African Journal of Biotechnology Vol. 10(3), pp. 337-343, 17 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.215
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Isolation and identification of differentially expressed genes between *Gossypium arboreum* and *Gossypium hirsutum* species

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Accepted 11 November, 2010

Plants have evolved sophisticated molecular defense mechanisms in order to survive disease conditions. So far, a number of pathogen resistance (*R*) genes have been reported in plants. These *R* genes are thought to be involved in activating the signals that lead to disease resistance. The structural specificity of *R* genes products makes it possible to isolate these genes from plants by homology based techniques. Knowledge regarding molecular organization of *R* genes in cotton is limited and other resistant gene analogues (RGAs) still need to be identified for Cotton Leaf Curl Disease (CLCuD) resistance, particularly from *Gossypium arboreum.* This study has, therefore, been designed to identify the natural resistance related genes from *Gossypium arboreum* against CLCuV. In addition to *G. arboreum* (resistant to CLCuV)*, G. hirsutum L. var S12* (highly susceptible to CLCuV), *G. hirsutum L.* var. CP15/2 and LRA (partially resistant to CLCuV) were used as reference controls to facilitate identification of defense related genes. This paper describes several differentially expressed transcripts through DDRT-PCR on total RNA from *G. arboreum* and some other tetraploid cotton species. The nucleotide sequence of these genes, when compared to the reported database, indicated that some of the differentially expressed transcripts had homology to the reported R-genes.

Key words: Pathogen resistance genes (R-genes), *G. arboreum*, differential display, cotton leaf curl virus (CLCuV), RT-PCR.

INTRODUCTION

Cotton contributes a major proportion towards foreign exchange earnings of Pakistan. It accounts for 7.3% of the value added in agriculture and about 1.6% to its GDP (Pakistan economic survey, 2008-09). Cotton leaf curl virus (CLCuV) is the major problem in achieving high cotton production. Cotton Leaf Curl Disease (CLCuD) is caused by whitefly transmitted monopartite begomovirus having β-satellite as the infectious agent. (Briddon and Markham 2000). The disease symptoms may be characterized by upward or downward curling, vein thickening and appearance of enation on the underside of the leaf (Mansoor et al., 1993). The overall growth of the plant becomes stunted. The disease resulted in 40-80% yield losses and caused loss of US $5 billion between 1992-97 (Briddon and Markham, 2000). Due to the emergence of new Burewala strain of cotton leaf curl virus (BSCV) in 2002-2003, almost all commercial varieties that were developed to resist cotton leaf curl got susceptible to BSCV. After emergence of this new strain of virus, the disease has increased from 2.4% to 73.4 % during 2001-2007 (Arshad and Anjum, 2008). At present, there is not even a single cultivar of *Gossypium hirsutum,* which shows complete resistance against BSCV. Virologists believe that the re-current viral infections may result into the development of virulent variants through natural recombination (Varma and Malathi, 2003). The emergence and re-emergence of CLCuV (Burewala strain) of new geminivirus strains is a continuous threat to

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cotton production in Pakistan.

Desi cotton (*Gossypium arboreum)*, also known as "Old World" cotton is a diploid A-genome containing specie of Malvacea family. This *Gossypium* specie is naturally resistance to CLCuD. *G. arboreum* is believed to be evolved from its wild ancestor *G. herbaceum* (Hutchison et al. 1984). Both *G. arboreum* and *G. herbaceum* are assumed to be Asiatic cotton species and are being successfully planted in some regions of Pakistan and India (Guo et al., 2006). *G. arboreum* shows a considerable amount of immunity not only against CLCuV/BSCV but also against a number of diseases like cotton rust and bacterial blight (Mahbub, 1997, Blank and Leather 1963, Knight 1950). However, most extensively cultivated cotton species are allotetraploid *G. hirsutum L.* (upland cotton) and *G. barbedense L.* These species are known as "New World Cotton", which have been evolved by interspecific hybridization of closely related species including *G. herbaceum* or *G. arboreum* and an American diploid, *Gossypium raimondii* or *Gossypium gossypoides* (Smith and Cothren, 1999). The allotetraploid cotton has somehow lost resistance against a number of pathogens. Since the genetic diversity and variability provides the basis of genetic improvement, the *G. arboreum* may prove to be a diverse resource to be explored through biotechnological tools.

