Recent Developments in Cotton Biotech Research in Pakistan

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Pakistan ranks fourth in cotton lint production and is one of the largest producers of cotton yarn in the world. Cotton production affects both rural and urban economies in the country. Cotton faces many challenges, including diseases, pests, climate change, planting seed quality, weed infestation, competition with other crops, rising cost of production, prices received by farmers and the need to increase production to supply the local spinning and textile industry. Domestically-produced cotton is insufficient to provide a reliable supply of raw material for the textile industry. So, there is a future need for quality enhancement in cotton fiber and use of niche areas where longer and finer cotton can be grown. Therefore, the simultaneous improvement of yields and lint quality is an imperative future challenge. Biotechnology offers solutions to some of these issues, while progress in cotton genomics has created a window of opportunity to understand the complex biological processes involved in fiber development and plant responses to biotic and abiotic stress. At present, genetically-engineered cotton containing a gene (Cry1Ac) for resistance against bollworms has been commercialized in Pakistan and is grown on around 90% of the area under cotton cultivation. There are several institutions in the country that have varying capacity to work on cotton biotechnology. The National Institute for Biotechnology and Genetic Engineering (NIBGE), located in Faisalabad, and the Center of Excellence in Molecular Biology (CEMB), in Lahore, are the leading centers with trained manpower and well-equipped research facilities. These centers have the capacity to understand the key limiting factors for the cotton crop and to generate synthetic and optimized gene constructs transformation in cotton. In recent years, several traditional cotton research institutes have developed research collaborations with institutes specialized in biotechnology to introgress genetically engineered traits in cotton.

Cotton Leaf Curl Virus Complex: The Disease and Resistance

Cotton leaf curl disease is a continuous threat to cotton crops and is caused by a complex of whitefly-transmitted geminiviruses (Family Geminiviridae, genus Begomovirus) (Mansoor *et al*., 2008). Although the disease has been known to occur for a long time, it emerged as a major limiting factor in the early 1990s. The disease was found to be associated with several distinct begomoviruses that were able to produce disease symptoms when co-inoculated with a disease-specific DNA satellite called Cotton leaf curl Multan betasatellite (Mansoor *et al*., 2003). Two sources of resistance (LRA5166 and CP15/2) were identified in germplasm collections. The dominant source of resistance was introgressed into local elite cotton lines in the late 1990s. Unfortunately, resistance was quickly broken due to the emergence of the resistance-breaking "Burewala strain" (Mansoor *et al*., 2003; Amin *et al*., 2006; Amrao *et al*., 2010). The disease complex has been constantly evolving by recombination, component capture and mutations that help the virus complex to overcome host resistance strategies (Mansoor *et al.*, 2003; Mansoor *et al*., 2006; Zaidi *et al*., 2016). The virus is rapidly evolving to make newly developed and introduced cultivars susceptible to new CLCuV species. Strategies are therefore required that intelligently combine natural and genetically-engineered resistance, based on the concept of broad-spectrum and high-level resistance (Ilyas *et al*., 2011). Foreign and endogenous resistance sources are being characterized for combined use in cotton improvement. A new source of natural resistance has been identified in cotton germplasm imported from the USDA germplasm collection. The complete genome of the cotton line named Mac7 is being sequenced and the genetics of disease resistance are being investigated.

Efforts at genetically-engineered resistance are employing strategies aimed at creating broad-spectrum resistance. Triple gene constructs (either microRNA or siRNAi) targeting different regions of CLCuD components (begomovirus and betasatellite) were characterized in tobacco and are being introduced into cotton in both Coker 312 and local elite cultivars. There are other efforts aimed at creating broad-spectrum resistance based on the expression of single-stranded DNA binding proteins (G5 and VirE2) or a protein that binds with geminate particles called GroEL (Rasool *et al.*, 2016; Yousuaf *et al.* 2015). Several gene constructs based on genome editing tools, such as zinc fingers, TALENS and CRISPR-Cas9, are also being investigated, either alone or in combinations of two or three genes. Transgenic cotton expressing truncated Rep and antisense Rep gene of cotton leaf curl virus showed tolerance to CLCuD under field conditions (Hashmi *et al.*, 2011; unpublished data).

