VECTORS CONSTRUCTION FOR THE DEVELOPMENT OF COTTON LEAF CURL DISEASE AND INSECT RESISTANCE IN COTTON

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By

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CERTIFICATE

This thesis submitted by Muhammad Arshad is accepted in its present form by the National Institute for Biotechnology and Genetic Engineering (NIBGE) and Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Biotechnology.

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RdRP	RNA dependent RNA polymerase
Rep	Replication associated protein
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAase `	Ribonuclease
RNAi	RNA interference
si RNA	small interfering RNA
SL	Stem Loop
ss DNA/RNA	Single stranded DNA/RNA
SV40	Simian virus 40
T-DNA	Transfer DNA
TEs	Transposable elements
TEV	Tobacco etch virus
TGMV	Tomato golden mosaic virus
TGS	Transcriptional gene silencing
TLCV-Pk	Tomato leaf curl virus Pakistan
TYLCV-Aus	Tomato yellow leaf curl virus Australia
TMV	Tobacco mosaic virus
TPCTV	Tomato pseudo curly top virus
TYLCV	Tomato yellow leaf curl virus
V	Virion
VIGS	Virus induced gene silencing
WDV	Wheat dwarf virus
µl	Micro liter
Bt	Bacillus thuringiensis
ICP	Insecticidal crystal protein
PEP	Phosphoenolpyruvate carboxylase
IPM	Integrated pest management
GUS	Glucuronidase synthase
GC	Guanine, Cytosine
CDPK	Calcium dependent Protein Kinase
cry	Crystal gene
Cry	Crystal protein
IR	Insect resistant
FH	Faisalabad hybrid
CIM	Cotton research institute Multan
DGR	Director General Research

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Abstract

Cotton is a high value agricultural product, adapted mainly for textile fiber and also for production of vegetable oil. Cotton leaf curl disease is caused by whitefly-transmitted begomovirus, which require additional satellite DNA components like DNA β to induce symptoms. AC1 gene (replication associated protein) is absolutely required for replication of viral DNA. In addition to virus, cotton plant is attacked by a number of insect pests; the major ones are the cotton bollworms and cotton aphids that cause extensive damage. The insects are mainly controlled by chemical sprays, so the main objective of this study was to engineer cotton against leaf curl disease and insect pests (bollworm) complex by transforming with both AC1 (virus) and *cry1Ab* (bacteria) genes.

Initially, primers were designed to amplify the truncated rep (AC1) gene from DNA A of cotton leaf curl virus (CLCuV). Amplified rep gene (757 bp) was first cloned in plant expression vector pN6 in sense and antisense orientation. AC1 based RNAi cassette was then lifted from pN6 along with CaMV35S promoter and poly A tail and subcloned into the plant transformation vector pBS389. For insect (bollworm) resistance, *cry1Ab* (2.1kb) of *Bacillus thuringiensis* was cloned in plant expression vector pJIT 60 under constitutive CaMV35S promoter and then subclonrd into plant transformation vector pGA482. Both AC1 based RNAi and Bt (*cry1Ab*) constructs were transformed into *Agrobacterium tumefaciens* strain (LBA 4404). *Agrobacterium* (using equal concentration of *Agrobacterium* cultures) mediated transformation of hypocotyls of 7-8 days old plants of Coker-312 was carried out in four batches. Overall callusing and embryogenic efficiency of cotton calli were 53% and 20% respectively. Molecular analysis of the 5 randomdly selected embryogenic calli was performed. Total genomic DNA was isolated and PCR was performed on DNA isolated from five calli, which indicated the presence of both specific (truncated AC1 and *cry 1Ab*) and *nptII* genes. These plants will be challenged to virus/insect in containment facility. It is expected that transgenic plants produced in this study will resist against CLCuD and insect attack.

Introduction
And
Review of literature

Introduction and Review of Literature

Cotton (*Gossypium hirsutum* L.) is one of the most important commercial crops, playing a vital role in the economic, political and social affairs. Chiefly a fiber crop, it has been estimated to contribute US $ 15-20 billion to the world agricultural economy with over 180 million people depending on it for their livelihood (Benedict and Altman, 2001). In Pakistan, it is grown over an area of 2.79 million hectares with total production of 10.21 million bales of lint giving average of 621 kg/hec (Economic survey of Pakistan, 2002-03). It provides about 90% raw material to more than 350 textile mills and is the source of more than 60% of total foreign exchange earnings. Cotton crop not only provides livelihood to farmers and workers of ginneries, textile mills and garment factories but also 60% of edible oil for human consumption is produced from cotton seed. Whereas, pressed cake/oilseed cake, a by-product of cottonseed serves as additional food supplement for dairy animals. During the past few years, cotton production has been showing a remarkable downward trend mainly due to attack of cotton leaf curl disease (CLCuD). The disease is caused by whitefly transmitted geminivirus and has charscteritic symptoms including, growth stunting, leaf curling, vein swelling, vein darkening and enations on the main vein which frequently develop into leaf like structures (Briddon and Markham, 2001). During the year 1990 and 199,1 it damaged cotton crop considerably and caused losses from 22.3 to 68.5% in the fields depending on the cotton variety, time of infection and the environmental conditions (Khan *et al.*, 1998).

Another major constraint to cotton production in Pakistan is insect pest complex which largely comprises of sucking (Homoptera) and chewing (Lepidopterna) insects. To control insects, Pakistan imports pesticides of 10 billion rupees every year and 70% of these are consumed for cotton (Economic survey of Pakistan, 2002-03). The level of these loses is such that cotton protection represents about 24% of the world insecticide market (Colliot and Le Rpux de Bretange, 1993).

Geminiviruses

Geminiviruses are large, diverse family of plant viruses that infect a broad variety of plants and cause significant crop losses worldwide (Harrison *et al.*, 1999; Mansoor *et al.*, 2003)). They are characterized by twin icosahedral capsid and circular, single stranded DNA (ssDNA) genomes (Stanley, 1995) that replicate through double stranded intermediates in infected cells (Saunders *et al.*, 1991). The viral genome consists of one or two small circular single stranded DNA (ssDNA) molecules (2.6-3.0 kb) (Rybicki *et al.*, 2000). Geminiviruses fall into four groups, mastrecurviruses, curtoviruses, begomoviruses and topocurviruses based on their genome structure, host ranges and taxonomy of insect vectors. Group I (Mastreviruses) include Leafhopper transmitted viruses with monopartite genome and infects both monocots and dicots. *Maize streak virus* (MSV) and *Wheat dwarf virus* (WDV) are well-studied examples. GroupII (Curtoviruses) is leafhopper transmitted viruses with monopartite genome and Group III topocurviruses dicot infecting, treehopper transmitted viruses with monopartite genome, infect dicots. *Beet curly top virus* (BCTV) is one of the well studied examples. Group IV (Begomoviruses) is dicot infecting, whitefly transmitted viruses with bipartite genome. Famous examples are *Cotton leaf curl virus* and *Tomato golden mosaic virus* (Van Regenomortel *et al.*, 2000).

Conserved Features of Geminiviruses

Geminiviruses have different genome organizations, reflecting the biological diversity. However there are features that are conserved among geminiviruses. These conserved features are listed below followed by the differences among different subgroups of geminiviruses. (1) Genes are encoded in both the virion sense and the complementary sense DNA. (2) Transcription occurs bidirectionally. (3) Existence of an intergenic region (IGR) between the first open reading frames of virion sense DNA and complementary DNA. IGR is identical in DNA A and DNA B of a single but is very different among different geminiviruses. A nonanucleotide sequence (TAATATTAC) within intergenic region is conserved in all subgroups of geminiviruses and is positioned at the loop region of a stable stem loop in all geminiviruses. (4) A capsid protein gene is

encoded on the sense DNA and replication protein is encoded on complementary sense DNA (Lazarowitz, 1992).

Begomoviruses

Begomoviruses are whitefly-transmitted, dicot-infecting viruses. Majority of members of the geminiviridae, genus begomovirus, have bipartite genome i.e. DNA A and DNA B (Rybicki, 2000). Genes are arranged bidirectionally on each DNA component. DNA A encodes in the virion strand a single, large open reading frame (ORF) for capsid protein (AV1) and a few uncharacterized ORFs. In the complementary strand DNA A encodes four open reading frames (AC1, AC2, AC3, AC4) along with a few uncharacterized ORFs. All the proteins essential for the replication of begomoviruses are encoded on the DNA A. DNA B contains two large ORFs, one each on the virion sense DNA and on the complementary sense DNA. These two ORFs encode proteins necessary for the movement of virus and symptom development in infected plants. Both DNA components of begomoviruses contain different sequences except for a common region (CR) of 200 base pairs (bp). The CR is nearly identical in DNA A and DNA B of a particular virus (Fig. 1). It includes promoters origin of replication. A small hairpin loop forming sequence in CR is highly conserved among all geminiviruses (Hanley-Bowdoin *et al.*, 1999). *Tomato-Leaf curl virus India* (TLCV-India) and *Tomato golden mosaic virus* (TGMV) are examples of Begomoviruses with bipartite genomes.

In contrast to bipartite begomoviruses only a single genomic component that resembles DNA A of bipartite has also been isolated for several begomoviruses including *Tomato yellow leaf curl virus Australia* (TLCV-Aus), *Ageratum yellow vein virus* (AYVV) and *Cotton leaf curl virus* CLCuV). Cloned DNA A components of *Tomato yellow leaf curl virus* and TLCV-Aus are infectious in tomato, the crop species from which they were first isolated, as well as in permissive laboratory hosts such as *Nicotiana benthamiana* and *N. tobacum*. It is evident that monopartite begomoviruses can in some way compensate for the lack of a DNA B component (Briddon *et al.*, 2000). The functions of well-characterized genes of begomoviruses and their homologues in the other two subgroups are summarized below in a table (Hanley-Bowodoin *et al.*, 1999).

Genes	Appr. sizes	Functions
AC1	40 kd	Required for replication, site-specific nuclease, ligase, autosuppressor and DNA sequence-specific binding protein, cell cycle regulatory protein
AC2	15-20kd	Transactivator for AV1 and BV1 gene expression.
AC3	14-16kd	Increase replication efficiency of geminiviruses.
AC4	15 kd	Disease symptom and movement?
AV1	27-30kd	Capsid protein vectors specificity and may regulate the ss/ds DNA ratio.
AV2	13kd	Functions in virus movement in groups II
BC1	34kd	Virus movement (N.A. cell to cell trafficking), host ranges and symptom development.
BV1	30kd	Virus movement (Transport N.A. out of nuclei) and host ranges

Recombination among Begomoviruses

Geminiviruses may contain monopartite or bipartite genomes and consequently recombination may occur in two different ways: by exchange of viral chromosomes (interchromosomal recombination, or "pseudorecombination" as plant virologist call this type of reassortment) (Sung *et al.*, 1995; Unseld *et al.*, 2000a; Unseld *et al.*, 2000b) or by crossover of chromosomes (intrachromosomal recombination) (Briddon *et al.*, 1996; Gibson *et al.*,1996; Zhou *et al.*, 1998; Padaidam *et al.*, 1999; Fondong *et al.*, 2000; Sanz *et al.*, 2000; Berrie *et al.*, 2001). Moreover, geminiviruses are able to adopt satellite like-DNA circles which has an additional impact on pathogenesis (Dry *et al.*, 1997; Saunders et al., 2000; Briddon *et al.*, 2001). Recombination has been found to occur in recently identified *East African cassava mosaic Virus* (EACMV) isolate from Cameroon, in both DNA-A (AC2 and AC3 ORFs) and DNA-B (BC1 ORFs) components (Fondong *et al.*, 2000), indicating the possibility for recombination in both genomic components of begomoviruses. In comparison to RNA containing plant viruses, geminiviruses are relatively prone to recombination and harbor frequent footprints of recombination events within their genomes (Padaidam *et al.*, 1999). This phenomenon is conceivable on the

basis of a recombination-dependent replication mode of geminiviruses (Jeske *et al.*, 2001).

Replication of Geminiviruses

Geminivirus DNA replication follows a rolling circle (RCR) model (Saunders *et al.*, 1991). Four genes encoded by the complementary sense strand of DNA in case of dicot infecting and two spliced open reading frames in case of monocot-infecting geminiviruses have been implicated in the regulation of replication and transcription (Palmer *et al.*, 1998; Hanley Bowdoin *et al.*, 1999). Following the uncoating of the virion ssDNA, the first step is the synthesis of the complementary strand on single stranded DNA template leading to the production of transcriptionally active dsDNA intermediates. This complementary DNA synthesis is accomplished entirely by host proteins since naked ssDNA is infectious when introduced into plants. This synthesis is primed by a short stretch of ribonucleotide complementary to nucleotides in the intergenic region (IGR). In the monocot infecting geminiviruses (subgroup I), the primer for complementary DNA synthesis are encapsidated in the virions. The primer DNA fragments of *Maize streak virus* (Donson *et al.*, 1984) and *Wheat dwarf virus* (Hayes *et al.*, 1988) are about 80 nucleotides containing 5′ terminal ribonucleotides. Such primers within subgroup III have not been found in virions and they may be synthesized by de novo immediately after the uncoating of the viral ssDNA. An RNA primer processing the synthesis of the complementary DNA of *African cassava mosaic virus* (ACMV) has been identified in the DNA replication intermediates from ACMV infected tobacco plants (Saunders *et al.*, 1991).

At the onset of sense DNA synthesis, a nick is introduced in the plus strand within the nonanucleotide TAATATT/AC (slash, nicking site) at the origin of replication identical among all geminiviruses. This function is provided by AC1/C1 proteins. After cleavage, the rep protein remains bound to the 5′ end of the cleaved strand. The 3′prime terminus of the nicked DNA serves as a primer for DNA synthesis, displaying the original virion sense DNA as the template (complementary) strand is copied. The enzyme that synthesize the virion strand continuously circles around the complementary DNA

template, hence, by the rolling circle replication models, unit length virion strand is synthesized, it is cut and ligated to form a closed circular ss virion DNA. Laufs *et al.*, (1995) reported that the Rep (AC1) protein also has a joining activity, suggesting that it acts as terminase, thus resolving the nascent viral single strand into genome sized units. This close circle ss DNA can either serve as template for another round of replication or can be encapsidated into virions. If the unit length virion DNA is released immediately, concatamers of viral DNA is formed. The cleavage activity is initiated by tyrosine-103 and this tyrosine is a physical link between the rep protein and its DNA origin.

The rolling circle replication (RCR) model explains easily how viral ssDNA is produced from double stranded template at the final stage of multiplication cycle. However model is unusual as geminiviruses transcribe bidirectionally, thus risking collision between replication and transcription complexes (Brewer, 1988). Being aware of this discrepancy, it was conformed to the replication of *Abutilon mosaic virus* (AbMV) DNA by two-dimensional gel analysis and BND (Benzoylated naphthoylated DEAE) chromatography that geminivirus rolling circle replication is multitask (Preiss *et al.*, 2003).

Importance of AC1 gene for pathogen derived resistance

AC1 is the only viral encoded protein, which is absolutely required for viral DNA replication because it plays key roles in geminivirus DNA replication and transcription. AC1 protein specifically binds to double stranded DNA during origin recognition and acts as endonucleases and ligase to initiate and terminate rolling circle replication (Stanley *et al.*, 1995; Laufs *et al.*, 1995). AC1 hydrolyzes ATP during an uncharacterized step of viral replication and interacts with itself and viral replication enhancer, AC3. The functional domains for DNA binding, DNA cleavage/ligation oligomerizations have been mapped to the N-terminal half of the AC1 protein (Orozco *et al.*, 1997). AC1 is a multifunctional protein that binds double stranded DNA, catalyzes cleavage and ligation of single stranded DNA, and forms oligomers. The region of TGMV AC1 necessary for DNA binding maps to N-terminal 181 amino acids of the protein and overlaps the DNA cleavage (amino acids 1-20) and oligomerization (amino

acids 134-181) domains. A series of site directed mutations was induced in conserved sequence and functional motifs in the overlapping DNA binding and cleavage domains and analyzed their impact on AC1 function in vivo and in vitro. Only two of the fifteen mutant proteins were capable of supporting viral DNA synthesis in tobacco protoplasts. In vitro experiments demonstrated that a pair of predicted alpha helices with highly conserved charge residues are essential for DNA binding and cleavage. Three sequence motifs conserved among geminivirus AC1 proteins and initiator proteins from other rolling circle systems are also required for both activities. Truncated AC1 proteins fused to a heterologues dimerization domain to show that the DNA binding domain is located between amino acids 1 and 130 and that binding is dependent on protein dimerization. In contrast, protein monomers were sufficient for DNA cleavage and ligation. Together these results established that the conserved motifs in the AC1 N-terminus contribute to DNA binding and cleavage with both activities displaying nearly identical amino acid requirements. However DNA binding was readily distinguished from cleavage and ligation by its dependence on AC1/AC1 interactions (Hanley-Bowdoin, 1999). *Tomato leaf curl virus* (TLCV-Aus) rep gene was expressed in *Escherichia coli* as a histidine-tagged fusion protein and purified to homogenity in non-denaturing form. The fusion protein was used in in-vitro binding experiments to identify the Rep binding elements within the origin of replication of TLCV. Electrophoretic mobility shift assays demonstrated that the Rep binds specifically to 120-bp fragment within the TLCV intergenic region. Fine resolution of the binding regions within the 120 bp fragment, using DNase I footprinting, demonstrated two footprints covering the sequences GCAATTGGTGTCTCTCAA and TGAATCGGTGTCTGGGG containing a direct repeat of the motif GGTGTCT. Results suggest that the repeated motif is involved in virus specific Rep-binding, but may not constitute the entire binding element (Akbar, *et al.*, 1998).

