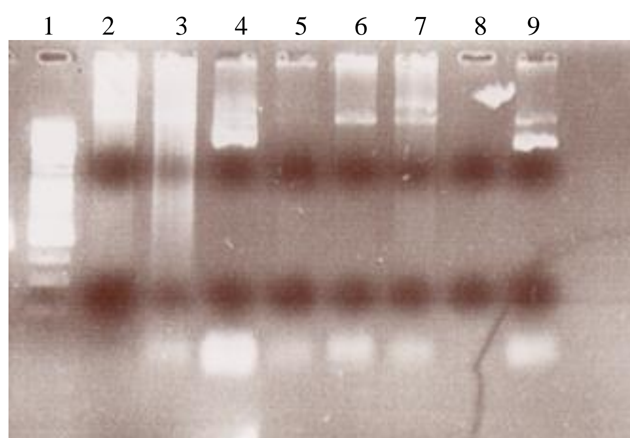
1, 1 Kb DNA Ladder, 18,19 control, 17 was P-Loop-7+GLPL-5

Fig. 1. PCR amplification with P-Loop-7 and 17 different primer combinations.



1 and 20, 1Kb Ladder, 19 control, 2 is P-Loop-5+ MHD-1

Fig. 2. PCR amplification with P-Loop-7 and 17 different primer combinations.



1.1 Kb DNA Ladder, 8-control, 2 to 7 plasmids for different colonies

Fig. 3. Plasmids for P-Loop-5 and MHD-1 primer combination.

In second round of PCR amplification Kinase-2 considered as forward primer in combination with all other reverse primers (MHD, WMA, CFA, and GLPL). In one set of PCR the same 19 combinations including 2 controls were practiced. In total 4 PCR sets (19 combinations for each) were performed in second round of PCR i.e. 76 PCR reactions.

In an attempt to increase the specificity of PCR one or two internal primers. Products amplified from genomic DNA in the first two rounds of PCR were used as template DNA in the third round of PCR amplification with different forward and reverse primer's combinations. Some third round PCR reactions yielded prominent products of expected size (Fig. 3).

In the three rounds of PCR amplifications, forty nine products of the required size according to the primers were eluted by the gels and some were directly precipitated from the PCR reactions (Table 2). This approach produced RGL sequences some of which could be similar to already known disease resistance genes loci. So, all of the forty nine RGLs are not resistance genes analogs (RGAs). However, some of equal size RGLs may be used as markers to identify the resistance genes.

PCR products of equal sized candidate RGLs were purified and precipitated and DNA fragments of the appropriate size were excised and purified and cloning of the required size PCR products was performed (Fig. 4).

The clones were sequenced and analyzed. Comparisons with the other reported disease resistance genes were conducted by Blast Search (Gish & States, 1993). This revealed no similarity with the other reported resistance genes. However, some of the sequences show similarity with other proteins, kinases etc.

In addition, same primers were used to amplify fragments from another diploid species *Gossypium ramnodii* L. which is highly susceptible to CLCuD. Fragments of 1500 and 2000bps were present only in *G. arboreum* L. These may be for other diseases and insect pests' resistance because this A genome diploid species was resistant to most of diseases and insect pests. It was also amplified in *G Raimondi,* which is highly susceptible to CLCuD, therefore, the fragment of 600 bps may be an RGA for some other disease resistance gene. A tetraploid AD genome cotton LRA-5166 which is tolerant to CLCuD was also used for amplification. This study provided a valuable insight to the characterization of A and D genomes and establishment of DNA markers for disease resistance to CLCuD.

Discussion

It was found that the NBS-containing genes as expected are present in cotton, some of which may be involved in disease resistance in cotton. Variation in sequences is may be due the possibility that these may be belongs to different classes of genes. This information of mapping RGAs will be useful for identification of markers linked to known resistance genes and possibly in identifying candidate resistance loci.