Resistance Against Whiteflies/ Sucking Pests

The whitefly is not only a vector for cotton leaf curl disease, but is also an important sucking pest (Oliveira *et al.*, 2001; Mansoor *et al.*, 2008). It is directly involved in disease transmission and the severity of infestation due to the availability of other host plants makes it a serious pest of cotton. Several distinct biotypes/species of whitefly (*Bemisia*

Table 1: Insect Bioassay of Transgenic Cotton (Coker 312) Using the Armyworm (*Spodoptera litura*)

S. No.	Genes	Event or Cotton Line	Plants (T_0)	Leaves	Larvae (1st instar 5 larvae per leaf)	Insects Mortality	Insects Mortality (%)
1	Cry1Ac+Cry2Ab+EPSPS	C3- E2	P1	3	15	15	100
2	Cry1Ac+Cry2Ab	CC15- E49	P1	3	15	13	87
3	Cry1Ac+Cry2Ab	CC15- E49	P2	3	15	13	87
4	Control (Non-GM cotton)	Coker 312		3	15	3	20

tabaci) are found in cotton and have been characterized over time and space in Pakistan, using the cytochrome C oxidase (CO1) gene as a marker (Ahmed *et al.*, 2011; Ashfaq *et al.*, 2014). Efforts are also under way to completely sequence the AsiaII1 biotype, the dominant biotype/species in Pakistan. The use of RNA interference (RNAi) to silence whitefly genes has shown promising results. Sequence diversity analysis has demonstrated that the target genes are conserved among the whitefly biotypes found in the region and their silencing by RNAi is promising. Three gene constructs targeting whiteflies (one micro-RNA and two RNAi constructs) were characterized in tobacco. Transgenic tobacco showed resistance against whiteflies (Raza *et al.*, 2016). These DNA constructs have been introduced into cotton Coker 312.

Another approach is based on the expression of insecticidal proteins under a phloem-specific promoter isolated from the 'Banana bunchy top virus.' Two insecticidal proteins, namely Hvt (a gene isolated from the Australian funnel web spider) and onion leaf lectin, were shown to provide broad-spectrum resistance against sucking pests in a model plant (Javed *et al.*, 2016). Efforts are now underway to transform the gene construct into cotton.

Resistance Against Cotton Bollworms

In Pakistan, a complex of chewing insects, such as *Helicoverpa armigera*, *Earias vittella* and *Pectinophora gossypiella*, which require significant insecticide use for proper management, attack cotton. Natural sources of resistance to these pests are limited in the cotton gene pool; therefore, Bt (biotech) cotton was formally approved for commercial cultivation in the country in 2010 (Robert *et al*., 2012). Although providing effective protection against bollworms, most of the Bt cotton varieties grown have a comparatively low expression level of Cry1Ac and appropriate refuge plans have not been followed. Widespread adoption of Bt crops without proper refuges imposes high selection pressure for development of resistance in target insect pests and the planting of Bt cotton expressing low levels of toxin may limit its efficacy. Time is therefore needed to fuse two different genes with unique mode of actions in order to develop biopesticides with long-lasting resistance to insect pests.