Cotton Leaf Curl Virus: a member of a new group of Begomoviruses

Cotton leaf curl disease (CLCuD) of cotton has been identified to be associated with a begomovirus based on symptoms, mode of transmission and polymerase chain reaction based diagnostic tests. The virus of this disease, cotton leaf curl virus (CLCuV)

is transmitted by whitefly (*Bemicia tabaci*) in a persistent manner. Characteristic symptoms of CLCuV-infected cotton include upward or downward curling of leaves, vein distortion, thickening and enations on underside of leaves (fan like outgrowths) (Fig. 2). It has been found that leaf curl disease of cotton is associated with four variable geminiviruses (Zhou *et al.*, 1998). These viruses which are most diverse have been named as *Cotton leaf curl virus* Pk1 (CLCuV-Pk1) and *Cotton leaf curl virus* Pk2 (CLCuV-Pk2) (Zafar *et al.*, 1997) and correspond to CLCuV-Pk type 26 and CLCuV-Pk type 72b, respectively as described by Zhou *et al.*, 1998. This is indeed a unique example where four variable geminiviruses are associated with the same disease in the same geographical area. During the course of investigation nanovirus like DNA 1 associated with CLCuD in Pakistan has also been reported (Mansoor *et al.*, 1999). DNA 1 may represent one component of multipartite virus that belongs to a novel nanoviridae genus comprising member that are transmitted by whitefly *B. tabaci*. It differs from nanovirus components in two main respects. Firstly, it is whitefly transmitted, whereas all known nanoviruses are transmitted by either aphids or plant hoppers (Grylls and Butler, 1959; Julia, 1982). Secondly, it is significantly larger than the multiple components of Banana bunchy top virus (BBTV), *Faba bean necrotic yellow virus* (FBNYV) (Boevink *et al.*, 1982; Katul *et al.*, 1997; Sano *et al.*, 1998). This is the first demonstration of a disease involving two single stranded DNA components, which show no sequence homology (Mansoor *et al.*, 1999). Recently, a novel single-stranded DNA of approximately 1350 nucleotides in length has also been identified and isolated which when co-inoculated with the begomovirus to cotton, induce symptoms typical of CLCuD. This molecule, termed DNA β, requires the begomovirus for replication and encapcidation (Zafar *et al.*, 2003). The CLCuV/DNA β complex together with a similar complex previously identified in *A. conyzoides*, represent members of an entirely new class of infection disease causing agents (Briddon *et al.*, 2001). Thus such virus complexes are widespread in which distinct DNA β and DNA 1 homologues are associated with monopartite begomovirus infections of *A. conyzoides*, cotton in Pakistan and hollyhock in Egypt (Mansoor *et al.*, 1999; Briddon *et al.*, 2001; Mansoor *et al.*, 2003). Moreover, geminiviruses exploit the replication-recombination-repair connection to replicate more efficiently to overcome host plant defense (Von Hippel *et al.*, 2000).

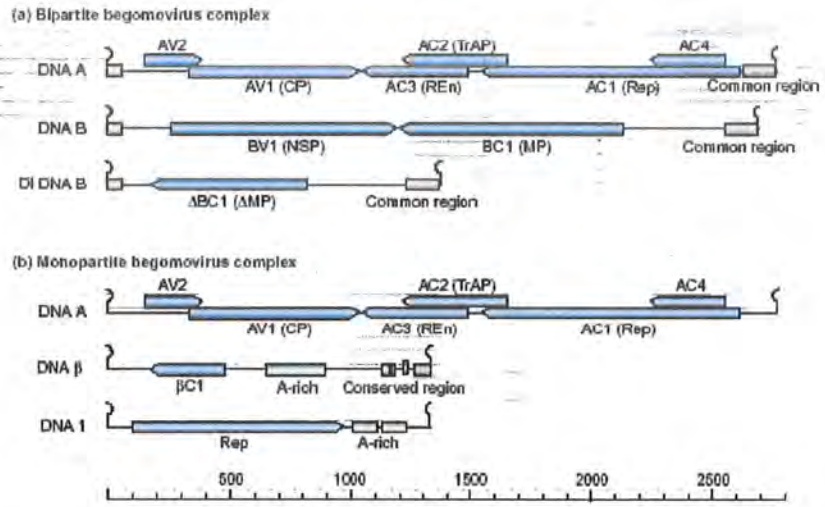


Figure 1: Genome organization of Old World (a) bipartite and (b) monopartite Begomoviruses, and their associated defective and satellite components. Some monopartite Begomoviruses are associated with helper-dependent satellite components (DNA β) and autonomously replicating nanovirus-like components (DNA 1). Both DNA β and DNA 1 contain an A-rich region, which is thought to maintain their size of approximately half that of begomovirus component. A highly conserved region found in all DNA β components is indicated. For simplicity, circular components are indicated as linear DNA starting at the nick site for the initiation of replication located within a ubiquitous stem-loop motif (Mansoor *et al.*, 2003).
Scale is given in nucleotides.

Figure 2: Symptoms of cotton leaf curl disease

Pathogen derived resistance

Pathogen-derived resistance is mediated either by the protein encoded by the transgene (protein-mediated) or by the transcript produced from the transgene (RNA-mediated). Resistance to viral infection in transgenic plants caused by expression of transgene that produce viral coat protein is termed coat protein-mediated resistance. Powell-Abel *et al.*, (1989) showed that transgenic tobacco expressing the coat protein gene of *Tobacco mosaic virus* (TMV) was resistant to TMV and that the resistance was due to the expressed coat protein. Recent research indicates that pathogen-derived resistance to viruses is mediated, in most cases, by an RNA-based post-transcriptional gene silencing mechanism. This plant defense system results in degradation of mRNA produced both by the transgene and the virus. In general, protein-mediated resistance provides moderate protection against a broad range of related viruses while RNA-mediated resistance offers high levels of protection only against closely related strains of a virus (Lomonosoff, 1995; Baulcombe, 1996; Dawson, 1996).

A. Protein mediated resistance

1. Coat protein mediated resistance

Coat protein-mediated resistance (CP-MR) refers to resistance of transgenic plants that produce virus CP to infections against the virus from which the CP is taken. It has been observed that transgenic expression of *Tobacco Mosaic Virus* protein confers resistance against *Tobacco mosaic virus infection* (Bendahmane *et al.*, 2002). Coat protein is the determinant of the specificity of virus transmission (Briddon *et al.*, 1990) and, in some virus-host systems, appears to be associated with symptom development (Gardiner *et al.*, 1988). It has also been proposed that the coat protein is necessary for the systemic movement of bipartite geminiviruses in hosts in which the virus is not well adapted. The coat protein is the viral protein to which a function in vascular movement has been most frequently ascribed (Guevera-Gonzalez *et al.*, 1999). However, there is a fewer reports of CP mediated engineered resistance to geminiviruses. The CP of the bipartite viruses is dispensable for systemic infection in an adapted host (Pooma *et al.*, 1996), although it can aid the movement or pathogenecity of such viruses. Success has

been reported with expression of geminivirus transgene of modified and unmodified coat protein (AVI) (Hong *et al.*, 1996; Kunik, *et al.*, 1998). The very acidic N-terminal extension of the viral CP precursor inhibits nuclear targeting of the protein and hence the precursor is localized in the cytoplasm. It has been suggested that this provides a control mechanism that ensures that the CP precursor is used for virus assembly in the cytoplasm and that only mature virus particles reach the nuclear pore (Karsees *et al.*, 2002).

2. Replicase-Mediated Resistance

Transformation of plants with plant viral replicase sequences, resulting in induction of resistance was first reported in 1990, employing the 54-kDa read-through region of the TMV replicase gene transformed into *Nicotiana tabacum* (Golomboski *et al.*, 1990). The characteristic features of "replicase-mediated resistance" are the high degree of resistance shown both to viral and to RNA inocula, and the narrowness of the specificity (i.e., resistance is shown only to the virus from which the replicase gene was derived and to very closely related strains or mutants). In most cases examined, there is a substantial inhibition of virus replication in initially inoculated cells and some limited cell-to-cell movement, but the infection does not spread from the inoculated leaf and no systemic disease develops.

Transgenic tobacco plants expressing an altered form of the 2a replicase gene from the Fny strain of *Cucumber mosaic virus* (CMV) exhibit suppressed virus replication and restricted virus movement when inoculated mechanically or by aphid vectors (William *et al.*, 2000). Translatability of transgene itself facilitates replicase-mediated resistance to *Cucumber mosaic virus* (CMV) in tobacco (Wintermantel and Zaitlin, 2000).

3. Movement Protein-Mediated Resistance

Many plant virus genera encode a triple gene block (TGB), a specialized evolutionary conserved gene module involved in the cell-to-cell and long distance

movement of viruses (Morozov *et al.*, 2003). It has generally been considered that viral systemic infection of a host plant occurs in two steps. First the virus enters a wounded cell, and viral protein including a nonstructural movement protein (MP) are synthesized. Movement protein allows viral movement among cells through plasmodesmata (short-distance or cell to cell movement). The second step occurs when, via short-distance movement, a virus reaches the vascular system and the host plant becomes systematically infected (long-distance movement. It has also been reported that plant virus transport systems recruit and modify normal plant intra- and intercellular pathways for movement of viral proteins and nucleic acids (Leisner, 1999; Lazarowitz, 1999; Lee *et al.*, 2000; Jackson, 2000). The potential use of virus movement proteins as source of genes for resistance to virus infection was predicted following research in the late 1980s that identified viral genes that are responsible for local virus spread (Deom *et al.*, 1992).

B. RNA mediated resistance

1.Sense RNA suppression

Plants with high transcription rates generate RNA levels that exceed a certain threshold level, this then activates a cytoplasmic-based, cellular process that specifically targets this overexpressed RNA for elimination and results in low steady state levels of the transgene mRNA. If the transgene shares homology with a plant virus, the plants are phenotypically resistant the challenging virus (Smith *et.al,* 1994).

This phenominon observed was later termed sense suppression where the overexpression of a specific mRNA in the plant cell cytoplasm induces machienery to eliminate the deleterious RNA. (Flavell *et.al.*, 1994; Napoli *et.al.*, 1990; Smith *et.al.,* 1994).

A little revolution took place in 1992 with the demonstration that untranslatable sense RNA could provide protection against *Tobacco etch potyvirus* (TEV) (Lindbo and Dougherty, 1992). Similar observations were reported for PVY (potato virus Y) (Farinelli, 1992). Recent experiments with *Potato virus* X (PVX) demonstrated that virus-resistance can result from a more general process of homology-dependent gene

silencing (English *et al.,* 1996). This silencing (or co-suppression), (Finnegan and Mcelroy, 1994) model may also explain the TEV experiments as well as a large part of the reported cases of pathogen-derived protection.

Very recently, Asad *et al.*, (2003) engineered transgenic tobacco plant expressing sense and antisense RNAs of AC1, AC2 and AC3 of *cotton leaf curl virus* showing resistance to serious viral pathogens causing cotton leaf curl disease.

2. Antisense RNA technology

The antisense RNA has a polarity opposite to that of mRNA (Bourque, 1995). For several constructs, the coat protein gene was inserted either in sense or antisense orientation.However, experiments with CMV, TMV, PVX and PVY showed that the antisense constructs conferred a weaker protection than the sense coat protein constructs (Cuozzo *et al.,* 1988; Powell *et al.,* 1989; Hemenway *et al.,* 1988). Similar results were reported for the geminivirus *Tomato golden mosaic virus* (Day *et al.,* 1991). The antisense RNA is thought to interfere with the viral replication or translation by hybridizing with the sense genomic RNA (or DNA) or by competition for viral or host components needed for replication. One would think that regions that differ from the coat protein gene would be more suitable to provide protection, but this is apparently not the case. For CMV, antisense genes corresponding to the genomic regions of the putative replicase, movement protein and 3' site of replication initiation were used for plant transformation (Rezaian *et al.,* 1988). Only one line expressing relatively low amounts of the antisense RNA corresponding to the replicase region showed some protection against CMV. Similarly, the antisense RNA of the TMV 54 K gene did not confer protection (Golemboski *et al.,* 1990).

3. Defective interfering and satellite RNA

Satellite RNAs are "parasite" RNAs which need a helper virus to replicate in host plants, and which differ from defective interfering RNA by having no sequence

homology with the helper virus (Simon, 1988; Courtice, 1987). Some of the satellite RNAs attenuates the symptoms caused by the helper virus, whereas others increase them (Simon, 1988).

By expressing a satellite RNA in transgenic plants, it is possible to obtain resistance to the corresponding virus (Yie and Tien, 1993). Experiments with CMV showed that the plants produced large amounts of satellite RNA upon infection with a satellite-free inoculum of CMV and, as a result, the satellite became transmissible as a component of the virus culture. The satellite RNA decreased the CMV replication and largely suppressed the symptoms. The related *Tomato aspermy virus* (TAV) likewise induced the CMV satellite RNA synthesis accompanied by symptom suppression but with little decrease in TAV replication (Harrison *et al.*, 1987).

4. RNA Silencing

RNA silencing also known as RNA interference, posttranscriptional gene silencing or quelling is broadly conserved across the eukaryotes (Fire *et al.*, 1998; Hannon *et al.*, 2002) and implicated in functions ranging from transcriptional silencing (Mette *et al.*, 2000; Jones *et al.*, 2001; Volpe *et al.*, 2002) to develpmental regulations (Waterhouse *et al.*, 2001; Plasterk *et al.*, 2002). In many species RNA silencing acts to supress the expression of transcripts corresponding to " Non-self genes" potentially harmful elements such as those encoded in viruses or transposons (Hamilton *et al.*, 2002; Lee *et al.*, 2002) This process parallels the operation of the vertebrate-specific immune system: Similar to immune system, RNA silencing guards against expolitive parasitic elements by (i) Identifying non self elements (ii) Generating target specific responses against these foreign elements (iii) Rapidly amplifying these responses to clear or otherwise in activate the threat (Bergstrom *et al.*, 2003).

dsRNA as an inducer of RNA silencing

In a broad range of eukaryotic organisms, RNA silencing is triggered by dsRNA, which may be naturally derived from the transcription of inverted-repeat loci of

replicating exogenous RNAs by host or viral encoded RNA dependent RNA polymerase (RDRP) (Sijen *et al.*, 2001).

Viruses and transgenes are two main exogenous factors including the formation of dsRNAs in plants during RNA silencing. The viral replication by a virus encoded RDRP in most single stranded RNA viruses generates a dsRNA intermediate, which contains the sequence-specific information to guide a plant to protect itself by degrading viral RNAs (Sijen *et al.*, 2001). Comperatively, the generation of dsRNA initiated by transgenes may have distinct mechanisms according to the transgene structure and its integration status in the plant genome. RNA silencing caused by the transgene overexpressing an endogenous mRNA requires a putative plant-encoded RDRP, SGS2/SDE1 *((Arabidopsis)* for example, which synthesizes dsRNA intermediates from the template of aberrantly expressed single-stranded RNA (Tang *et al.*, 2003). However, the requirement for RDRP is bypassed if a tansgene is designed to create a double stranded structure, or multiple copies of a transgene integrated as inverted repeat insertions in the genome, in which dsRNA is generated by read-through transcription (Wang and Waterhouse, 2000).

Recent studies have also revealed that dsRNA could induce sequence-specific hypermethylation of genomic DNA, which suggests an alternative function of dsRNA in TGS, and indicates that RNA silencing and TGS might be mechanistically related in targeted gene silencing (Bender, 2001).

Mechanism of RNAi

The mechanism of RNA silencing induced by double stranded RNA can be simplistically summarized as having two major steps, viz., Initiation and effector steps (Cerutti, 2003).

Initiation step

The initiation step involves the cleavage of the triggering dsRNA into siRNA of 21-26 nucleotides with 2-nucleotide 3′ overhangs which correspond to both sense and antisense strands of a target gene (Fig. 4). The processing of long double stranded RNA

into small interfering RNA is mediated by an RNase-III-like dsRNA-specific ribonuclease, designated Dicer, initially in Drossophila (Bernstein *et al.*, 2001). The members of the Dicer protein family, which may be functionally conserved in fungi, plants and animals (Tijsterman *et al.*, 2002), generally contain N-terminal RNA helicase motif, a PAZ (for 'Piwi/Argonute/Zwille') protein-protein interaction domain, at least on dsRNA binding domain, and two tandem RNase-III domains that may directly regulate the endonucleolytic cleavage of dsRNA into siRNAs, which is an ATP-dependent process.