G. arboreum is the native cotton cultivar of Pakistan. This diploid cotton specie is naturally resistant to the CLCuV and many other pathogens (Briddon et al., 2000; Rahman et al., 2001; Lapidot and Friedmann, 2002). It can withstand heavy loads of virus without development of infection symptoms (Rahman et al., 2002; Rahman et al. 2005). The *G. arboreum*, therefore, provides an ideal source for exploring the *R*-genes and may help in understanding the mechanism of natural resistance against CLCuV infection. This diploid cotton specie along with other tetraploid cotton lines having partial resistance to CLCuV infection (*G. barbedense*, *G. hirsutum* line LRA and CP15/2) along with a susceptible line (*G. hirsutum* var. S12), were selected to explore the differentially expressed transcripts through our modified technique of differential display (Iqbal et al., 2008). We selected 9 differentially expressed transcripts from the selected cotton species. The differentially expressed transcripts were cloned in TA based cloning vector and sequenced. Nucleotide sequence similarity was searched in the GenBank. Most of the clones indicated their alleged role related to plant defense mechanism.

MATERIAL AND METHOD

Plant Material

Seeds of *G. arboreum, G. barbedense, G. hirsutum L.* Var. S12, LRA and CP15/2 were collected from Central Cotton Research Institute (CCRI) Multan, Pakistan. Seeds were sown in the field area of National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan, under natural environment and normal conditions suitable for cotton cultivation. Fresh leaves samples were collected from these plants and kept frozen in liquid nitrogen.

Total RNA Extraction

Total RNA was extracted from young sprouting leaves of fore mentioned cotton species by using plant RNA purification reagent (Invitrogen, USA) following the manufacturer's instructions. Several precautionary measures that were mandatory for RNA work had been considered, while extracting the total RNA. Agarose gel electrophoresis was used to test the integrity and purity of the extracted RNA.

Isolation of messenger RNA and cDNA synthesis

Messenger RNA was isolated from total RNA by using oligo dT cellulose columns (MRC, USA) according to manufacturer's instructions. The mRNA loaded columns were washed twice with binding buffer and mRNA was eluted in elution buffer. The eluted mRNA was precipitated and dissolved in DEPC treated water. Messenger RNA was reverse transcribed to cDNA using Revert Aid H- First Strand cDNA synthesis kit (Fermentas, USA). Three reverse transcription reactions were carried out for each mRNA using three anchored ($T_{11}M$) primers (where M may be G, C or A). The products of reverse transcription (cDNA) were stored at -20^{o}C. This cDNA was later used as template for differential display PCR amplifications.

Differential Display RT-PCR

Differential display RT-PCR was performed using the same anchoring primers as used in cDNA synthesis and random (arbitrary) decamers supplied by (GeneLink, USA). PCR was carried out in a final reaction volume of 50µl containing 2.5µl (50ng/µl) of first strand cDNA, 5 µl of 10x PCR buffer, 4µl MgCl2 (25mM), 1µl of dNTPs (10mM each) 2 µl of anchored primer (250ng/µl) 8µl of arbitrary primer (100ng/µl), 0.5µl of *Taq* DNA polymerase (5U/µl; Fermentas, USA), 27µl double distilled PCR grade water. The PCR amplification profile included first cycle at 94^{o}C for 4 min; 30^{o}C for 2 min; 72^{o}C for 2 min followed by 35 cycles at 94^{o}C for 1min; 30^{o}C for 1min and 72^{o}C for 10 min. The amplified products were resolved on 1% agarose gel and stained with ethidium bromide.

Cloning and sequencing of differentially expressed transcripts

The differentially expressed bands were excised from the gel and extracted by QIAGEN gel extraction kit. The eluted DNA fragments were cloned using InstaClone TA cloning kit (Fermentas, USA) and transformed in *Escherichia coli* TOP10 competent cells. The plasmid DNA from the purified clones were sequenced using M13 (-20) forward and M13 reverse primers using BigDye terminator v 3.1 cycle sequencing kit on ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA).