Transgenic cotton containing double Bt Cry1Ac and Cry2A genes, developed by the CEMB, has been commercialized, while cotton containing glyphosate resistance and Bt genes is under trials (Puspito *et al.*, 2015; Latif *et al.*, 2015). The NIBGE has developed a number of synthetic genes for insect resistance and herbicide tolerance. Transgenic cotton Coker 312 with double (Cry1Ac + Cry2Ab) and triple (Cry1Ac + Cry2Ab+ EPSPS) gene constructs has been developed. Cry1Ac has been found to be very effective against the cotton bollworm (*H. armigera*) and ineffective against the armyworm (*Spodoptera littoralis*). The transgenic cotton expressing Cry2Ab was exposed to insect bioassays using armyworm. Some lines exhibited 80-100% mortality of 1st instar larvae of the armyworm within 48 hours of exposure. The NIBGE has also developed a novel technology, the ω-ACTX–Hv1a toxin gene (Hvt), based on the Australian funnel web spider. Most spider venoms are rich sources of insecticidal compounds and their primary role is to kill or paralyze arthropod prey. Hvt is a 37 amino acid, insect specific calcium channel antagonist peptide, which is toxic to a range of agriculturally-important arthropods in the orders *Coleoptera*, *Lepidoptera* and *Diptera*, but has been reported to have no effects on a number of mammals. The transgenic cotton expressing Hvt gene was tested against the armyworm (*Spodoptera litura*) and *Heliothis armigera* and 80-100% mortality was recorded in 1st instar larvae within 96 hours. The results of both genes are significant for the development of transgenic elite cotton cultivars against the armyworm, as well as the management of resistance against the cotton bollworm (*H. armigera*) and the pink bollworm. The transgenic cotton overexpressing Hvt showed stable inheritance and insect resistance in subsequent generations, making it a valuable germplasm source for breeding insect-resistant cotton, and is being used for introgression of the spider gene into local elite cultivars, which can be shared with breeders subject to biosafety clearance. Moreover, it should also be combined with Bt genes to further enhance the life and scope of Bt cotton for long-term and broad-spectrum insect control. (See Table 1 above).

Transgenic Cotton for Abiotic Stress Tolerance

Abiotic stresses, such as heat, drought, salinity and flooding, are important challenges in global warming and climate change scenarios. Unpredicted patterns of rainfall and heat waves have further complicated abiotic stresses, highlighting the need to develop cotton cultivars with improved abiotic stress tolerance. The AVP1 (Arabidopsis pyrophosphatase type I)

gene is well known to confer tolerance against abiotic stresses, such as drought and salinity. (Yu *et al.*, 2016). Transgenic cotton Coker 312 expressing AVP1 gene also showed improved seed germination, extensive root growth, efficient photosynthesis, nutrient uptake and, ultimately, plant vigor as well as increased cotton yields (Gaxiola *et al.*, 2016; Pasapulla *et al.*, 2011). It also produced superior quality fiber by bringing significant improvement in fiber characteristics, particularly spinning consistency index (SCI), micronaire values, maturity index and ginning out turn (GOT). Transgenic AVP1 cotton germplasm can be an important source of genetic information for improving plant response against abiotic stresses when used as breeding parents. The transgenic cotton Coker 312 germplasm has been shared with breeders at NIBGE, NIAB (National Institute for Agriculture and Biology, Faisalabad) and NIA (Nuclear Institute of Agriculture, Tandojam) and is being introgressed into local elite cultivars, which could be a vital base source for developing climate resilience in cotton

Enhancement of Seed Germination

Major abiotic stresses, namely salinity and drought, are the key factors that adversely affect the germination of cotton seeds. Seed viability is a major problem in ensuring optimum plant population in the field for sustainable cotton production in Pakistan. The seed germination reported in most cotton varieties is around 50-60% (Nabi *et al.*, 2001). 10% polyethylene glycol-based seed germination analysis showed high and enhanced seed germination potential in transgenic cotton seeds in comparison with non-transformed control plants. The AVP1 protein is also known to be directly involved in facilitating auxin transport and plays a vital role in organ development, hyperplasia, and other developmental events. In addition to proton electrochemical gradient (PEG), AVP1 determines the abundance and distribution of P-adenosine triphosphatase and Pi formed auxin efflux facilitator affecting auxin distribution and organogenesis (Li *et al.*, 2005). AVP1 transgenic cotton tolerant to abiotic stress exhibits vigorous growth, higher biomass and yield. The seed germination of transgenic (AVP1) cotton shows earlier germination in comparison with control plants. Early germination ensures that plants emerge from the soil at the same time and show faster growth. Thus, AVP1 cotton has the potential to improve seed germination and enhance plant vigor. The problem of low seed germination may be solved by introgression of a high seed germination trait from AVP1 transgenic cotton into other local cotton varieties, which may also help optimize the number of plants required per unit of area for sustainable cotton yield.