Effector step

In the effecter step, the small interfering RNAs are recruited into a multiprotein complex referred to as the RNA-induced silencing complex (RISC) (Fig. 3), in which the degradation of target mRNAs occurs with the siRNAs as a guide (Zamore *et al.*, 2000). Each RISC appears to have a single siRNA, an RNase (and may even have two separate Rnases—exonucleolytic and endonucleolytic), and an mRNA-homology-recognition and binding domain.

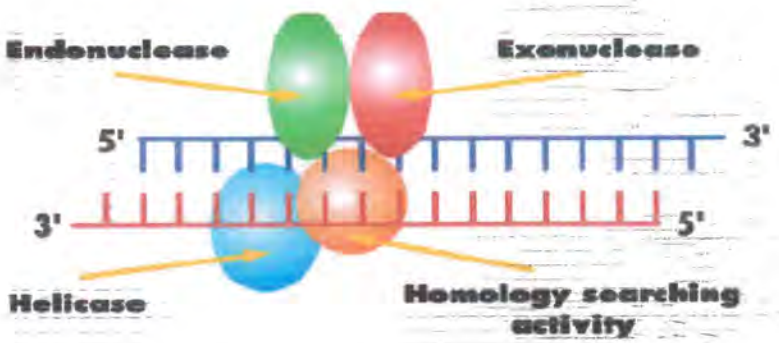


Figure 3: RISC complex

Further, there are two size classes of siRNA in plants (Tang *et al.*, 2003), namely long 'siRNAs' of 24-26 nucleotides that are involved in triggering systemic gene silencing, and the short 'siRNAs' (21-22 nucleotides) correlated with sequence-specific degradation of target mRNAs. Such cleavage of the target mRNA occurs almost all the middle of the siRNA within the relevant RISC (guide RNA), resulting in over 90% inhibition of target gene expression (Yu *et al.*, 2003)

RNA silencing has become major tool to engineer plants against viruses by transforming genes derived from pathogens (viruses). Hairpin RNA genes constructs in which the two complementary region of the gene are separated by an intron are extremely efficient in triggering PTGS (Tenllado *et al.*, 2003). It has been shown that gene constructs encoding intron spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes. These constructs could prove valuable in reverse genetics, engineering of metabolic pathways and protection against pathogens (Smith *et al.*, 2000).

The role of methylation and chromatin remodeling in Plants

DNA methylation and chromatin structure have an integral role in transcriptional gene silencing (TGS). In this form of silencing, the promoter and sometimes the coding region of the silenced transgenes are densely methylated (Kooter *et al.*, 1999). Methylation or methylation-associated chromatin remodeling, of promoter sequences is thought to prevent binding of factors necessary for transcription. The coding sequence of PTGS-inducing transgenes are also frequently found to be methylated. PTGS can be established in plants with a mutant methyltransferase (*metl)*, but during growth, the silencing becomes impaired, reactivating the silenced gene in sectors of the plant (Morel *et al.*, 2001).

The mechanisms of PTGS and TGS may have more in common than was previously thought. In PTGS, the short RNAs derived from the transcribed region of the

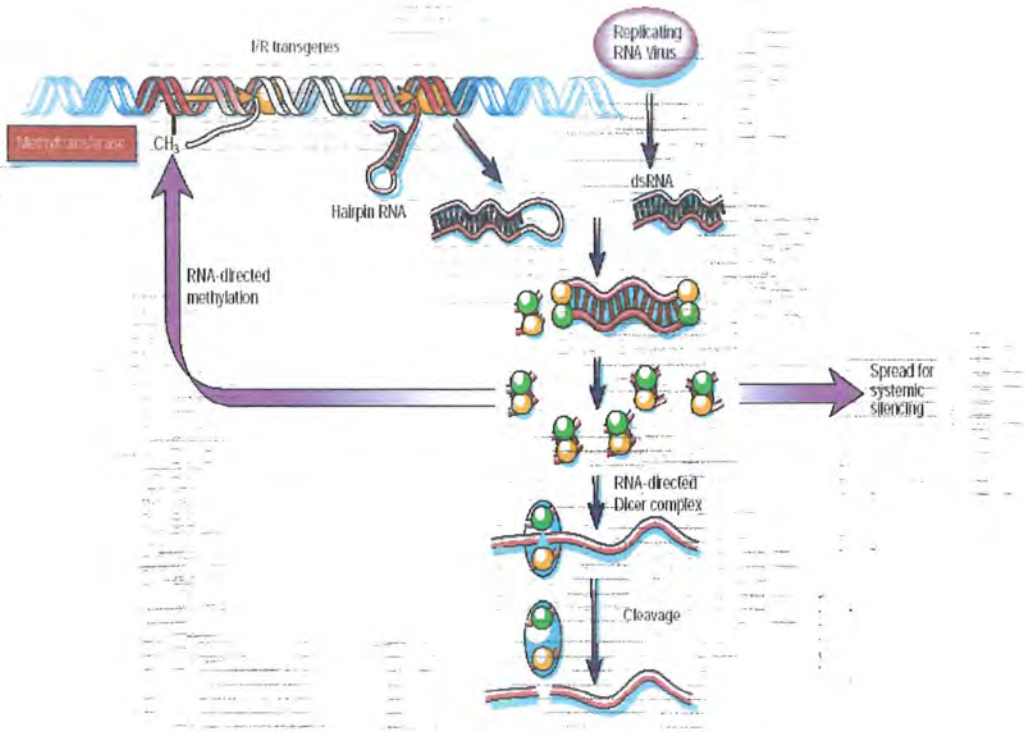


Figure 4: A model for the initation and operation of Posttranscriptional gene silencing (PTGS) (Waterhose *et al.*, 2001).

transgene act as guides for siRNPs to degrade target ssRNA. In TGS plants, hpRNAs (hairpin RNAs) containing promoter-region sequences are processed into short dsRNAs, and seem to direct methylation (Mette *et al.*, 2000). Similarly, virus-replicated RNAs direct sequence specific DNA methylation and are associated with short dsRNAs (Wang *et al.*, 2001). It is possible that the steps of PTGS and TGS are, in fact, the same and differ only in their target sequences: hpRNA or dsRNA is cleaved by the plant homologue of Dicer-1 into ~21-nucleotide dsRNAs to guide specific ssRNA degradation in the cytoplasm, and a similar ribonucleoprotein complex passes into the nucleus to direct chromatin remodeling/methylation of homologous DNA. The methylation of coding region DNA in PTGS and the potential degradation of promoter sequence transcripts in TGS would be irrelevant by-products, as methylated coding regions are readily transcribed and promoter sequences are not usually transcribed.

It seems unlikely that the DNA methylation mechanisms associated with PTGS and TGS is involved directly in protecting plants against most RNA viruses. The vast majority of these viruses have exclusively cytoplasmic lifecycles and no homologous DNA sequences in plant genomes. It is possible that dsRNA-directed methylation is involved in inhibiting the handful of known plant retroviruses or pararetroviruses during their DNA phases within the nucleus. It is even more likely that the mechanism is primarily for defence against transposable elements (TEs) (Waterhouse *et al.*, 2001).

PTGS can spread systemically through a plant

PTGS has three phases: initiation, maintenance and remarkably, spread (Voinet *et al.*, 1998). Transgenes and viruses can initiate PTGS, as can exogenous DNA delivered by bombardment or *Agrobacterium* infiltration, and grafting of unsilenced scions onto silenced rootstock. These last methods give localized delivery points for PTGS that spread from these points into other tissues. It seems to spread by a non-metabolic, gene specific diffusible signal that is capable of traveling both between cells through plasmodesmatal, and long distances via phloem (Fig. 5). An extra dsRNA specific movement protein complex may be required for the spread and amplification of the

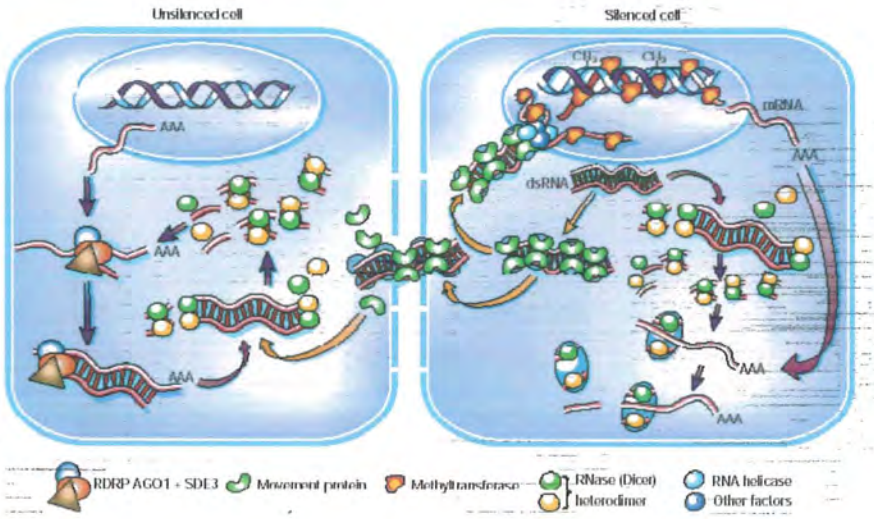


Figure 5: A model for mobile PTGS and methylation signals. (Palauqui *et al.*, 1997)

PTGS signal. This complex transports the dsRNA into the nucleus via the nuclear pore, and into adjacent cells (ultimately the phloem) by means of plasmodesmatal (Cerutti, 2003). In the nucleus, the dsRNA is unwound by a helicase and directs methylation of homologous DNA sequences. In situation such as grafting of an unsilenced scion on to a silenced rootstock, the dsRNA complex entering the unsilenced cell of the scion is recognized and degraded releasing some dissociated short RNAs. These RNAs act as primers on a target mRNA for a host RDRP (for example SGS2) with a helicase (for example, SDE3) and initiating factors (for example, AGO1) to synthesize complementary RNA. This generates dsRNA that is recognized by the components of the movement and degradation complexes, thus initiating degradation of homologous ssRNA and amplifying the mobile signal (Waterhouse, 2001).

Insect pests of cotton

Cotton is attacked by a complex of insects including plant bugs, aphids, whiteflies, and boll weevil; however, on a worldwide basis, the main pests are the diverse set of Lepidoptera (Fig. 6) that feed on the cotton buds or bolls and against which most of the insecticides are targeted ((Luttrell *et al.*, 1994)

Whitefly (*B.tabaci*) is polyphagous and has been reported on more than 500 host plants from different parts of world (Greathead, 1986). It is one of the most destructive pest and causes considerable yield losses to cotton crop in Pakistan. Intensive and indiscriminate use of insecticides has created high level of resistance resulting poor control of this pest (Ahmad, 1996). As a result of elimination of natural enemies with insecticides may even aggregate the problem as also reported in America (Miller, 1986).

Major insects pest include pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Erias insulana*), and american bollworm (*Helicoverpa armigera*) attack cotton crop at larval stage with chewing mode of damage.The genus Heliothis and Helicoverpa contain some of the most destructive species. More insecticide is used to control these species than all other species combined. The high reproductive potential, strong flight habits and

Pink bollworm

American bollworm

Spotted bollworm

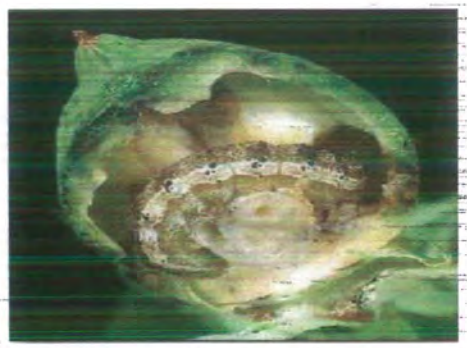


Figure 6: Bollworm complex of cotton

ability to develop high resistance to pesticides makes them very formidable enemies (Leukefahr, 1991).

After application of excessive commercial pesticides for longer times, these pests have become resistant inviting our attention towards alternate methods of control as a high priority in Agricultural research programmes (Annonymous, 1992). About 96 insects and mite pests have been reported in Pakistan which enjoy unique importance as limiting factor in accelerating cotton production and caused yield losses varying from 39-40%. The sucking insect pests were reported to cause 4.6% losses in yield of seed cotton where as bollworm complex is responsible to cause 30-40% loss in the yield of seed cotton (Ahmad, 1980).

Methods of insect control

Insect control includes everything that makes life hard for insects and tends to kill them and to prevent their increase or spread over crop plants. The insect control implies the regulation of insect activity in the interest of man. Important methods to control insects are the following:

(a) Development of host plant resistance
(b) Biological control
(c) Chemical control
(d) Integrated pest management (IPM)

Transgenic Bt Cotton

Transgenic plant expressing insecticidal proteins from the bacterium, *Bacillus thuringiensis* (Bt), are revolutionizing agriculture (Shelton *et al.*, 2002). Conventional host-plant resistance to insects involves quantitative tracks at several loci and as a result the progress has been slow and difficult to achieve. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert genes into the plant genomes that confer resistance to insects (Benedict, 1996).

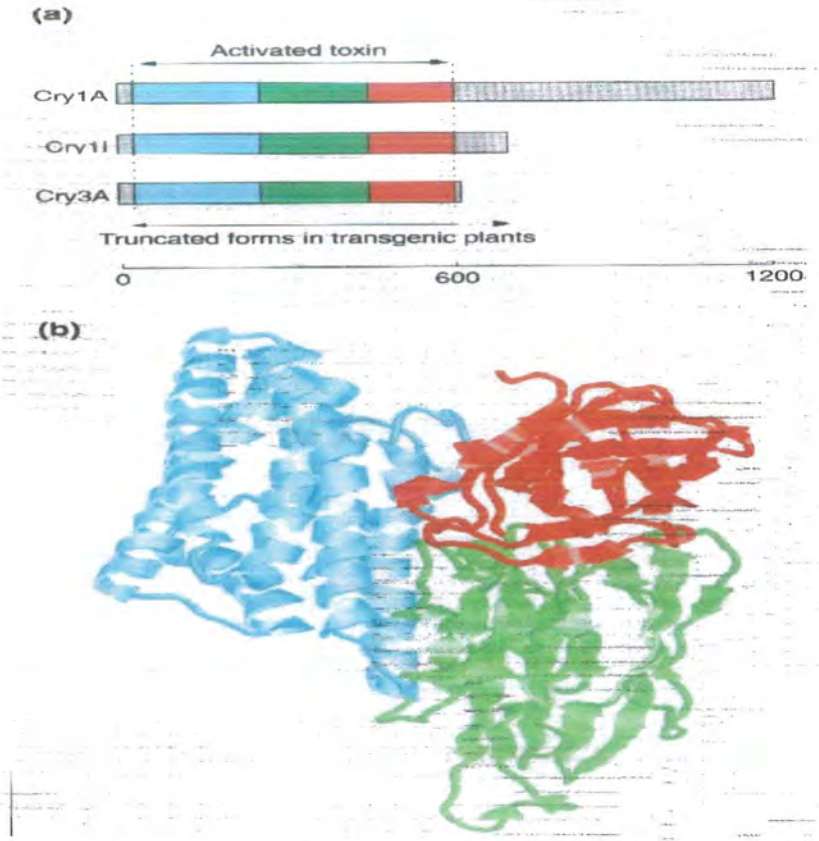


Figure 7: (a) Structure of a typical *Bacillus thuriengesis* toxin (Scale is given in amino acids).

(b). Three dimensional structure of activated Bt toxin

- **Domain I**
- **Domain II**
- **Domain III (Grochulski, *et al.*)**

Genes from bacteria such as Bacillus thuringiensis (Bt) and Bacillus sphaericus (Gill *et al.*, 1992. Charles *et al.*, 1996) have been the most successful group of organisms identified for use in genetic transformation of crops for pests control on commercial scale. Protease inhibitors, plant lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from Bt and related species, and small RNAs viruses can also be used alone or in combination with Bt genes to generate transgenic plants for pest control (Hilder and Boulter, 1999). Transgenic plants rarely result in 100 % control but tend to retard insect development and growth (Estruch *et al.*, 1997).

Considerable progress has been made in developing transgenic crops with resistance to the target pests over the past decades (Hilder and Boulter, 1999). Such transgenic plants have shown good promise in reducing insect damage both in laboratory and field conditions. Successful control of pink bollworms (*Pectinophora gossypiela*) has been achieved through transgenic cotton (Wilson, 1992). In transgenic cotton, the mean percent injury has been observed to be 2.3 in flowers and 1.1 in capsules compared to 23 and 12 percent in Coker 213 respectively (Benedict *et al.*, 1996). Significant variation in insecticidal activity has been observed in transgenic plant at different growth stages during the season and in different parts of the cotton plant (Zhao *et al.*, 1998). The efficacy of Bt toxin in leaf and squares was high during the second generation of the insect but declined in the third and forth generations in north china. The surviving third and fourth generation larvae after feeding on flowers of Bt cotton fed on the bolls until pupation, which could cause selection in the field population of *H. armigera*. The increase resistance was 7.1 fold-after 17 generation of selection in the laboratory with an average of mortality of 67.2 % in each generation the resistance grade of Bt cotton declining from high resistant against a none-selected population to medium levels of resistance against the selected population, indicating potential problem of development resistance on insects to Bt cotton.