The inability to sequence RGA loci similar or related disease resistance loci may be due to the fact that all resistance genes in cotton do not belong to with only NBS-LRR class. But this has generated a possibility to identify more RGAs especially in cotton. In *Arabidopsis thaliana*, NBS-LRR like resistance gene RPM-1 identified in one line was absent from some lines (Grant *et. al*., 1995), suggesting the possibility that different lines of plant species contain different sets of NBS-LRR gene class. Similar situation is already reported in cotton, in which two dominant segregating loci were detected in an F_2 population, were completely absent in other segregates of same population. More the variants lines used for mapping of RGAs exploring the possibility of greater number of loci.

Results of cloning of PCR products amplified from *G. arboreum* L., showed that 600, 800 and 900 bp size fragments occurred frequently whereas 1200, 1500 and 2000 bps also occurred but as they were of not required size according to primer combinations, so attention was not focused on them but still these were valuable fragments. In genome characterization 600bps products were amplified in *G. arboreum* L., LRA-5166 (Tetraploid cotton) and *G. raimondii.* Whereas 800 and 1200 bps amplified from both *G. arboreum* L and LRA-5166, while 1500 and 2000 bps were amplified only in *G. arboreum* L. Zambounis *et al*., 2016 used similar approach based on conserved motifs and amplify 38 RGAs from two upland cotton cultivars differing in their susceptibility and identified RGA markers were closely linked to R genes.

From these studies, 800 and 1200 bps fragments were identified and suggested that these can be used as markers for resistance to cotton leaf curl virus, because these were present in A genome *G. arboreum* L. and AD genome LRA-5166 which are both resistant to CLCuV. These were not present in D genome *G. thurberii* and *G raimondii* which were susceptible to CLCuV and also were not amplified from tetraploid AD genome *G. hirsutum* L. (S-12) which was highly susceptible to CLCuV. We named these markers as *MCLCuV-1* and *MCLCuV-2*. Earlier, Kim *et al.*, 2017 used similar combined approach of of screening, PCR and cloning techniques and identified DM-resistance candidate genes in maize. Thu cloning and sequencing approach corresponding to conserved motifs may also be applied to identify genes for resistance to other diseases in crop plants.

Table 2. Size of PCR products amplified from various primer combinations in regular & Nested PCR.

	Regular PCR-size of PCR products (bp) amplified														
G. arboreum L (Diploid)	250	300	350	400	450	500	**600**	700	**800**	850	900	1000	**1200**	**1500**	**2000**
G. hirsutum L (LRA-5166)	-	300	-	-	450	-	**600**	700	**800**	850	900	-	**1200**	-	-
G. thurberii (Diploid)	250	-	350	400	450	500	**-**	700	**-**	850	-	-	**-**	-	-
G. raimondii (Diploid)	250	300	-	-	450	500	**600**	700	**-**	850	-	-	**-**	-	-
	Nested PCR- size of PCR products. (bp) amplified														
G. arboreum L (Diploid)	250	300	350	400	450	-	**600**	700	**800**	-	900	1000	**1200**	-	-
G. hirsutum L (LRA-5166)	-	300	350	400	450	-	**600**	700	**800**	-	900	1000	**1200**	-	-
G. thurberii (Diploid)	250	-	350	400	450	500	**-**	-	-	-	900	1000	-	-	
G. raimondii (Diploid)	250	300	-	-	450	500	**600**	-	-	-	900	1000	-	-	-