Fiber Length Improvement

Cotton fibers are differentiated unbranched epidermal cells. The development of fibers involves complex processes that can be divided into four overlapping stages: fiber initiation, elongation, secondary cell wall synthesis and, finally, maturation. The elongation phase is perhaps the best-studied period of fiber development. The developing fiber cells have cytoplasmic connections (plasmodesmata) to the neighboring epidermal cells. The fiber length of cotton genotypes correlates with the closure time period of plasmodesmata (PD). The closure of PD generates and maintains a high turgor pressure to improve fiber elongation. Fiber specific β-1,3-endoglucanase (*GhEG*) gene degrades the callose deposition at the fiber base plasmodesmata (Pd), resulting in the re-opening of PD, which ultimately terminates the elongation phase of developing fiber (Ruan *et al.,* 2004; Ruan, 2007). To improve cotton fiber length, NIBGE has developed transgenic cotton 312 by down-regulating this gene through RNAi. The closing of PD by callose deposition and reduction in the expression of *GhEG* in trangenic cotton Coker 312 lines resulted in a 2-3 mm increase in fiber length.

Marker-assisted Cotton Breeding

In Pakistan, efforts were made to understand, as a potential buffer against the spread of disease, the extent of genetic diversity among the available cotton germplasm using a number of markers, including RAPD, SSRs and SNPs—unanimously declared narrow genetic base (Rahman *et al.*, 2002, 2008; Shaheen *et al.*, 2010, 2014, 2016; Tabassam *et al.*, 2014). The information generated was extensively used for planning crosses and also bringing new alleles from untapped genetic resources, including diploid cotton species and old genotypes. For example, introgression work assisted by SSR markers was started—culminating in the development of new germplasm. This germplasm is being extensively used in the development of new cotton cultivars. Similarly, accessions introduced through the USDA-assisted project are being utilized in the development of new cultivars using conventional or molecular breeding procedures.

Cotton leaf curl, a disease of viral origin, has been found detrimental to cotton production worldwide, including in Pakistan. A number of genotypes showing resistance and/or high tolerance were identified and used for identifying DNA markers associated with resistance to the disease. For example, RAPD markers OPO-$19_{460,}$ OPQ-14_{325} and OPY-2_{1080}, as well as SSR markers JESPR-151 and CM-3, associated with resistance to the old strain of virus were identified (Rahman *et al.*, 2009). Similarly, markers associated with resistance to CLCuBuV disease were identified (Abbas *et al.*, 2015). Two QTLs, i.e. QCLCuD25 and QCLCuD26, associated with CLCuD resistance were also identified (Rahman *et al.*, 2014). In another population, a total two QTLs (one at 40.1 cM away from BNL-1421 and located on chromosome number 7; and the second at 44.1 cM away from PR-1360 and mapped on chromosome number 25) were mapped (Hussain *et al.*, 2016). Similarly, a number of DNA markers associated with nectarines, hairiness and red leaf color were identified (Rahman *et al.*, 2002, 2003; Ali *et al.*, 2009). Also, six QTLs impacting the productivity traits were identified using a diploid mapping population (Shaheen *et al.*, 2013).

Drought, another important stress, has become especially alarming after the onset of climate change in Pakistan. For initiating molecular breeding programs, seven QTLs (osmotic potential 2, osmotic adjustment 1, seed cotton yield 1, number of bolls/plant 1, boll weight 1 and plant height 1) were identified. These QTLs can be deployed in MAS aiming at the development of drought-tolerant cotton cultivars (Saeed *et al.*, 2011).