Tissue specific regulation of Bt *cry1A (b)* gene has been utilized to achieve high and regulated expression in the leaves and pollen grains, the promoter derived from PEPC controls the expression of *cry1A(b)* in green tissues (Hudspeth and Grula, 1989). While the promoter derived from calcium dependent protein kinasae (CDPK) gene is

pollen specific (Estruch *et al.*, 1994). Combination of green tissue specific PEPC and pollen specific CDPK tissue promoters provides high *cry1A(b)* genes expression and pollen where it is most effective against European corn borer (*Ostrinia nuvilalis*). The intron 9 of maize PEPC is located between *cry1A(b)* structural genes and the 35S terminator and its presence increased the pesence of Bt genes (Hudspeth and Grula, 1989). Three untranslated termination sequences from CaMV35S are present adjacent to the PEPC intron 9 and provides the polyadenylation site (Rothstein *et al.*, 1987).

A number of vectors were developed for transferring the genes of interest into crop plants. This system involves a marker gene for resistance to antibiotics or herbicide (Phosphoincithrin), replication site and a multiple cloning site (MCS) with several restriction sites for DNA insertion. Foreign DNA can be inserted in the vector using restriction enzymes that recognize a specific DNA sequence. Insertion of foreign DNA interrupts gene expression of an identifiable protein product to indicate DNA incorporation. Construction of DNA sequence for incorporation into vectors consist several components, e.g. in case of the Bt gene should be first converted from AT rich (typical of bacteria) into GC-rich (typical of higher plants) to increased toxin expression. Most changes were made to the codon thereby minimizing changes in the amino acid sequence and increased the expression of Bt toxin by 10-100 fold (Perlak *et al.*, 1991). For expression of the Bt gene in the higher plants, recognizable promoter and terminator sequence must bracket the Bt gene. Popular constitutive promoters includes cauliflower mosaic virus (CaMV35S) and ubiquitin tissue specific promoter includes PEPC (Phosphoenolpyruvate carboxylase for green tissue) and maize pollen specific promoter (Koziel *et al.*, 1993). The size of the vector ranges 5000 to 11000 bp depending on the Bt gene and the promoter incorporated into the vector (Koziel *et al.*, 1993). Delivery of the vector into the nucleus has been achieved by using *agrobacterium* mediated transformation and biolistic methods (Koziel *et al.*, 1993).

An artificially synthesized *Bacillus thuringiensis* insecticidal protein gene, co-constructed with the GUS reporter gene (Bt/GUS), was transferred into fertilized ovaries of elite cotton (*Gossypium hirsutum)* cultivars simian 3 and Zhongmiansuo 12 via the pollen tubes, and transgenic cotton plants were recovered from the seeds of treated bolls.

Histochemical analysis of the GUS enzyme activity showed that the GUS gene was expressed in the transgenic R1 plants of the two recipient cultivars. PCR results of the GUS-positive R1 plants showed the existence of the Bt gene, and the same results appeared in the R2 and R5 plants, indicating the stable integration of the Bt gene into the recipients. Resistance to the cotton bollworm (*Heliothis armigera*) was identified in these transgenic plants. In R1, 5 plants of simian3 and 3 zhongmiansuo12 were highly toxic to insects with larval death rates of upto 93.8%. Insect-resistant R5 cotton lines were derived from selfed R1 transgenic insect-resistant plants (Wanchao and Zhenlin, 1999). By the pollen tube pathway method, the plant expression vector pGB121S4ABC, harboring both synthesized (*Bacillus thuriengiensiss*) Bt insecticidal protein gene and modified CpT1 gene, was introduced into the elite cotton (*G. hirsutum*) cultivars Shiyuan 321, Zhongmiansou 19, 3517 and 541. Resistance of transgenic plants to cotton bollworm (*Helicoverpa armigera*) was identified. The results showed that some cotton plants were highly toxic to the insect with larval death rates of upto 96%. The results of PCR, PCR-Southern, RT-PCR analyses confirmed the integration and expression of the two genes in these insect-resistant cotton plants (San Dui *et al*., 1999).

Mechanism of action of Bt toxin

Bt insecticides, whether in the form of a spray or a Bt crop, do not function on contact as most insecticides do, but rather, as midgut toxins. Following ingestion of activated toxin expressed in tissues of transgenic plants, the toxin binds to specific receptors on the cell membranes of the midgut epithelial cells, inserts itself into the membrane, and forms pores that kill the epithelial cells (and eventually the insect) by colloid osmotic lysis (Knowles *et al.,* 1993). There are two widely accepted models (penknife model and Umbrella model) for insertion of Bt toxins into insect plasma membrane, based on the CryIIIA structure as model toxin. The α helices (1-7) in domain 1 are numbered from the N-terminus (Grochulski *et al*., 1995; Li *et al*., 1991). As domain II identifies the epithelial receptor, the conformational change triggered by binding could be transmitted from domain II to domain I via helice 7 initiating membrane insertion of two or more helices. According to the "penknife" model modified from Hodgman and Ellar (1990): helices α5 and α6 flip into the membrane as a helical hairpin.

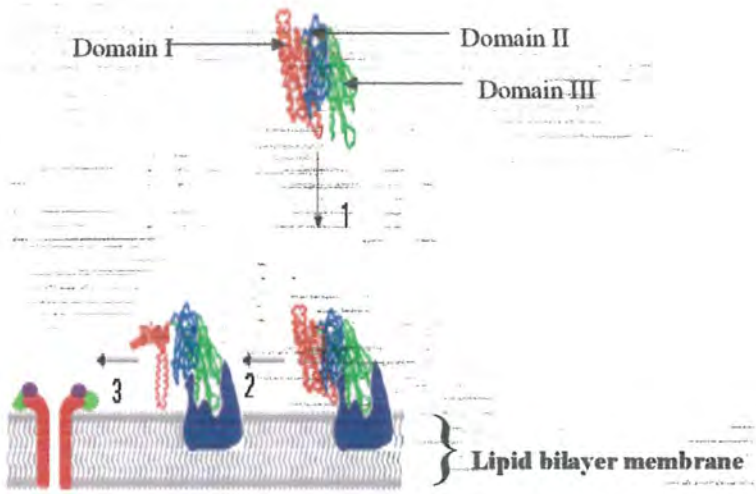


Figure 8: Mode of action of Bt toxin on insect midgut membrane (Li, *et al.*, 1991).

(1) Ingestion of activated crystal toxin by insect expressed in tissues of transgenic plant.
(2) Interaction with cell surface binding proteins.
(3) Conformational change exposing α 4-5 helical hairpin, followed by oligomerization and insertion in memebrane to form pore.

But "umbrella" model modified from Li *et al.*, (1991) explains that helices α4 and α5 drop down into the plasma membrane as helical hairpin, and other helices flatten out on the membrane surface, their hydrophobic faces towards the membrane (Fig. 8). In both models insertion of a pair of helices may be followed or accompanied by formation of an oligomeric barrel-stave pore with a central aqueous channel.

Table1. Cotton varieties/strains transformed for Bt gene in Pakistan (Anonymous, 2002)

Sr.No	Variety	Status	Institute
1	IR-FH901	DGR-Trial	NIBGE, Fsd
2	IR-CIM-448	DGR-Trial	NIBGE, Fsd
3	IR-CIM-443	DGR-Trial	NIBGE, Fsd
4	IR-FH1000	Under Transformation	NIBGE, Fsd
5	IR-VF-Karishma	Under Transformation	NIBGE, Fsd
6	NIAB-98	Under Transformation	NIBGE, Fsd
7	CIM-443	Transformed	CCRI, Multan
8	CIM-448	Transformed	CCRI, Multan
9	CIM-473	Transformed	CCRI, Multan

Bt Cotton and integrated pest management (IPM)

In recent years, introduction of insect resistant Bt cotton have become the major tool in the integrated management of cotton insect pests (Xia *et al.*, 1999). Specifically, the benefits of using Bt cotton in integrated pest management systems worldwide are as follows (Obando-Rodriquex, *et al.*, 1999):

1. Provides control of Lepidopteran species such as tobacco budworms, which in some have become resistant to some broad-spectrum insecticides.

2. Provides control of cryptic lepidopteron species that burrow into cotton tissues such as pink bollworms and cotton leaf perforater, which are difficult to control with conventional broad-spectrum insecticides.
3. Reduces use of broad-spectrum insecticides that complement integrated pest management program and natural biological control of insects and mite pests.
4. Reduces insecticide exposure for farm workers and the agricultural community.
5. Improves cotton yields and farm benefits when compared with use of conventional broad-spectrum insecticides in traditional pest management program, depending upon pest species.
6. Increases sustainability of cotton production systems by reducing broad-spectrum insecticide use, pest resistance, pest resurgence and secondary pest outbreaks, and by increasing the effectiveness of biological control of pests.

Cotton transformation and regeneration

Plant transformation technology has become a versatile platform for cultivar improvement as well as for studying gene functions in plants (Hansen *et al.*, 1999). Cotton traditional breeding programmes has produced steady improvement in agronomic traits, but the lack of useful economic characters in commercial cotton cultivars still remains a big challenge. Recently, however cotton has entered biotechnology arena, which, by advances in genetic transformation technology promises to meet this challenge by incorporating foreign genes for the desired agronomic traits. Resistance to pests, a successful evolutionary survival strategy used by most wild plant species, including cotton, had been dramatically increased through the techniques of modern biotechnology (Addisson, 1999) such that plants developed through biotechnology have been bred to provide insecticidal activity against cotton pests. Cultivation of Bt transgenic cotton plants has resulted in a reduction in the use of chemical insecticides, thereby reducing environmental pollution (Benedict and Altman, 2001).

Although transgenic offer a sustainable solution for insect pest management, transformation of cotton plants remains the bottleneck in the introduction of various Bt genes that have been isolated and characterized against cotton pests (Leelavathi *et al.*,

2004). Various protocols have been explored for the transformation of cotton plants such as meristem transformation (Zapata *et al.*, 1999), Particle bombardment (Rajaeskaran *et al.*, 2000) and *Agrobacterium*-mediated transformation (Umbeck *et al.*, 1987). *Agrobacterium* mediated transformation of cotton is very efficient process and has not been considered a limiting factor in generating transgenic plants. This was quite nicely demonstrated very recently using GFP (jellyfish green flourescent protein) as a means to track and monitor the transformation process in cotton (Sunilkumar *et al.*, 2001). The first report of genetically engineered cotton was in 1987 (Umbeck *et al.*, 1987). Rajaeskaran *et al.* (1996), reported successful regeneration of cotton (G. *hirsutum*) plants from cryopreserved embryogenic callus and cell suspension cultures. The cryoprotectant mixture consisting of a modified MS medium with sucrose, DMSO and glycerol gave the highest survival (70%). Regenerated plants from cell suspension and emryogenic callus cultures exhibited normal morphology, growth and boll set upon transfer to soil. Callus characteristics – color, texture, friability and size- all play a major role in successful regeneration of cotton via somatic embryogenesis. The best callus initiated on MCIM (Maxxa based callus induction medium) and later becoming embryogenic, was light parrot-green in color and featured a grainy, nodular texture (Mishera *et al.*, 2003). The regeneration potential for any given genotype requires formation of a friable callus from explants that undergo successful morphogenesis to produce somatic embryos. The first step to successful regeneration is callus formation. The coker callus induction medium supports formation of friable, embryogenic callus on a limited number of genotypes, including a few Acala cottons (Wilkins, *et al.*, 2000). Application of a polybrene-spermidine treatment for gene transfer into cotton cells greatly enhanced DNA uptake and recovery of transgenic cotton as indicated by Gus gene expression and southern hybridization (Sawahal, 2001).

Hypothesis for the study

The hypothesis, which we can formulate from the previous study, is:

1. AC1 gene is the only conserved viral gene absolutely required for viral DNA replication so it is attractive target to engineer virus resistant plants through RNA silencing.
2. Bt genes, *cry1Ab* has significant insecticidal activity.

Objectives

1. Construction of plant expression cassettes containing AC1gene for cotton leaf curl disease resistance and *cry1Ab* gene for cotton lepidopteron pest resistance.
2. Transformation of AC1 and *Cry1Ab* genes into cotton Coker 312.
3. Molecular analysis of transgenes in putative transgenic cotton calli/ embryoids.

Keeping in view, the importance of cotton and potential dangers from begomovirus complex and insect pest complex, present study was undertaken to engineer cotton against cotton leaf curl virus and insect pests.

Materials And Methods

Materials and Methods

This research work was conducted at the National Institute for Biotechnology and Genetic Engineering (NIBGE), during January 2003 to January 2004. Plant Biotechnology Division (PBD) provided experimental facilities and technical support.

1. PCR amplification of truncated replicase gene (AC1) of CLCuV

Designing of primers

Primer designing was undertaken to amplify the 757 bp of AC1 gene from a plasmid harboring full length of cotton leaf curl virus DNA A. Primers were designed in such a way that restriction sites of *Xho* I and *Eco* RI were introduced in forward and reverse primers for sense cloning and *Bam* HI and *Hind* III restriction sites for antisense cloning in the same vector respectively.

Following criteria were selected for primer designing.

1. Determination of particular restriction sites on vector as well as in the primers.
2. Confirmation that same restriction site is not present within the coding region of the target gene.
3. Primers should not be complementary to each other in order to avoid primer dimmer formation.
4. Primers should not contain less than 50% GC content.
5. Annealing temperature of primers should be considered.

Primers were designed complementary to flanking regions of target gene (Forward primer has same sequence while reverse primer has complementary sequence).

The sequences of all four primers for amplification of truncated AC1 gene are as follows.

Primer 1. Sense forward.

5′ GAC***CTCGAG*** AGAGCTTCACGAAGATGGGAC 3′

Xho I

Primer 2. Sense reverse.

5′ GAC***GAATTC***CGCCTTTAATTTGAACTG 3′

Eco R 1

Primer 3. Antisense forward.

5′ GAC***AAGCTT***ACGCCTTTAATTTGAACTG 3′

Hind III

Primer 4. Antisense reverse.

5′ GAC***GGATCC***AGAGCTTCACGAAGATGG 3′

Bam H I

Polymerase Chain Reaction (PCR)

PCR for truncated AC1 gene in sense orientation

The optimum reagents for the PCR are given below.

Template DNA	2µl	(50ng)
dNTPs (10mM)	1µl	(0.2mM)
Buffer (10X)	5µl	(1.0 X)
$MgCl_2$ (25mM)	3µl	(1.5 mM)
Primer 1 (forward)	1µl	25 ng
Primer 2 (reverse)	1µl	25ng
Taq polymerase (5U/µl)	0.5µl	(2.5U)
Deionized d.d.H2O	36.5µl	
Total volume	50µl	

Optimization of PCR profile

Segment 1

Initial denaturation temp.	94 ^{0}C
Time	5 min
Cycles	1

Segment 2

Denaturation temp	94 ^{0}C
Time	1min
Annealing temp	50 ^{0}C

Time	1min
Extension temp	72^0C
Time	1 min
Cycles	40

Segment 3

Extension Temp	72^0C
Time	10min
Cycle	1

Hybaid (Touch down) thermal cycler was used for the PCR reaction. The amplified products were analyzed by electrophoresis on1% agarose gel.

Cloning of truncated AC1 in plant expression vector in sense orientation

Digestion of PCR product

The truncated AC1 gene was amplified through PCR by using forward and reverse primers. PCR products were run on I% agarose gel and the amplified DNA fragments showing correct size was eluted by CONCERT^{TM} rapid gel extraction system (Invitrogen, USA; Appendix XI). The purified PCR product was digested with *Xho* I and *Eco* R I enzymes. The optimized reaction reagents were as follows.

DNA (PCR product)	16µl
Reaction buffer (10X)	2µl
Xho I (10U/µl)	1µl
Eco R I (10U/µl)	1µl
Total volume	20µl

The digestion mixture was incubated at 37 ^{0}C for 1 hour. Digested fragment was precipitated by adding 1/10th 3M sodium acetate and 2 volume ethanol.

Digestion of pN6 RNAi vector

Plant expression vector pN6 DNA was also digested with the same enzymes i.e. *Xho I* and *EcoR I*. The optimized reagents for the reaction were as follow.

DNA fragment	16µl
Reaction buffer (10X)	2µl
Xho I (10U/µl)	1µl
Eco R I (10U/µl)	1µl
Total volume	20µl

The digestion mixture was incubated at 37 ^{0}C for 1 hour. The digested plasmid was treated with phenol-chloroform to inactivate enzymes and precipitated by 2.5 volume ethanol. The digested plasmid (pN6) and truncated AC1 sense PCR fragment were run on 1% agarose gel along with 1kb DNA marker. Restricted PCR fragment and pN6 plasmid were eluted by Concert^{TM} Rapid Gel extraction Kit (Appendix XI)

The digested and purified PCR fragment of sense gene and plasmid DNA were ligated. The ligation reaction was optimized as follows.