Analysis of differentially expressed transcripts

The nucleotide sequences for each of the cloned transcripts were translated to derive the coded amino acid sequence using translate tool available at www.expasy.ch. The sequences were then BLAST searched at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The identity of the clones was determined from their similarity in the GenBank.

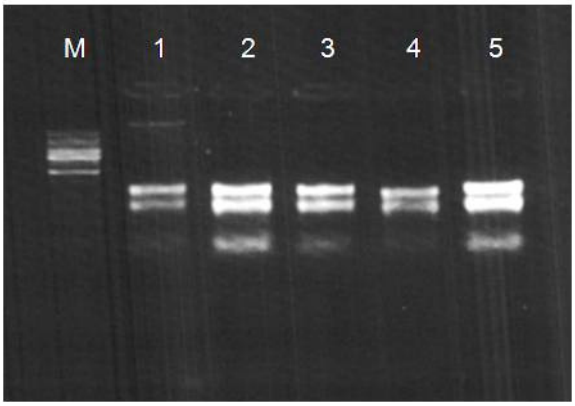

Figure 1: Total RNA isolated from five different cotton lines.
Lanes 1 through 5 represent total RNA isolated from; 1) *G. arboreum*; 2) *G. barbadense*; 3) *G. hisutum* var. S12; 4) *G. hirsutum* line CP15/2; and 5) *G.hirsutum* line LRA 5166. Line L represents 1Kb DNA ladder.

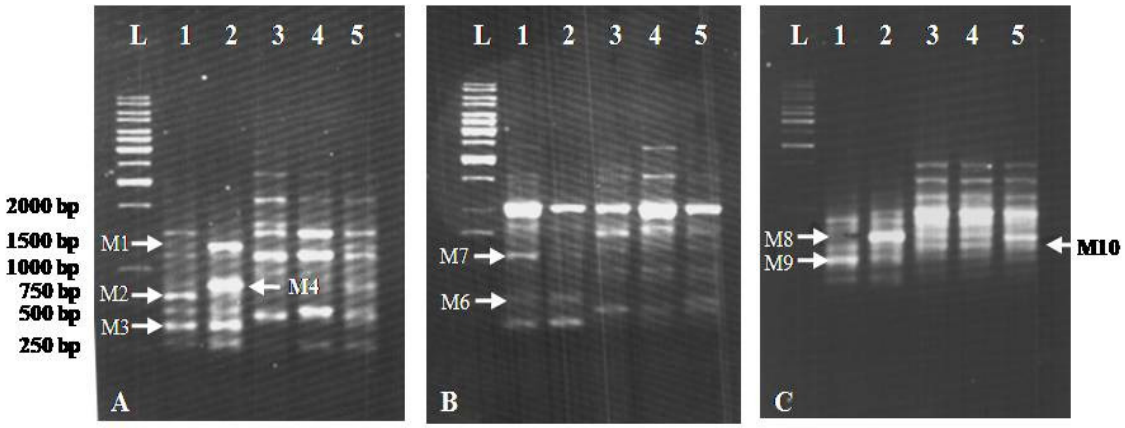

Figure 2. Results of DDRT-PCR for the identification of differentially expressed transcripts in five cotton varieties. Panel A, B and C represent amplifications using 3 different arbitrary primers (one specific to each panel) but same anchoring primer (all the three panels). The lanes 1 through 5 represent the DDRT-PCR from five different cotton lines in the same sequence: 1) *G. arboreum*; 2) *G. barbadense*; 3) *G. hisutum* var. S12; 4) *G. hirsutum* line CP15/2; and 5) *G.hirsutum* line LRA 5166. Line L represents 1Kb DNA ladder. The differentially expressed transcripts are indicated by arrows (M1 to M10).

Results and Discussion

Differential display reverse transcriptase RT-PCR technique was used to detect differentially expressed genes from *G. arboreum*, which is naturally resistant to CLCuD in comparison with different *G.hirsutum* lines susceptible or partially resistant to CLCuV. Total RNA was isolated from the leaves of all varieties indicated in Figure 1. Nine differentially amplified fragments were selected from five cotton cultivars using single anchored and random decamers. The DNA fragments from the nine differentially expressed transcripts (DETs) were isolated from gel and cloned in the pTZ57R/T cloning vector (Figure 3).