Also, DNA markers associated with certain fiber traits were identified in multiple studies using interspecific and intraspecific populations. For example, 75 marker-trait associations were identified for different fiber traits [13 SSR markers for average boll weight, 18 for GOT percentage, eight for micronaire value, 18 for staple length, three for fiber bundle strength and 15 for uniformity index (Atif, 2016)]. Other new procedures, for example, TILLING (targeted induced local lesions in genome)—a reverse genetic approach—, have been used to explore phenotypic diversity, followed by associating these variations with mutations in the cotton genome. The mutation frequency was found to be in the range of 0.94% to 5.6%. The development of mutagenic cotton populations will enrich breeding resources for the production of sustainable cotton candidates in the future (Hasan, 2014).

Cotton Genomics

There are exciting developments in cotton genomics that can be used to understand gene functions and regulatory pathways in cotton. Virus-induced gene silencing (VIGS) is a reverse genetic approach used for the characterization of gene functions by repressing the transcript level of a target gene (Padmanabhan *et al.*, 2009). This approach utilizes plant defense mechanisms against invading viriods and viruses and knocks down the expression of the target gene by post-transcriptional gene silencing. VIGS is activated by the infection of engineered virus vectors having partial sequences of the target genes to be silenced. When infected with the virus vector, the plants produce virus-related siRNA. These siRNA mediate the degradation of related endogenous gene transcripts, resulting in silencing the target gene expression (Baulcombe, 1999; Waterhouse *et al.*, 2001).

The entire genome sequences of *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense* (Li *et al.*, 2014; Li *et al.*, 2015; Yuan *et al.*, 2015; Paterson *et al.*, 2012) are publicly available. The VIGS system provides an alternative and attractive approach to identify the function of many genes involved in fiber development, biotic and abiotic stresses, and tolerance and resistance to verticillium, by suppressing the target genes and bypassing the need for the traditional transformation approach, which is usually time-consuming and laborious.

Recently, successful optimization of a TRV-based VIGS system in three cultivated cotton species (*G. hirsutum*, *G. arboreum* and *G. herbaceum)* has been reported from Pakistan (Mustafa *et al.*, 2016). Researchers used a TRV-based VIGS assay to determine the role of GhECR in resistance to the *V. dahliae* strain (king isolate, field JR2, VdLS.17) and the *Fusarium oxysporum f. sp.* vasinfectum strain (FOV CA10, FOV5, RBHI) in *G. hirsutum* cultivar FM9160 that is partially resistant to *V. dahliae.* GhECR-silenced plants showed more susceptibility in comparison to non-silenced plants and produced wilting, chlorosis, defoliating symptoms. Moreover, it was also shown that reduced expression of GhECR produced a phenotype typical of cell death/necrotic lesion in leaves of *G. hirsutum.* Collectively these results suggest that the GhECR gene has a key function in disease resistance and cell viability in *G. hirsutum* (Roma Mustafa manuscript in preparation).

Deep sequencing of short RNA has been utilized to characterize miRNAs in *G. arboreum* under abiotic stresses, such as heat, drought and salt tolerance. NIBGE results have identified several miRNA that were up-regulated or down-regulated during abiotic stresses. The target genes of these miRNAs included cellular factors, transurases, structural proteins and stress-related proteins. From the total of 1303 miRNA families expressed commonly under all stresses, 197 families are considered to be highly abundant (i.e. have a copy number greater than 60). miR166 was the most abundant family and was up-regulated in the case of all three stresses. During this study we also came across 29 novel miRNA families and identification of their targets is currently under way (manuscript in preparation).

Several cDNA libraries have been developed from *G. arboreum* and *G. hirsutum* to clone useful genes and to understand their functions. Some of the cloned genes are being tested for their potential to improve fiber quality. In the future, whole genome sequencing or exome sequencing will be able to be done on novel germplasm resources in cotton.

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