Digested DNA fragment	6µl
Plasmid DNA (digested)	2µl
Ligase buffer	2µl
Ligase (enzyme)	2µl
Double distilled H_2O	8µl
Total volume	20µl

The ligation reaction mixture was incubated for overnight at 16 ^{0}C.

Preparation of electrocompenent cells of *E. coli* (DH5α)

Electrocompetent cells of *E. coli* were prepared according to protocol given in appendix (VIII).

Transformation of recombinant pN6 in *E.coli* by electroporation

Protocol followed for the transformation of recombinant pN6 in *E. Coli* (DH5α) is given in Appendix (IX).

Plasmid Isolation from *E. coli*

For the isolation of recombinant plasmid from *E.coli* the protocol followed is given in appendix (X).

The obtained DNA was digested with *Xho* I and and *Eco* R I enzymes. The reaction reagents were as follows.

Plasmid DNA	4µl
Reaction buffer (10X)	2µl
Xho I (10U/µl)	1µl
Eco R I (10U/µl)	1µl
RNase	1µl
Doubled distilled water	11
Total volume	20µl

The digested DNA samples were run on 1% agarose gel along the standard 1kb DNA marker and recombinant clone was marked. The samples having the correct inserted fragment i.e. of 757 bp were selected and marked as recombinant clones. The recombinant clone was further confirmed with other restriction sites present in the vector as well as through PCR amplification.

Cloning of truncated AC1 in plant expression vector in antisense orientation

For antisense cloning of truncated AC1 gene of CLCV DNA A into pN6 vector containing truncated AC1 gene cloned in sense orientation, same procedure was used as for sense cloning but here *Bam* H1 and *Hind* III restriction enzymes were used to restrict both pN6 and PCR fragment of AC1 amplified with specific primers.

Electroporation of recombinant pN6 (having truncated AC1-based RNAi cassette) in *E. coli*.

Protocol followed for the transformation of recombinant pN6 in *E. coli* DH5α is given in Appendix (IX).

Plasmid isolation from *E. coli*

For the isolation of recombinant plasmid from *E.coli,* the protocol followed is given in appendix (X).

The obtained DNA was digested with *Bam* H I and *Hind* III enzymes. The reaction reagents were as follows.

Plasmid DNA	16µl
Reaction buffer (10X)	2 µl
Bam H I (10U/µl)	1 µl
Hind III	1 µl
Total volume	20µl

The digestion mixture was incubated for 1 hour at 37 ^{0}C. Digested DNA samples were run on 1% agarose gel along the standard 1kb DNA marker and recombinant clone was marked. The binary clone was further confirmed by other restriction sites present in the vector as well. The cultures of samples having both sense and antisense fragments were grown in LB (Appendix VII).

Cloning of tAC1 RNAi cassette in plant transformation vector pBS389

Recombinant plasmid containing truncated AC1-based RNAi cassette and plant transformation plasmid PBS389 were cut with *Not* I and confirmed on 1% agarose gel and eluted fragments were ligated to pBS389 restricted with same enzyme. The ligation reaction was as follows;

Fragment DNA (eluted)	6µl
Vector DNA (Digested)	2µl
Ligase enzyme	1µl
Iigase buffer	2µl
dd H_2O	9µl
Total volume	20µl

The ligation mixture was incubated at 16 ^{0}C for 24 hours.

Preparation of electrocompenent cells of *Agrobacterium LBA 4404*

Protocol followed for the preparation of electrocompetent cells of *Agrobacterium* LBA 4404 is given in Appendix (VIII).

Transformation of pBS389 in *Agrobacterium tumefaciens* strain LBA4404 by electroporation

For the transformation of recombinant pBS389 in *Agrobacterium tumefaciens* strain LBA4404 protocol followed is given in (Appendix IX).

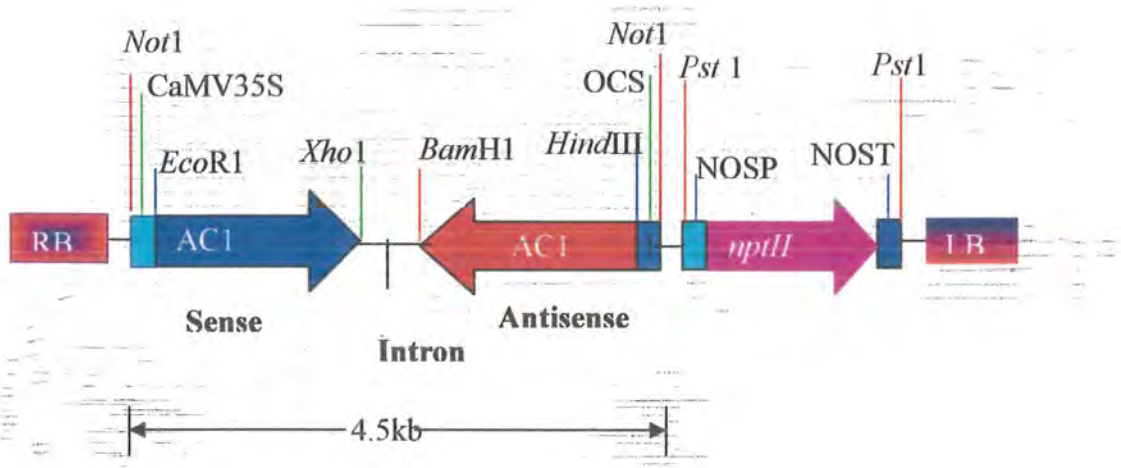


Figure 9: T-DNA map of binary vector pBS389-AC1

RB: Right border

LB: Left border

NptII: Neomycinphosphotransferase II

NOSP: Nopaline synthase promoter

NOST: Nopaline synthase terminator

CaMV35S: cauliflower mosaic virus promoter

OCST: Octopine synthase terminator

T-DNA: Transferred DNA

Cloning of Bt (*cry1Ab*) gene in pJIT 60

Synthetic *cry1Ab* gene was removed from puc 19 by cutting with *Bam* HI and *Eco* RI. The optimized reagents were the same as for restriction of pN6. The restriction mixture was resolved on 1% agarose gel and fragment was eluted by Concert ™ Rapid gel extraction kit (Appendix XI). The restricted pJIT60 and eluted Bt gene were ligated by the reaction given below.

Fragment DNA	6µl
Plasmid DNA	2µl
Ligase buffer	2µl
T4 DNA Ligase	2µl
dd H_2O	8µl
Total volume	20µl

The ligation mixture was incubated at 16^0C for 24 hours.

Electro-transformation of recombinant pJIT60 into *E. coli*

For the transformation of pJIT60 into *E. coli*, DH5α same protocol was carried out as described earlier (Appendix IX).

A single colony from freshly grown plate of DH5α was picked and transferred into 100 ml LB medium (Appendix VII) in 1L flask and incubated at 37^0C overnight with vigorous shaking. Vector DNA was harvested by miniprep according to protocol given in the (appendix X). After (*Kpn* I) restriction confirmation on 1% agarose gel, fragment was eluted (appendix XI) and ligated to pGA482 plasmid at *Kpn*I site. The optimized reagents for the ligation reaction were as follows;

Fragment DNA	6µl
Plasmid DNA	2µl

Ligase buffer	2µl
T4 DNA ligase	1µl
dd H_2O	9 µl
Total volume	20µl

The ligation reaction was incubated at 16 ^{0}C for 24 hours.

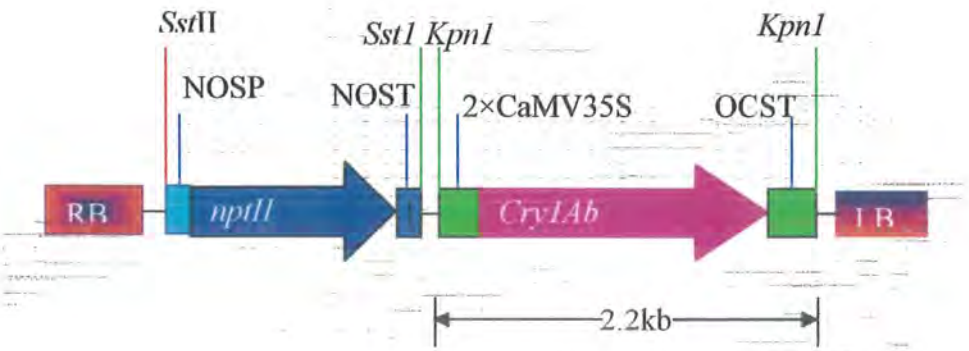


Figure 10: T-DNA map of binary vector pGA 482-*cry1Ab*

RB: Right border

LB: Left border

NptII: Neomycinphosphotransferase II

NOSP: Nopaline synthase promoter

NOST: Nopaline synthase terminator

CaMV35S: cauliflower mosaic virus promoter

OCST: Octopine synthase terminator

T-DNA: Transferred DNA

Transformation of pGA482 into *Agrobacterium* LBA 4404

For the transformation of pGA482 into *Agrobacteriumm,* same protocol was used as for transformation of pBS389 (Appendix IX).

Agrobacterium tumefaciens mediated transformation of Cotton (cultivar Coker-312)

Two weeks before transformation the following procedures were started.

Delinting and surface sterlizaton of Cotton seeds:

Cotton seeds were delinted by soaking in commercial conc. sulphuric acid @1L/10Kg for 3-5 minutes, then rinsed with autoclaved distilled water for 4-5 times.

Delintedseeds were surface sterilized by soaking in 2ml of 1% Mercuric chloride, 1ml of 1% SDS and 100 ml sterilized water through shaking for 5 minutes. Mercuric chloride and SDS were removed by washing with double distilled water for 4 times.

Seed germination

The 5-10 cotton seeds were cultured on MS0 medium (Appendix III) in the Dark room at 28 ^{0}C. When roots were emerged from the seeds, germinated seeds were shifted to the growth room at 28^{0}C (16hr-photoperiod) for 7-8 days.

***Agrobacterium*-mediated transformation**

***Agrobacterium* Culture:**

Agrobacterium tumefaciens strain LBA 4404 for genetic transformation was prepared as follows:

1 Pick one fine colony of *Agrobacterium* and float in 5-10ml of LB medium supplemented with the rifampicin (100mg/L) and spectinomycin (100µg/ml)

antibiotics for AC1 construct. Similarly 5-10 ml LB supplemented with rifampicin (100mg/L) and tetracycline (3mg/ml) antibiotics were inoculated for *cry 1Ab* construct.

2 Incubate at 28°C with vigorous shaking for 2 days

3 Incubate 1 ml of prestarter cultures in separate 25 ml of fresh LB with respective antibiotics for 4 hrs untill O.D reaches upto 0.6-0.8 at 600nm.

Agro-infections of Cotton hypocotyls

1. Cut cotton hypocotyl sections (5-6 mm) with sterile scalper.
2. Agroinnoculation of stem sections by immersing them in the culture of *A. tumefaciens* (1/2hr). Blot dried hypocotyl sections were placed on CCI medium incubate at 26-28°C with low light intensity 10 hr photoperiod for 2 days.
3. Transfer transformed explants on to CCI selection medium (kanamycin 50mg/L) and incubate at 30°C.
4. After 4-6 weeks of inoculation, callus initiation takes place at the wounded edges.
5. Separat, well proliferating calli from the explant and subculture as in IV.
6. Transfer 10 weeks old selected calli to embryo induction medium
7. Subculture after 10-15 days as in VII for 4-6 weeks.
8. Transfer embryoids to embryo germination medium for 3-4 weeks, transfer to fresh medium when embryos start germination.

Molecular analysis of putative transgenic cotton callus

Total Plant Genomic DNA Isolation *(*Iqbal *et al.*, 1997*)*.

1: Turn on the water bath, set at 65°C and preheat 2XCTAB.

2: Precool the autoclaved pestle and mortar with liquid nitrogen.

3: Take 1gm cotton callus; grind into a very fine powder in liquid nitrogen.

4: Transfer the tissue to a 50 mL blue cap tube.

5: Add 15 mL (at 65°C) 2XCTAB to the tube (prevent thawing of frozen powder).

6: Mix gently by inverting the tube then incubate at 65°C for half an hour.

7: Add 15 mL of chloroform/isoamylalcohol (24:1). Mix gently by inverting the tube

8: Centrifuge for 10 minutes at 9000 rpm.

9: Transfer the supernatant solution (the top aqueous phase) to a new tube

10: Repeat the steps 7 to 9.

11: Add 0.6 volume isopropanol to precipitate the DNA.

12: Centrifuge at 9000 rpm for 10 minutes and discard the supernatant solution.

13: Wash the pellet with 70% ethanol

14: Air dries the pellet and resuspend in 0.5mL 0.1X TE buffer or dH_2O.

15: Transfer the suspension into eppendorf and then gives RNase treatment at 37°C

16: Add equal volume of chloroform/ isoamylalcohol (24:1) and mix gently.

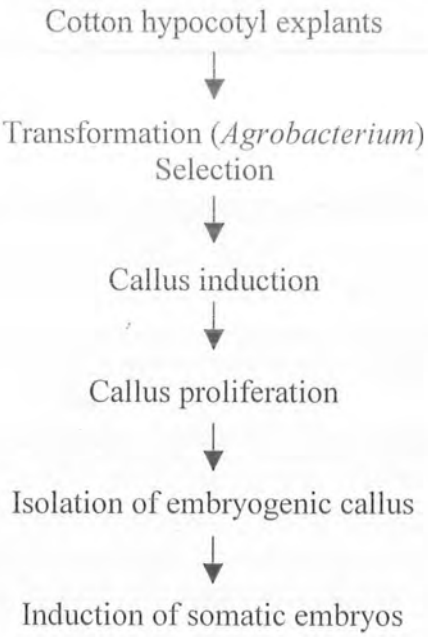
17: Spin for 10 minutes at 13000 rpm in the microcentrifuge and take the supernatant.

18: Precipitate the DNA with absolute ethanol (2 volumes), and 3M NaCl ($1/10^{th}$)

20: Spin at 13000 rpm for 10 minutes, discard the supernatant and wash the pellet with 70% ethanol.

21: Air dry and resuspend DNA pellet in 0.1X TE buffer or dH_2O.

22: Measure the concentration of the DNA by using DNA Quant 200 fluorometer.



Flow chart of protocol for cotton transformation

PCR analysis

PCR analysis was carried out to identify the presence of truncated AC1 & Bt transgenes. For PCR amplification, following reaction and profile was used.

PCR reaction		PCR profile	
10 X PCR Buffer	5µl,	95 °C	1 min
25 mM $MgCl_2$	3µl	55 °C	1min
10 mM dNTPs,	1µl	72 °C	1 min
Primer1 (Forward)	1µl		(35 cycles)
Primer2 (Reverse),	1µl	72 °C	10 min
Taq polymerase	0.5 µl of 5U/µl	105°C	Lid Temp.
ddH_2O	33.5µl	4°C	Stand by
Plant DNA	5µl		
Total	50µl		

The PCR product was resolved on 1% agarose gel and visualized on ethidium bromide stained gel for the detection of expected size DNA fragment on the gel.

Results

Results

PCR amplification of truncated AC1 gene of *Cotton leaf curl virus* (CLCuV) DNA A

To produce the RNAi expression cassette, two pairs of specific primers were used to amplify the truncated AC1 gene of CLCuV DNA A for cloning in sense and antisense orientation in pN6 vector. These primers included specific restriction sites at the both ends of the truncated AC1 gene for directional cloning as described in materials and methods. PCR was used to amplify these fragments. These fragments were fractionated on 1 % agarose gel containing ethidium bromide to visualize the DNA bands along with the 1 kb DNA marker. Figure 11 shows the amplification of the sense and antisense fragments of truncated AC 1 gene of CLCuV DNA A.