The DDRT-PCR indicated that many of the transcripts were similar in size from all the five cultivars of cotton. The similar sized amplification products were considered to be common transcripts in the 5 selected cotton lines and might represent the house keeping genes. Eight of the transcripts represented differentially expressed genes, while the ninth (M10) represented the transcript expressed in all the cotton lines (Figure 2). Although, *G.barbadense* has partial resistance against CLCuD, but this cultivar is not well adapted in Pakistan. The sequence analysis and BLAST searches of the cloned DETs indicated that all the DETs had similarity to pre-characterized genes (Table1). The nine DETs were, therefore, grouped into three categories. The group 1

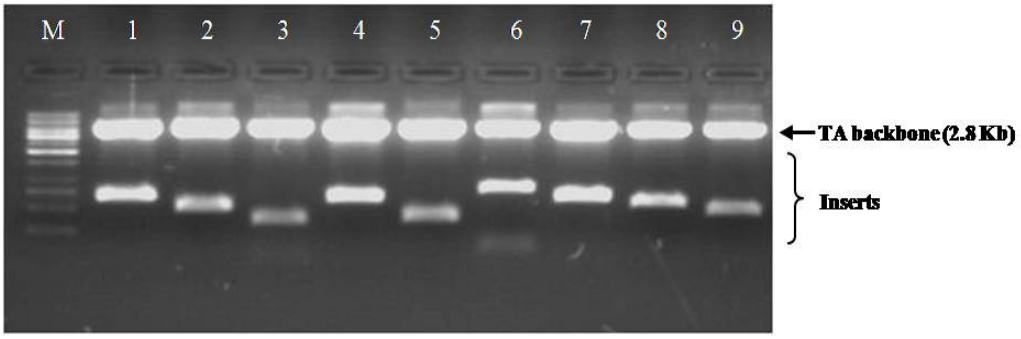

Figure 3. Restriction digestion of the clones having differentially expressed transcripts. The plasmid from each clone was digested with *EcoR*I and *Hind*III to excise the cloned fragment. L; (1Kb ladder), Lanes M1-M10 represent the backbone of TA vector having different insert sizes with respect to each clone of 9 DETs.

contained house keeping genes, group 2 comprised of resistance related genes (R-genes), while group 3 contained special function genes.

House keeping genes

M10 transcript was detected in all the five cultivars of cotton used in this study. This fragment of gene showed similarity with NADH dehydrogenase gene of *Nicotiana tobacum*. NADH dehydrogenase is usually involved in cellular respiration, which is the process of oxidizing food molecules like glucose to carbon dioxide and water. During this oxidation reaction energy is released in the form of ATP, which is utilized by the cell in all energy consuming activities. Energy is the basic requirement of every living cell for performing various functions. It is presumed that due to this reason NADH dehydrogenase gene is detected in all the cultivars of cotton (Dzelzkalns et al., 1994).

Resistance related genes (R-genes)

M3 gene, expressed in *G. arboreum* and *G. barbadense,* was found to have similarity with rhodanese family protein of *A. thaliana*. The existence of rhodanese family proteins (thiosulfate:cyanide sulfurtransferase) in plants has been highly controversial (Hatzfeld and Saito, 2000). Plants may achieve the defense against pathogens through various mechanisms, which may involve signal transduction pathways or alternatively by production of metabolites or hormone like substances. In plants, these products are quite diverse and range from alkaloids to terpenes, phenolics, steroidal, cyanogenic and mustared oil glycosides. Phenolics are ubiquitous in seed plants; alkaloids occur in several thousand species and cyanogenic glycosides occur in only a few hundred species (Abou and Diekert, 1971; Acedo and Rohde, 1971; Levin, 1976). Rhodaneses catalyzes the transfer of sulfane sulfur from thiosulfate or thiosulfonates to thiophilic acceptors such as cyanide and dithiols (Ray et al. 2000), which makes these plants unsuitable host for various pathogens.