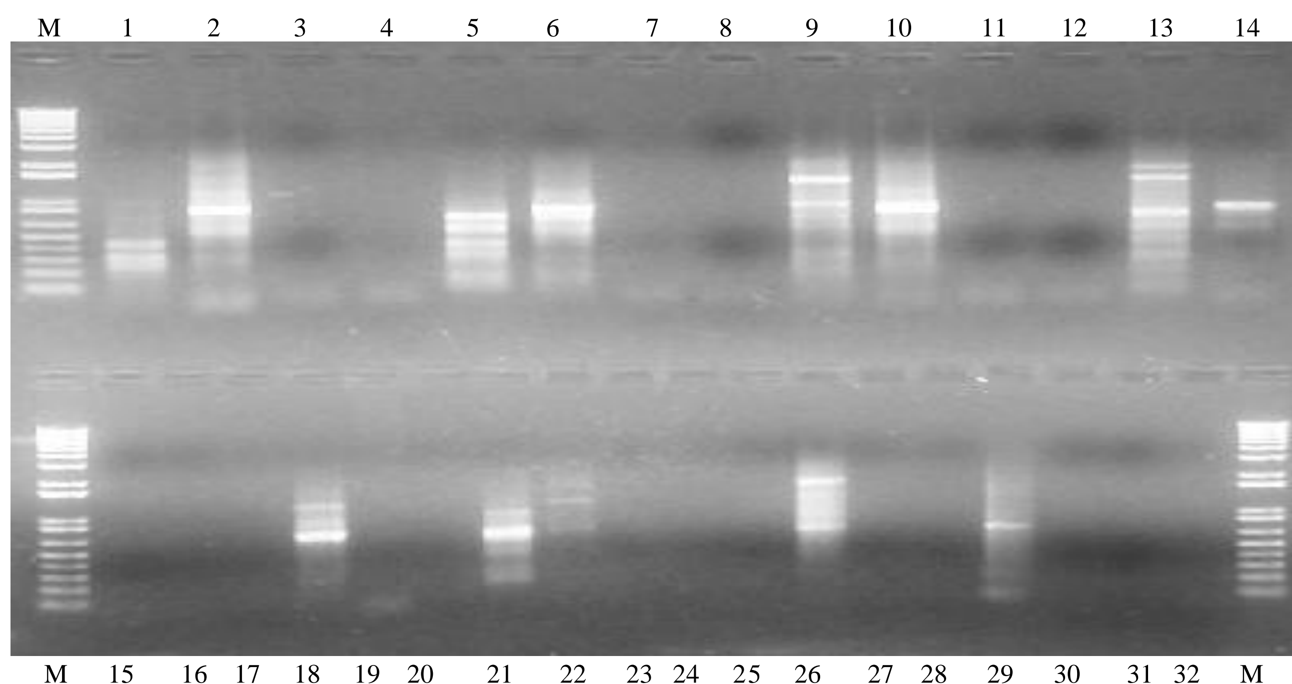


Fig. 4. PCR amplification with P-Loop4 to 5 x MHD 1 to 4 primer combinations using genomic DNA of four cotton species.

PCR with P-Loop4 to 5 x MHD 1 to 4 primer combinations using DNA of *G. arboreum* L, LRA-5166 (*G. hirsutum* L.), *G. thurberii* and *G raimondii.* M = 1Kb Marker; Lane 1, 5, 9, 13, 17, 21, 25, 29 = *G. arboreum* L; Lane2, 6, 10, 14, 18, 22, 26, 30= *G. hirsutum* L; Lane 3, 7, 11, 15, 19, 23, 27, 31 = *G. thurberii.* ; Lane 4, 8, 12, 16, 20, 24, 28, 32 = *G raimondii.* Lane 1 to 4 = P-Loop 4 x MHD 1; Lane 5 to 8= P-Loop 4 x MHD-2; Lane 9 to 12 = P-Loop 4 x MHD-3; Lane 13 to 16 = P-Loop 4 x MHD-4; Lane 17 to 20= P-Loop 5 x MHD 1; Lane 21 to 24 = P-Loop5 x MHD 2; Lane 25 to 28 = P-Loop 5 x MHD 3 and Lane 29 to 32 = P-Loop 5 x MHD -4 primer combinations.

Conclusion

Establishment of DNA markers for CLCuV is an important effort for the development of cotton genotypes resistant to this disease. With these molecular markers, the cotton breeders will be able to select plants resistant to CLCuV. Sequencing of these cotton leaf curl virus resistance markers may provide the opportunity for the PCR based identification of CLCuV resistant plants and provide the basis for rapid screening of segregated populations in breeding programs. Further, linked DNA markers, such as described for cotton leaf curl virus, are an initial step for cloning of genes for which there is a little information on its specific biochemical functions. The possibility of cloning of CLCuV resistance genes would open up new opportunities to better understand this plant virus interaction at the molecular level.

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