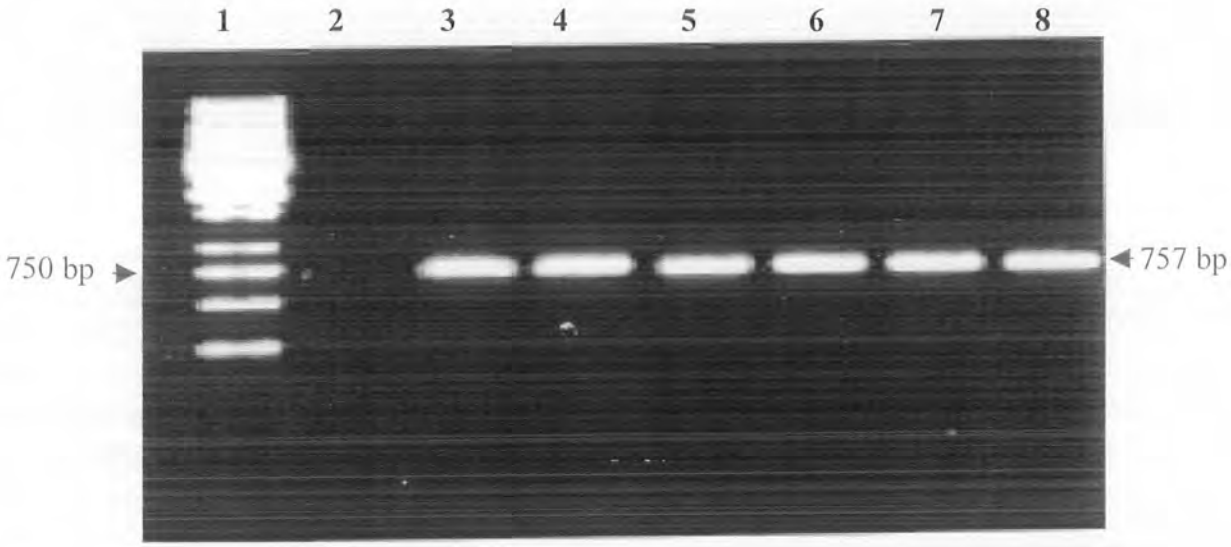


Figure 11: PCR amplification of truncated AC1 gene of CLCuV DNA A

Lane 1, DNA marker

Lane 2, negative control (no plasmid DNA)

Lanes 3-5, amplified fragment of ca 757 bp of truncated AC1 gene for sense cloning

Lanes 6-8, amplified fragment of ca 757 bp of truncated AC1 gene for antisense cloning

Cloning of truncated AC1 genes in RNAi based plant expression vector pN6

Plasmid pN6 is a plant expression vectors containing CaMV35S promoter, multiple cloning site, a poly A tail and ampicillin resistant gene. Purified, digested truncated AC1 gene fragments were ligated into plasmid pN6. The verification of the clones in sense and antisense orientation in plasmid pN6 was carried out by digesting the plasmid with *EcoR* I/ *Xho* I and *BamH* I/ *Hind* III respectively as described in materials and methods. These digested fragments were separated on 1 % agarose gel electrophoresis along with 1 kb DNA marker. Figure 12 shows the verification of the cloned truncated AC1 gene in sense and antisense orientation.

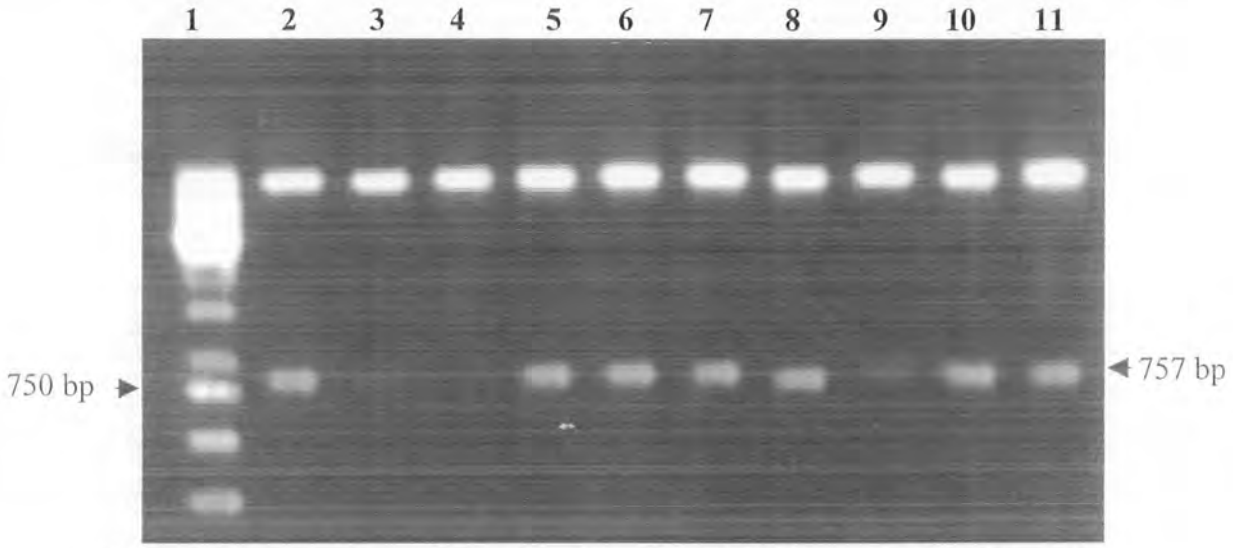


Figure 12: Verification of the cloned truncated AC1 gene in RNAi based vector pN6

Lane 1, DNA marker

Lanes 2, 5, 6, truncated AC1 cloned in sense orientation (with released of ca 757 bp band)

Lanes 3 and 4, no cloning of truncated AC1 cloned in pN6 in sense orientation (no release of band)

Lanes 7, 8, 10, 11, truncated AC1 cloned in pN6 in antisense orientation (with release of ca 757 bp band)

Cloning of RNAi based plant expression cassette in plant transformation binary vector pBS 389

The RNAi based plant expression cassette carrying sense and antisense truncated replicase genes from plasmid pN6 was separated by digesting with the restriction enzyme *Not*1 and then subcloned at the same restiction site of plant transformation binary vector pBS389 as described in materials and methods. The verification of the clones in plasmid pBS 389 was carried out by digesting the plasmid of transconugants with *Not* I. These digested fragments were separated on 1 % agarose gel along with 1 kb DNA marker. Figure 13 shows the verification of the cloned plant expression cassette of truncated AC1 gene in binary vector.

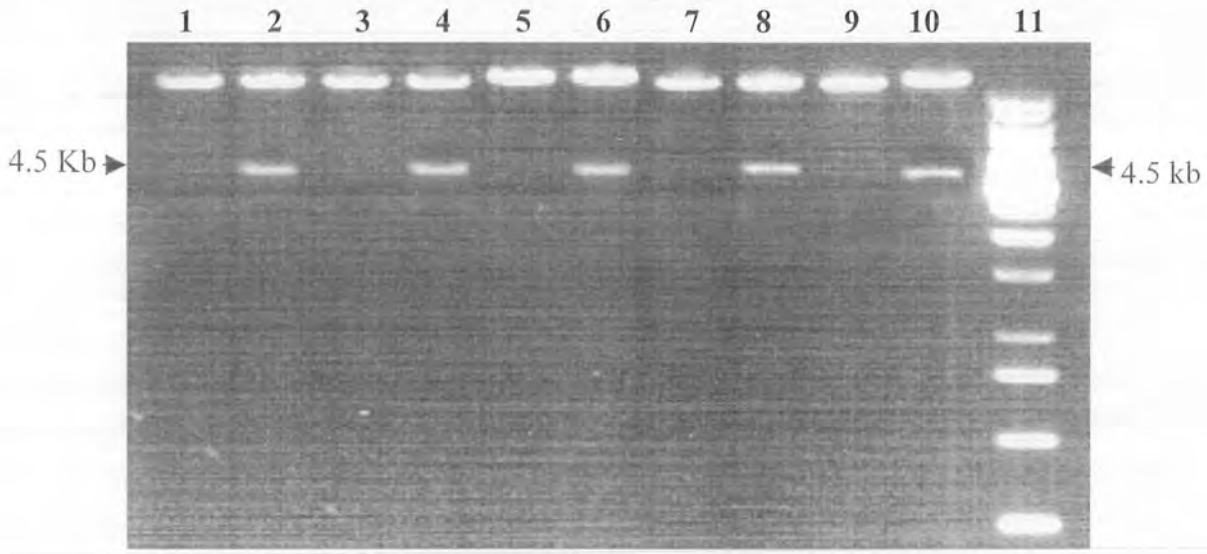


Figure 13: Restriction analysis of plant binary vector pBS 389 for the verification of plant expression cassette

Lanes 1, 3, 5, 7 and 9 absence of cloning of RNAi expression cassette (no release of band)

Lanes 2, 4, 6, 8 and 10 RNAi expression cassette released with *Not*1 (ca 4.5 kb band)

Lane 11, DNA marker

Transformation of recombinant plant transformation vector pBS 389 into *Agrobacterium tumefaciens* strain LBA 4404

The recombinant plasmid pBS389 containing RNAi based plant expression cassette of truncated AC1 gene was transformed in *Agrobacterium tumefaciens* strain LBA4404 as described in materials and methods. Transformed *A. tumefaciens* cells were individually grown in liquid LB medium containing spectinomycin and rifampicin (100mg/ml). PCR was performed on the overnight grown culture by using specific primers. Figure 14 shows the amplification of the truncated AC1 gene from the recombinant plasmid of pBS389 in *Agrobacterium tumefaciens* strain LBA4404.

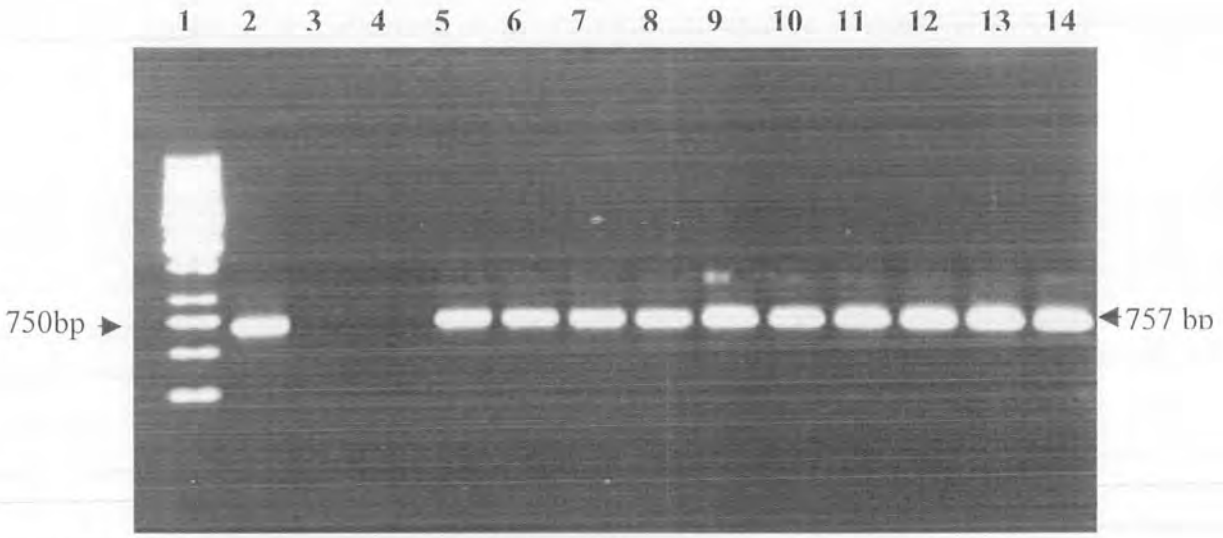


Figure 14: Verification of recombinant plasmid pBS 389 into *Agrobacterium tumefaciens* strain LBA 4404

Lane 1, DNA marker

Lane 2, Positive control with plasmid DNA as template

Lanes 3-4, Negative control without any template

Lanes 5-14 amplified fragment of ca 757 bp of truncated AC1 gene

Cloning of synthetic *cry1 Ab* gene into plant expression vector pJIT 60

Plamid pJIT60 is a plant expression vectors containing double CaMV35S promoter. Synthetic *cry1Ab* gene was cloned at *Bam*H I and *Eco*R1restriction sites of pJIT60 as described in materials and methods. Plasmids of transconjugants were digested by using same restriction enzymes and separated on 1 % agarose gel along with 1 Kb DNA marker. Pattern of restriction analysis of the transconjugants is shown in figure 15, which confirmed the cloning of *cry1 Ab* gene with the release of 2.2 kb fragment.

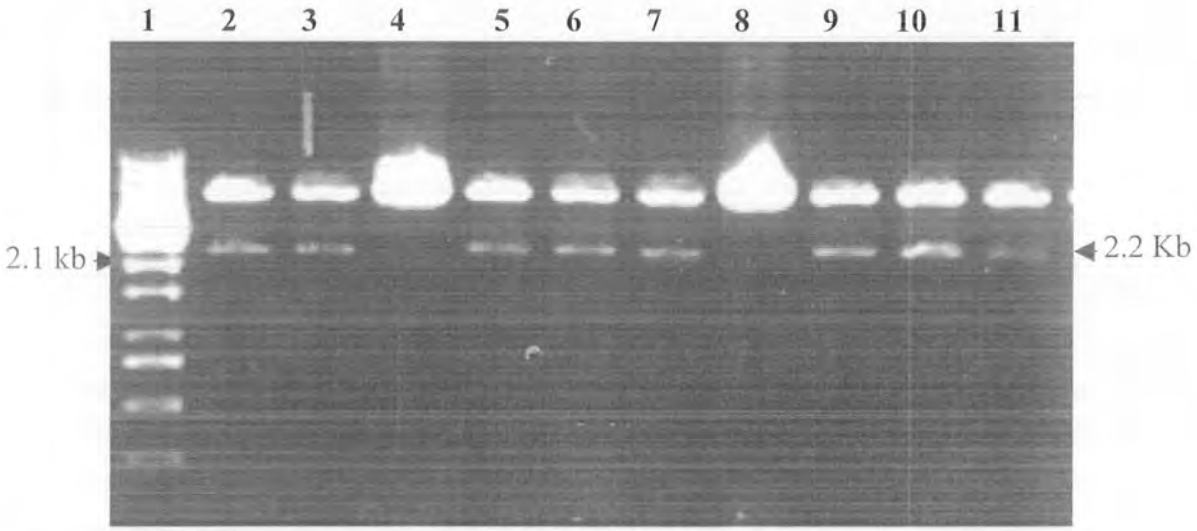


Figure 15: Restriction analysis of recombinant plasmid pJIT 60

Lane 1, Marker DNA

Lanes 2, 3, 5, 6, 7, 9, 10, and 11, recombinant plasmid pJIT60 releasing ca 2.1Kb fragment of *cry1 Ab* gene

Lanes 4 and 8, linearized plasmid without release of insert

Cloning of plant expression cassette of *cry1Ab* gene in plant transformation vector pGA482

The plant expression cassette containing CaM35 S promoter, nos terminator and synthetic *cry 1Ab* gene was removed from recombinant plasmid pJIT 60 with *Kpn* I and cloned at the same restriction site of plant transformation vector pGA482 as described in materials and methods. The verification of the clones in recombinant plasmid pGA482 was carried out by digesting the plasmid of transconugants with *Kpn* I. These digested fragments were separated on 1 % agarose gel electrophoresis along with 1 kb DNA marker. Figure 16 shows the verification of the cloned plant expression cassette of *cry I Ab* gene in plant transformation binary vector pGA 482.

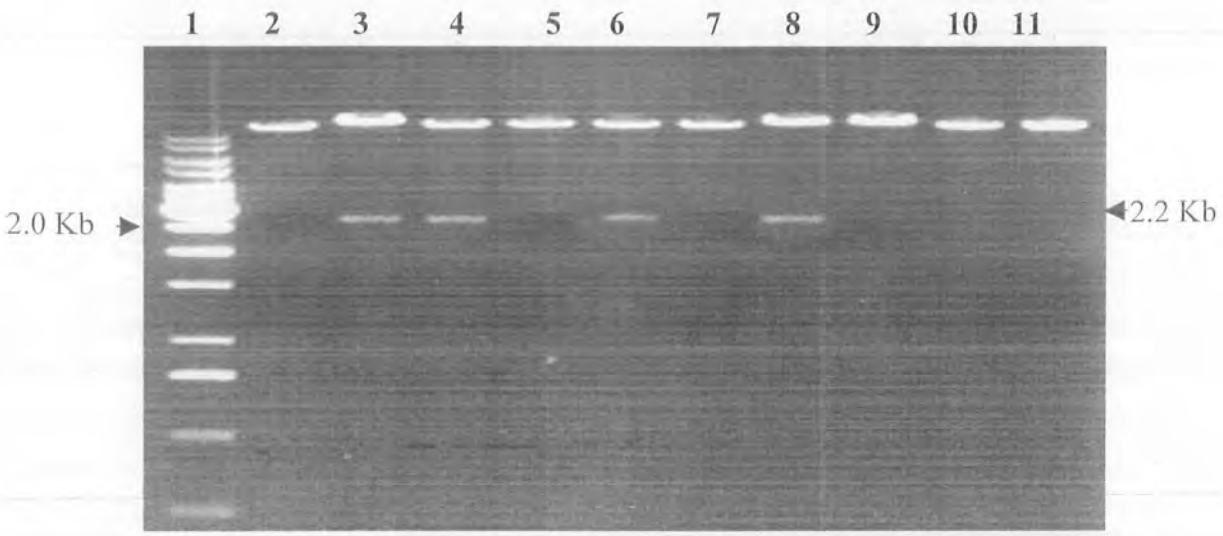


Figure 16: Restriction analysis of recombinant plant transformation vector pGA 482 for the verification of plant expression cassette

Lane1, DNA Marker

Lanes 2, 5, 7, 9, 10 and 11 absences of bands (no cloning)

Lanes, 3, 4, 6 and 8 *cry1 Ab* clones with release of ca 2.1 Kb fragment with *Kpn*1

Transformation of recombinant vector pGA482 into *Agrobacterium tumefaciens* strain LBA 4404

The recombinant plasmid pGA 482 containing plant expression cassette of synthetic *cry I Ab* gene was transformed in *Agrobacterium tumefaciens* strain LBA4404 as described in materials and methods. Transcongugants were individually grown in liquid LB medium containing antibiotics(tetracycline (3mg/ml) and rifampicin (50mg/ml)). PCR was performed on the overnight grown culture of seven transcongugants using specific primers. Figure 17 shows the amplification of the synthetic *cryI Ab* gene from the recombinant plasmid of pGA482 in *Agrobacterium tumefaciens* strain LBA4404.