M4 gene transcripts were observed in *G. arboreum* and *G. barbedense*. This gene was identified to have identity with transketolase through BLASTx. Transketolase is involved in calvin cycle and in oxidative pentose phosphate pathway. It produces erythrose-4-phosphate, which is the precursor to shikimate pathway leading to phenynpropanoid metabolism (Henkes et al, 2001). A family of transketolases directs the isoprenoid bio-synthesis via mevalonate independent pathway (Lange et al, 1998). The isoprenoids comprise the largest family of natural products with over 20,000 individual compounds described to date (Connolly, & Hill, 1991). The isoprenoids play numerous functional roles in plants as hormones (gibberelins and abscisic acid), photosynthetic pigments (side chain of phytol carotenoids), electron carriers (side chain of plastoquinone), and structural components of membranes (phytosterols). Isoprenoids also serve in communication and defense as attractants for pollinators and seed dispersers. They also acts as competitive phytotoxins, antibiotics, herbivore repellents and toxins (Harborne and Tomas-Berbean 1991). M4 gene transcript observed in *G. arboretum*, *G. barbedense* and *G. hirsutum* line LRA5166, might have some role in the production of metabolites conferring pathogen resistance.

M6 gene was observed in *G. arboreum* and *G. barbedense.* This gene has similarity with oxysterol binding protein (OBP) gene of *G. raimondii*. It represents a family of genes with a role in program cell death (PCD). In animals the oxysterol binding proteins (OBPs) are very well documented. Oxysterols are oxygenated derivatives of cholesterol that influence a variety of biological functions, including sterol metabolism, sphingolipid metabolism, lipid trafficking, apoptosis and necrosis in mammals (Anniss et al., 2002; Panini and Sinesky 2001; Schroepfer, 2000). Oxysterol-induced cell death in

Table 1: Analysis of the differentially expressed transcripts (DETs). The DDRT-PCR results are indicated as (+) for the presence of a differentially expressed transcript and (-) for the absence. The similarities of DETs to the genes reported in GenBank are indicated by their accession numbers. The E values indicate the error rate in similarity index.

DETs	Size/Accession No. of DDRT-PCR clone	*G .arboreum*	*G. barbedense*	S12	CP15/2	LRA5166	Similarity to known genes	E Value
M1	700 bp EF502024	+	+	-	-	+	Sporulation protein RMD5, putative (*Ricinus communis*) Accession no. EEF33478	1e-79
M2	470 bp EF502025	+	-	-	-	-	Gossypium hirsutum cotton fiber protein mRNA. Accession no.EF440359	0.0
M3	347 bp EF502026	+	+	-	-	-	Putative rhodanese family protein (*Arabidopsis thaliana*) Accession no.AAL69515	4e-40
M4	505 bp EF502027	+	+	-	-	+	Transketolase, putative (*Ricinus communis*) Accession no. EEF50359	3e-89
M6	349 bp EF502028	+	+	-	-	-	Oxysterol-binding protein, putative (*Ricinus communis*) Accession no. EEF47596	8e-46
M7	556 bp EF502029	+	+	-	-	-	Adenosine diphosphatase, putative (*Ricinus communis*) Accession no. EEF39928	1e-41
M8	631 bp EF502030	-	+	+	+	-	Receptor-like kinase (*Glycine max*). Accession no. BAH56602	2e-38
M9	543 bp EF502031	+	+	-	-	-	Glutathione-s-transferase omega, putative (*Ricinus communis*); Accession no. EEF37311	6e-53
M10	441 bp EF502032	+	+	+	+	+	*Carica papaya* mitochondrion, complete genome Accession no. EU431224	0.0

animals correlates with the activity of OBPs (Bakos et al., 1993). OBPs are in fact nuclear receptors that also function as transcription factors and coordinately regulate sterol catabolism, storage, efflux and elimination of pathogens (Repa et al., 2002). Although, a role for OBPs in plant defense response remains to be demonstrated, yet there is a connection between the sterol-binding capacity of elicitins, small cysteine-rich proteins secreted by *Phytophthora* spp. However, their ability to activate plant defense system through hypersensitive response (HR) in tobacco has been well documented (Blein et al., 2002; Osman et al., 2001).