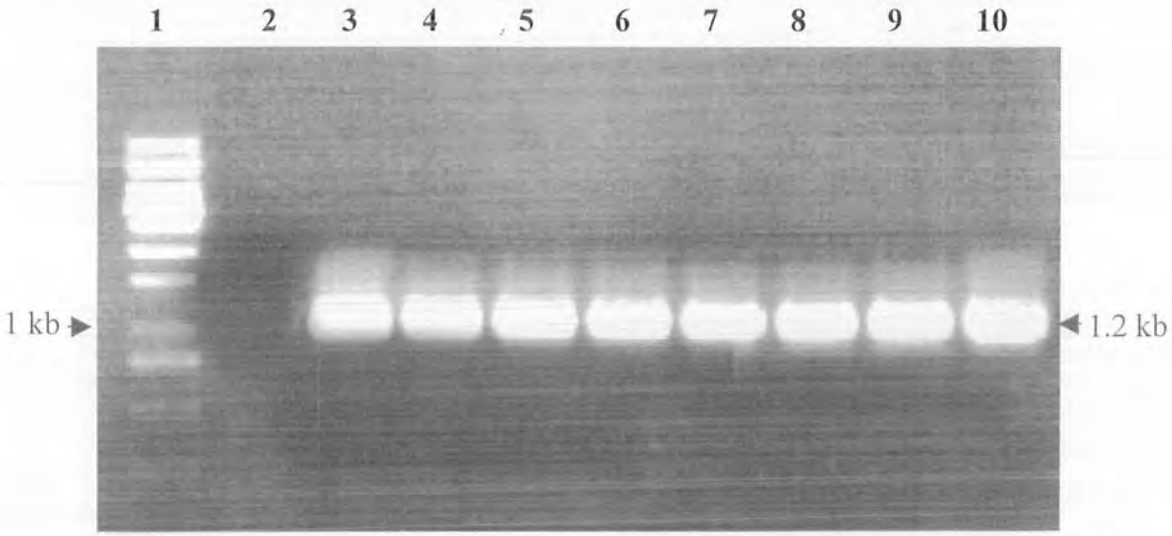


Figure 17: PCR amplification of *cry1Ab* gene from transconjugants of *Agrobacterium* tumefaciens strain LBA 4404

Lane 1, DNA Marker

Lane 2, Negative control without any template

Lanes 3-9, amplified product of *cry I Ab* gene (1.2 kb)

Lane 10, Positive control with plasmid DNA as template

Transformation of cotton Coker-312 with *Agrobacterium tumefaciens* LBA 4404

Seeds of cotton Coker-312 were placed on seed MS0 medium plates after delinting and surface sterilization. These plates were incubated at 28 ^{0}C in dark room (Fig. 18) Two days old germinating seeds were transferred to glass tubes containing same medium and kept at 28 ^{0}C in cotton growth room (Fig. 19). The hypocotyls sections of 7-10 days old cotton plants were used as explants. After inoculation and cultivation, these explants were placed on callus induction medium containing kanamycin and cefotaxime. The transformed calli were then transferred to embryo maturation medium containing kanamycin as selective agent (Figs.18-23).

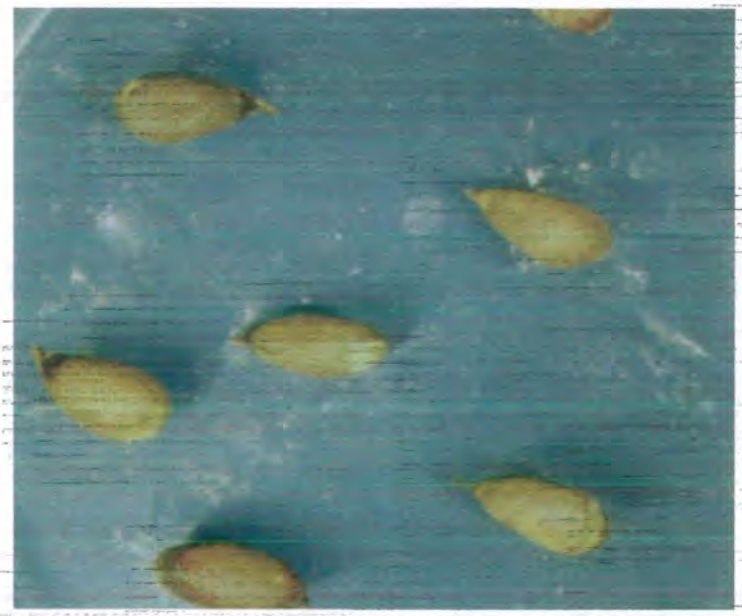


Figure 18: Seeds of cotton Coker-312 placed on germination medium

Figure 19: Cotton Coker-312 plants (7-8 days old)

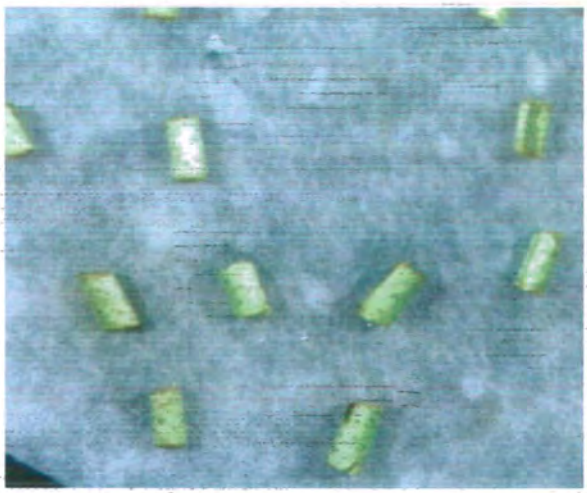


Figure 20: Co-cultivation of inoculated hypocotyls of Cotton Coker-312

Figure 21: Callus formation from the transformed hypocotyls of cotton Coker-312

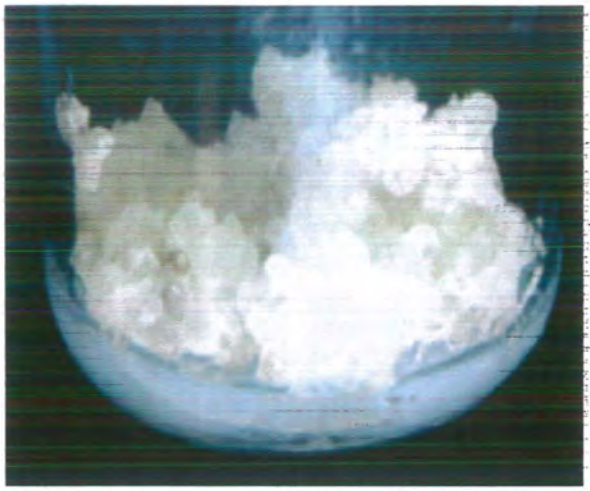


Figure 22: Proliferation of transgenic callus on selection medium

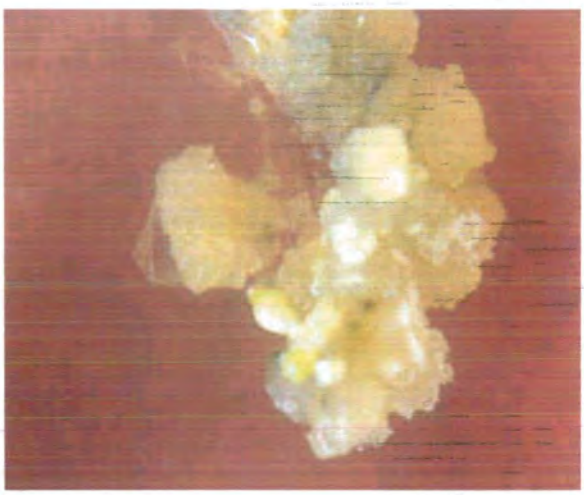


Figure 23: Embryogenic calli with matured embryoids.

Table. 2. **Embrogenic efficiency of transformed hypocotyls sections of cotton Coker-312**

Sr. No	No. of explants placed on callus induction medium(X)	No. of explants producing callus(Y)	Callusing efficiency (%)=Y/Xx100	Embryogenic callus (Z)	Embryogenic efficiency (%)=Z/Yx100
1	18	8	44	2	25
2	32	17	55	4	23
3	40	22	55	3	16
4	32	19	59	3	15
Total	30	16	53	3	20

Molecular analysis of Putative transgenic cotton calli

Total genomic DNA was isolated from kanamycin resistant calli of cotton Coker-312 as described in materials and methods. PCR analysis was carried out on the isolated genomic DNA, using *npt* II, truncated AC1, and *cry1Ab* gene primers. The amplified products of transgenes are shown in figs. 24, 25.

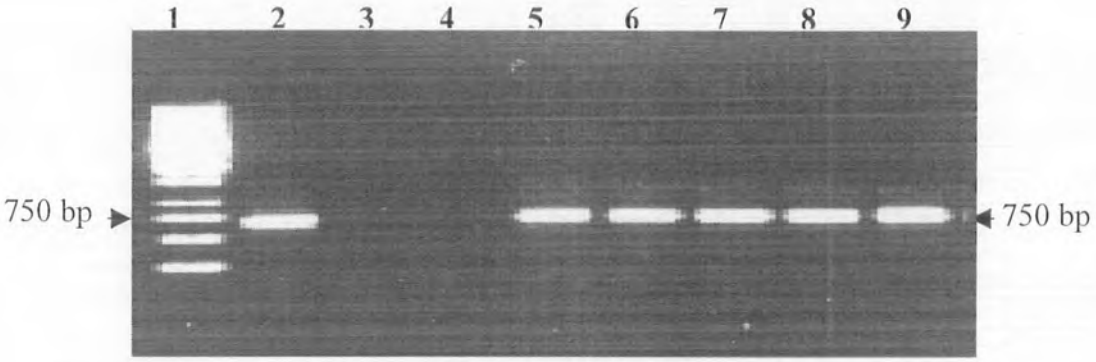


Figure 24: PCR Analysis of the kanamycin resistant calli for the presence of *npt* II gene

Lane 1, DNA Marker

Lane 2, Positive control amplified from cloned plasmid DNA

Lane 3, negative control

Lane 4, untransformed callus

Lane 5- 9, 750 bp *npt* II band amplified from the transgenic calli

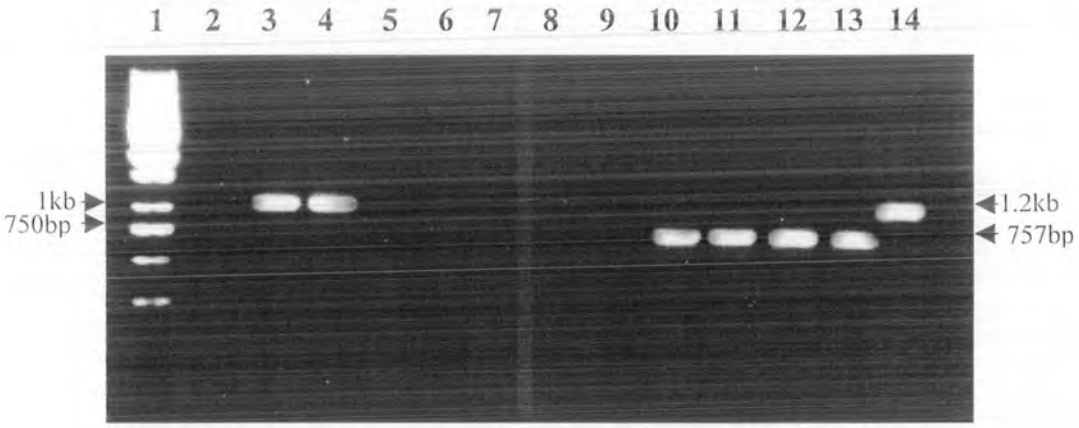


Figure 25: Detection of transgenes (truncated AC1 and *cry 1Ab*) in kanamycin resistant cotton Coker-312 calli

Lane 1, DNA marker

Lane 2, untransformed callus

Lane 3-4, presence of *cry 1Ab* gene

Lanes5-7, absence of *cry 1Ab* gene

Lanes 8-9, absence of truncated AC1 gene

Lanes 10-12, presence of truncated AC1

Lane 13, Plasmid template with cloned truncated AC1 gene (positive control)

Lane 14, Plasmid template with cloned *cry 1Ab* gene (positive control)

Discussion

Discussion

Crop improvement through genetic engineering is a reality (Dunwell, 2000). The ability of cotton regeneration coupled with the use of *Agrobacterium* to insert genes into cotton genome provides the cotton industry the tools necessary to take advantage of the knowledge developed through molecular biology on model organisms. Most of the cotton varieties transformed by *Agrobacterium* system are coker and coker-derived (Thomas, *et al*., 1995, Rajaeskaran, *et al* 1996, Agrawal, *et al*., 1997). *Agrobacterium* is naturally occurring pathogenic bacterium in the soil that has the ability to transfer its DNA into plant's genome. The engineered or constructed genes are inserted into the *Agrobacterium* vectors and enter the plant by bacteria's own internal transfer mechanisms. Transformation is typically done on a small excised portion of a plant known as explant. This small piece of transformed tissue is then regenerated into a mature plant through tissue culture techniques.

The present study deals with construction of vectors and their transformation into cotton Coker, 312. Four batches of genetic transformation cotton Coker 312 was performed by *Agrobacterium* method and overall 53 % callusing efficiency and 20% embryogenic efficiency was obtained as reported in the literature (Pennetier *et al*., 1997). The present study was conducted to engineer cotton against cotton leaf curl disease (CLCuD) and lepidopteron insect pests of cotton. For resistance against CLCuD, truncated rep (tAC1) gene was cloned in RNAi based construct to generate dsRNA, while in case of resistance against bollworm complex of cotton, *cry1Ab* (Bt gene) was cloned under strong CaMV35S promoter. Hypocotyl explants of cotton variety Coker-312 were co-transformed with *Agrobacterium* harboring recombinant vectors, pBS389 (tAC1) and pGA482 (Bt gene). The cotton calli were selected on kanamycin containing CCI medium and were regenerated into cotton embryods after transferring them to embryo formation MSK medium. Molecular analysis was performed to confirm the presence of transgenes in transgenic cotton calli.

CLCuD is endemic through out Pakistan and epidemic in western India (Mansoor *et al* 1993; Hameed *et al.*, 1994). This is because of virus genomic DNA variation that result from mutation, deletion, recombination and reassortment of viral genomic components, which is amplified by the acquisition of satellite DNA components (DNA β and DNA 1) (Mansoor *et al.*, 2003). These mechanisms generate new virus forms with enhanced biological fitness and help for maintaining flow of genetic material among different geminiviruses of the same geographical region (Harrison, 1999). The lack of natural resources of resistance sufficient for control of disease and insect pests has led to the exploration of genetically engineered resistance strategies (Hanson and Maxwell, 1999).

Pathogen derived resistance (PDR) concept states that it is possible to disrupt the normal pathogenic cycle by causing the host to express a pathogen gene at wrong time, in wrong amount or in a counter-functional form (Powell-Abel, *et al.*, 1989). Transgenic expression of pathogen-derived sequences has been extensively used to obtain virus resistant plants (Lucioli *et al.*, 2003). These strategies have variously explored and exploited general idea that transgenic expression of virus derived sequences may interfere with viral life cycle (Beachy, 1997). Native or altered virus–derived genes might be used to interfere with various stages in the viral life cycle as un-coating, transcription, translation, replication, cell-to-cell or long distance movement or vector-mediated transmission. A number of different constructs have been utilized, ranging from full length genes to mutated or truncated versions. In most cases constructs have been engineered to ensure that protein can be translated from the construct, but its translation may or may not be required. Three viral genes, i.e. coat protein, movement protein and replicase-associated genes have been used to engineer resistance against geminiviruses. Out of this rep gene is extensively used in different forms and by different procedures and showed promising results (Hanson and Maxwell, 1999) The replication associated protein is encoded by the C1 gene that is only viral protein absolutely required for viral replication (Elmer *et al.*, 1988; Etessami *et al.*, 1991). Therefore rep gene has been used for engineering resistance against CLCuD and related strains of multicomponent begomovirus complex.

Losses in crop yield due to insect alone amounts to nearly 20-30% of the global production inspite of the extensive use of chemical and biological pesticides (Estruch *et al.*, 1997; Peferon, 1997). The introduction of transgenic insect resistant plants in the market carrying Bt genes proved to be the first biotechnological breakthrough. Cloning of the crystal protein gene from *Bacillus thuringiensis* in *E. coli* led to the production of a potein highly toxic to tobacco hornworm larvae (Schnepof and Whiteley, 1981), thereby opening an avenue for their use in transgenic plants. Today more than hundred crystal protein genes sequences have been published each of which has its own specific insecticidal activity and spectrum. For example Cry1Ac protein is highly toxic to both tobacco budworm and cotton bollworms larvae but not to the European corn borer which is affected by the Cry1Ab protein. Apart from this, there are crystal proteins that appeared non-toxic when tested in bioassay with both insect and other invertebrate pests (Peferon, 1997).

Tissue culture process in cotton is multistep and slow (Kumar, *et al.*, 1998). So present study covered cotton tissue culture upto embryo formation. Transgenic cotton calli were confirmed by molecular techniques. Bioassay of transgenic plant is beyond the scope of the present work. However we can conclude the effectiveness of RNA interference in antiviral defence system and Cry1Ab proteins against cotton bollworms.

Asad *et al.*, (2003) suggested post-transcriptional gene silencing the possible mechanism to suppress the geminiviral DNA replication in transgenic tobacco plants expressing RNAs of truncated versions of AC1 (DNA replication), AC2 (a transcription activator) and AC3 (replication enhancer) genes. Sangare *et al.*, (1999) observed a delay in symptom appearance or presence of mild symptoms when transgenic plants expressing mutated *AC1* gene were infected with ACMV. In addition, resistant plants accumulated less viral DNA then plants that did not express the transgene. These results suggested constitutive expression of mutated gene affects viral DNA replication. It has been shown that transgenic expression of N-terminal 210 amino acids of the TYLCV Rep, rep 210 confers resistance to TYLCSV in both *N. bentamiana* and tomato plants (Brunetti *et al.*, 1997) and that Rep 210 acts as a transdominant negative repressing C1 gene transcription

(Brunetti *et al.*, 2001). Lucioli *et al.*, (2003) showed that rep 210 derived from *Tomato yellow leaf curl sardinia virus* AC1 confers resistance through two distinct molecular mechanisms depending on challenging virus. Resistance to homologues virus is achieved by the ability of rep-210 to tightly inhibit C1 gene transcription, while to that heterologues virus is due to interacting property of the rep-210 oligomerization domain.

The discovery of dsRNA as an inducer of RNA silencing has provided a scheme of dsRNA–mediated interference to direct gene specific silencing that is more efficient than antisense suppression or co-suppression by overexpression of target genes (Fire *et al.*, 1998; Kennerdell and Carthew, 1998; Waterhouse *et al.*, 1998: Vance and Vaucheret, 2001). The aberrant transcripts form local duplex structure that is synthesized into longer dsRNA by the action of a plant RNA dependent RNA polymerase in combination with other proteins. This dsRNA is then processed into 21 and 25 nt short RNA species that spread systemically in other cells (Vance and Vaucheret, 2001). The purest demonstration that dsRNA mediates gene silencing comes from work in which transgenic plants were established which expressed either sense or antisense of a gene of the potato virus Y (PVY). Both transgenic lines of tobacco were susceptible to PVY infection. However, crosses of these tobacco lines that expressed transgenes for both the sense and antisense orientation and thus could generate dsRNA became resistant to PVY. This suggests that the two complementary RNAs transcribed from unlinked loci were able to anneal in the nucleus and induce a gene specific suppressive state (Waterhouse *et al.*, 1998). Plant transformation vector capable of dsRNA formation were constructed by linking the gene specific sequences in both sense and antisense orientation under the control of strong viral promoter. These dsRNA-expressing constructs when delivered into *Arabidopsis* with *Agrobacterium*-mediated transformation, creates a heritable phenotypic series in the transformants showing both reduction and loss of function with gradually reduced expression of a specific gene which has proved to be an effective tool in studying genes involved in plant development and other processes essential for plant viability (Levin *et al.*, 2000; Yu *et al.*, 2002). It can also be made stably heritable by transgene constructs which express the dsRNA as a hairpin-loop structure from an inverted repeat (Chuang and Meyerowitz, 2000), raising the possibility that such sequences might also

occur naturally. Expression of self-complementary hairpin RNAs within two complementary regions separated by an intron elicits PTGS with high efficiency (Pandolfini *et al.*, 2003). PTGS has been achieved with high efficiency in transgenic plants expressing self-complementary hairpin RNA (Smith *et al.*, 2000). hpRNA have two complementary regions that form a double stranded region separated by a short loop. Hairpin constructs having a spliceosomal intron inserted into the loop region (ihpRNA) show an efficiency of upto 90% in eliciting RNA silencing (Wesley *et al.*, 2001).

The effectiveness of *Bacillus thuringiensis* crystal proteins against important agronomic pests and its safety towards beneficial organisms has been well documented (Koziel *et al.*, 1993). This supported by the isolation of Bt crystal protein genes has resulted in its extensive use in plants protection through biotechnology. Initially, δ-endotoxin encoding genes. such as, *cry1Aa*, *cry1Ab* and *cry1Ac* from the Bt sub-species Kurstaki was introduced into tomato (Fischoff *et al.*, 1987) and potato (Peferon, 1991) in their native and truncated forms using agrobacterium mediated transformation. Thus partial or complete resynthesis of these genes to contain a higher G/C content resolved this problem to a great extent. Sequence modification in areas of the genes with predicted mRNA secondary structures in combination with powerful promoters resulted in 100-fold increased level of insecticidal protein in transgenic cotton plants encoding the *cry1 Ab* or *cry1Ac* genes thereby leading to effective control of cotton pests (Perlak, *et al.*, 1990). Fields tests of transgenic cotton lines based on these improvements exhibited good protection against cotton bollworms (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*). In china, Bt cotton plants have provided 60-80 % decrease in the use of foliar insecticides ((Xia *et al.*, 1999). The cost of cotton production for small farmers was reduced by 20-33 % depending on the variety and location by using Bt cotton, and the net income and returns to labour to all Bt varieties were superior to all non-Bt varieties (Pray *et al.*, 2001). Potato plants have been genetically modified expressing *Bacillus thuringiensis* delta-endotoxin to resist attack by insect species belonging to the orders Coleoptera and Lepidoptera, through the insertion of a hybrid gene, *SN19*, (Naimov *et al.*, 2003). Very recently, Leelavathi, *et al.*, (2004) employed *Agrobacterium*-mediated transformation using embryogenic calli of cotton (variety coker 310) as a constant source

of explant to introduce the insecticidal toxin encoding *cryIIa5*, an isolate of Bt which gave complete protection against predation by American bollworm (*Heliothis armigera).*

Active research is going on to explore the mechanisms of virus resistance and insect pest resistance in transgenic cotton plants. Future plans will be the bioassay study against cotton leaf curl disease and cotton bollworm complex on transgenic cotton coker-312 and their use in cotton breeding programme as parents to study the inheritance and expression of transgenes as well as transfer of transgenes to elite cotton cultivars. This will add a very exiting era to widen our understanding for host encoded and virus/bacteria derived resistance genes. Future efforts will likely focus on combining host and pathogen derived genes to achieve a synergistic effect and ensure durability of protection against cotton leaf curl disease and cotton insect pests as an effective component of integrated pest management (IPM).

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APPENDICES

APPENDIX I

Components of MS Salt

Macro Elements	**mg/l**	**20x(g/l)**
KNO_3	1900	3.8
KH_2PO_4	170	3.4
$MgSO_4$	370	7.4
$CaCl_2$	440	8.8
NH_4NO_3	1650	3.3
Micro Elements	**mg/l**	**50xg/500ml**
H_3BO_3	6.2	0.31
$MnSO_4$	16.9	1.115
$ZnSO_4.7H_2O$	8.6	0.43
KI	0.83	0.0415
$NaMoO_4\ 2H_2O$	0.25	0.0125
$CuSo_4\ 2H_2O$	0.025	0.00125
$CoCl_2\ 6H_2O$	0.025	0.00125

APPENDIX II

Vitamin B_5

1	Nicotinic Acid	0.1g
2	Thiamine HCL	1g
3	Pyridoxine HCL	0.1g
4	Myo Inositol	10g

APPENDIX III

MSO Medium

(For seed germination)

pH 5.7-5.8 in dist. water

1	MS Salt	4.3g/l
2	Vitamin B_5	5ml/l
3	Gluocose	30g/l

APPENDIX IV

CCI Medium

(For callus induction & maintenance

pH 5.7-5.8 in dist. water

1	MS Salt	4.3g/l
2	$MgCl_2$	1.6g/l
3	Vitamin B_5	5ml/l
4	2,4-D	1ml/l
5	Kinetin	5ml/l
6	Glucose	30g/l

APPENDIX V

MSK Medium

(For embryo induction and maturation)

(pH 5.7-5.8; for 1000 ml) in dist. water

1	MS Salt	4.3g/l
2	Vitamin B_5	5ml/l
3	Glucose	30g/l
4	$MgCl_2$	1.6g/l
5	KNO_3	1.9g/l

APPENDIX VI

WASHING BUFFER

(pH 7.4; for 1000 ml) in dist. water:

NaCl	8.00 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.15 g
KCL	0.20 g
Tween 20	0.50 g
NaN_3	0.20 g

COATING BUFFER

(pH 9.6; for 1000 ml) in dist. water:

Na2 CO3	1.59 g
NaHCO3	2.93 g
NaN3	0.20 g

CONJUGATE BUFFER

(pH 7.4; for 1000 ml) in dist. water (pH adjusted with HCl)

Tris-(hydroxymethyl) aminomethane(TRIS)	2.40 g
NaCl	8.00 g
PVP (Polyvinylpyrrolidone) MW 24000	20.00 g
Tween 20	0.50 g
BSA (bovine serum albumin)	2.00 g
$MgCl_2 . 6 H2O$	0.20 g
KCL	0.20 g
NaN3	0.20 g

SUBSTRATE BUFFER

(pH 9.8; for 1000 ml) in dist. water (pH adjusted with HCl)

Diethanolamine	97.00 ml
NaN3	0.20 g

EXTRACTION BUFFER

(pH 7.4; for 1000 ml) in dist. water (pH adjusted with HCl)

Tris-(hydroxymethyl) ainomethane (TRIS)	2.40 g
NaCl	8.00 g
PVP (Polyvinylpyrrolidone) MW 24000	20.0 g
Tween 20	0.50 g
KCL	0.20 g
NaN3	0.20 g

APPENDIX VII

LB (LURIA-BERTINI) MEDIUM:

Tryptone	1.0 %
Yeast extract	0.5 %
NaCl	0.5 %
Agar	1.5 %

Adjust pH to 7.5 and autoclave.

APPENDIX VIII

PREPARATION OF ELECTROCOMPETENT CELLS OF *E. coli* DH5-α

1) A single colony from a freshly grown plate of DH5-α (GIBCO-BRL, USA) was picked and transferred into 100 ml LB medium in 1 liter flask and incubated at 37 ^{0}C overnight with vigorous shaking.
2) 2.5 ml of the overnight culture was taken and transferred to 250 ml LB in 1000 ml flask and shaken vigorously at 37 ^{0}C until O. D_{600} of 0.5-1.0 (**10^{10} cells/ml**).
3) The cells were transferred aseptically to sterile disposable 50 ml propylene tube. The culture was cooled by keeping on ice for 10 minutes.
4) The cells were pelleted by centrifugation at 5000 g at 4 ^{0}C for 15 minutes and resuspended in one of volume sterile cold distilled water.
5) The cells were pelleted by centrifugation at 5000 g at 4 ^{0}C for 15 minutes and resuspended in 0.5 volume of sterile cold distilled water.
6) The cells were pelleted by centrifugation at 5000 g at 4 ^{0}C for 15 minutes and resuspended in 0.02 volume of sterile cold distilled water.
7) The cells were again pelleted by centrifugation at 5000 g for 15 minutes and suspended finally in 0.002-0.003 volume sterile 10% cold glycerol.
8) The cells were stored in aliquots of 50 µl or 100 µl at -70 ^{0}C.

APPENDIX IX

TRANSFORMATION IN *E. coli* DH5- α BY ELECTROPORATION

For electroporation following protocol was used.

1. Electroporation cuvetts 2 mm gap were placed on ice.
2. Vials of frozen electrocompetent cells of DH5-α were allowed to thaw on ice.
3. 2 µl of ligation mixtures was pipetted into Eppendorf tube containing the competent cells and was mixed gently with the pipette tip.
4. The conditions for electroporation were:

Choose mode T	2.5 KV
Set resistance R	R5 (129 ohm)
Chamber gap	2 mm
Set charging voltage	2.45 KV

5. The electrocompetent cells containing the ligation mixture were transferred to electroporation cuvette.
6. Pulse was given and 1 ml of liquid LB medium was added immediately, mixed gently and transferred to a 1.5 ml Eppendorf tube and incubated at 37 ^{0}C for 45 minutes with vigorous shaking.
7. 100 µl of transformed culture was spread on solid LB medium having 100 mg/ml ampicillin.
8. After the liquid was absorbed completely. The plates were sealed with Para film and kept at 37 ^{0}C in incubation for over night.
9. Colonies were picked with sterile toothpicks and cultured in 3 ml liquid L.B medium containing 100 mg/ml ampicillin.
10. Culture tubes were kept at 37 ^{0}C in water bath for over night with vigorous shaking.

11. Plamid was isolated and checked on 1% agarose gel.
12. Plasmid DNA was digested with *Hind* III and *Bam* H I restriction enzymes for screening.

    Confirmed colonies were marked, preserved in 50% glycerol and stored at -70 °C for further use.

APPENDIX - X

MINI PREP SOLUTIONS:

Solution I (Resuspension buffer)

Tris (pH 7.4-7.6)	50 mM
EDTA	10 mM
RNAase A	100 µg/ml

Solution II (Denaturation solution)

NaOH	0.2 N
SDS	1 %

Solution III (Neutralization solution)

Potassium acetate (pH 4.8-5.0)	3M

PLASMID ISOLATION FROM *E.coli*: ALKALINE LYSIS METHOD (MINI PREP)

Following protocol was used for the isolation of plasmid DNA from E. coli.

1) A single *E. coli* colony was cultured in 3 ml liquid LB medium containing 100 mg/ml ampicillin or 50 µg/ml kanamycin and grown overnight at 37 °C.

2) The E. coli culture was centrifuged in 1.5 ml Eppendorf tube at 14000 rpm for 1 minute.

3) The supernatant was discarded and the pellet was allowed to dry on tissue paper.

4) 200 ml of solution I (Appendix X) was added to Eppendorf tube and the pellet was suspended in the solution with the help of vortex.

5) 200 ml of solution II (Appendix X) was added to Eppendorf tube and mixed well by inverting gently and incubate for 5 minutes at room temperature.

6) 200 ml of solution III (Appendix X) was added to Eppendorf tube mixed well and incubate for 5 minutes at room temperature then centrifuged at 14000 rpm for 5 minutes.

7) The supernatant was taken in fresh Eppendorf tube and two volume of 100% ethanol were added.

8) Eppendorf tube was kept at -20 °C for 20 minutes and then centrifuged at 14000 rpm for 10 minutes.

9) The supernatant was discarded and the pellet was washed with 70% ethanol and pellet was air dried.

10) 20 ml of sterile distilled water was added to the pellet to dissolve DNA and was stored at -20 °C.

The plasmid concentration and quality were detected by 1% agarose gel using stranded DNA markers.

APPENDIX XI

RAPID GEL EXTRACTION PROTOCOL

Perform all centrifugation at room temperature.

Before beginning preheats an aliquot of TE to 65 to 70 °C. Equilibrate a water bath or heat block to 50 °C. Verify that ethanol has been added to wash Buffer (L2).

1. **GEL SLICE EXCISION**: Cut the area of gel containing the DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.
2. **GEL SLICE WEIGHING**: Weigh the gel slice.

   a. For ≤ 2% agarose gel, place up to 400 mg of gel into a 1.5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

   b. For ≥ 2% agarose gel, place up to 400 mg of gel into a 5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 60 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

3. **GEL SOLUBILIZATION**: Incubate at 50 °C for ≥ 15 min. Mix every 3 min to ensure gel dissolution. After gel slice appear dissolved, incubate for 5 min longer.
4. **CARTRIDGE LOADING**: Place a spin cartridge into a 2 ml wash tube. Pipette the mixture from step 3 into the spin cartridge. Centrifuge the mixture in a microcentrifuge at > 12,000 rpm for 1 min. Discard the flow through.

   Note: Load no more than 400 mg agarose per cartridge.

5. **(OPTIONAL CARTRIDGE WASH)**: Place the spin cartridge back into the 2 ml wash tube. Add 500 µl Gel Solublization Buffer (L1) to the spin cartridge. Incubate at room temperature for 1 min, then centrifuge at > 12,000 rpm for 1 min. Discard the flow through.

6. **CARTRIDGE WASH:** Place the spin cartridge back into the 2 ml wash tube. Add 700 µl of Wash Buffer (L2) (containing ethanol) to the spin cartridge and incubate for 5 min at room temperature. Centrifuge at > 12,000 rpm for 1 min. Discard the flow through. Centrifuge again for 1 min to remove residual wash buffer.

7. **DNA ELUTION:** Place the spin cartridge into a 1.5 ml recovery tube (supplied). Add 50 µl of warm TE Buffer (TE) directly to the center of the spin cartridge. Incubate for 1 min at room temperature, then centrifuge at > 12,000 rpm for 2 min.