Glycine max receptor like kinase (RLK) gene (M8) was detected in *G. arboreum* and *G. barbedense.* Plant RLKs are grouped into a single family based on their configuration as transmembrane kinases with serine and threonine specificity. Many genes for plant resistance to bacterial, fungal and viral diseases encode receptor-like serine/threonine kinases (RLK), which specifically recognize the proteins of particular pathogenic strains and then trigger plant specific and nonspecific immune responses (Zaitsev et al., 2002; Cohn et al., 2001; Nürnberger and Scheel, 2001). Andrea et al., (2004) identified a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein (NSP). These proteins, designated LeNIK (*Lycopersicon esculentum*; NSP-interacting kinase) and GmNIK (Glycine max NIK) belong to the LRR-RLK (leucine rich-repeat receptor-like kinase) family. This protein family represents the major class of resistance gene analogues in plants and confers resistance response to pathogens.

Glutathione s-tranferase gene (M9) was detected in *G. arboreum* and *G. barbedense* in this study. This protein has been reported to play a key role in constitutive and inducible plant defense mechanism (Damien et al., 2003). The soluble glutathione transferases (GST) are encoded by a large and diverse gene family in plants, which can be sub-divided on the basis of sequence identity into the phi, tau, theta, zeta and lambda classes (David et al., 2002). GSTs are predominantly expressed in the cytosol, where their GSH-dependent catalytic functions includes the conjugation and resulting detoxification of herbicides, the reduction of organic hydroperoxides formed during oxidative stress and the isomerization of maleylacetoacetate to fumarylacetoacetate. Recent studies have also implicated GSTs as components of ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis (David *et al*., 2002).

Special function genes

M1 gene was found to have its protein sequence similarity with catabolic degrading protein of RMD family. RMD5 protein is very well studied in *Saccharomyces cerevisiae.* It is a cytosolic protein required for sporulation and for the ubiquitination of gluconeogenetic enzyme fructose-1, 6- biphosphatase, which in turn degrades rapidly after switching from gluconeogenesis to glycolysis (Regelmann et al., 2003). The identification of gene transcripts for RDM5 in *G. arboreum* and *G. barbadense* indicates that this gene might be involved in some specialized function in the two cotton species.

M2 gene expression was detected in *G. arboreum* and *G. barbedense*. This gene has similarity with the *Gossypium hirsutum* L. cotton fiber protein mRNA. Cotton fiber is a unique unicellular architecture. A number of genes which are active in fiber cells are similar to those in leaf, ovule or root tissues (John and Laura, 1992). *Gossypium hirsutum* L. fiber protein mRNA identified in leaf tissues of *G. arboreum* and *G. barbedense* might have some similarity with the reported fiber related genes.

M7 gene transcript was detected both in *G. arboreum* and *G. barbedense*. This gene exhibited strong similarity with adenosine diphosphatase of *Ricinus communis.* Under normal conditions, the ADP molecules generated during the metabolic processes regulate the metabolic pathways by feedback inhibition in conjunction with inorganic phosphate molecules. The breakdown of ATP to ADP and AMP with the release of P(i) acts as modulators of the activity of different glycolytic enzymes (Buchanan et al., 2000; Liguzinski and Korzeniewski, 2006). The detection of specific adenosine diphosphatase in leaf tissues of *G. arboreum* and *G. barbedense* indicates that these plant species have diverse adenosine diphosphatase as compared to other cotton species included in this study.

This study was designed for the identification of differentially expressed genes between the CLCuV susceptible and resistant cotton lines. This study revealed several candidate genes with respect to the *G. arboreum* specific R-genes. Moreover, the diversity of the sequences between the common transcripts among the tested cotton species was also observed. The presented data sports a strong need to explore the CLCuV resistant cotton lines at the genomic scale in further gene discovery programs. A large scale study might reveal more R-genes associated with CLCuV resistant cotton lines. It is concluded that *G. arboreum* may serve as an important genetic resource for isolating the pathogen resistance genes, as it is well adapted to the environmental, climatic and geographical location of Pakistan.

Acknowledgements

This study was financially supported by a project grant (CS152) from Pakistan Agricultural Research Council under the Agricultural Linkages Program (PARC-ALP).

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