Molecular Characterization of Melon Leaf Curl Disease and Development of Control Strategies

A dissertation submitted to Quaid-i-Azam University, Islamabad in partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

By

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“We, the supervisory committee, certify that the contents and form of thesis submitted by Mr. Aamir Humayun Malik have been found satisfactory and recommend that it be processed for evaluation by the External Examiners for the award of the degree.”

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This Humble Effort is Dedicated
To
My Mother & Father
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### Acronyms and Abbreviations

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<tr>
<td>AbMV</td>
<td>Abutilon mosaic virus</td>
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<td>ACMV</td>
<td>African cassava mosaic virus</td>
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<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AYVV</td>
<td>Ageratum yellow vein virus</td>
</tr>
<tr>
<td>BBTV</td>
<td>Banana bunchy top virus</td>
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<tr>
<td>BCTV</td>
<td>Beet curly top virus</td>
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<tr>
<td>BDMV</td>
<td>Bean dwarf mosaic virus</td>
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<tr>
<td>BGYMV</td>
<td>Bean golden yellow mosaic virus</td>
</tr>
<tr>
<td>BYDV</td>
<td>Bean yellow dwarf virus</td>
</tr>
<tr>
<td>CabLCuV</td>
<td>Cabbage leaf curl virus</td>
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<tr>
<td>CLCrV</td>
<td>Cotton leaf crumple virus</td>
</tr>
<tr>
<td>CLCuD</td>
<td>Cotton leaf curl disease</td>
</tr>
<tr>
<td>CLCuMV</td>
<td>Cotton leaf curl Multan virus</td>
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<tr>
<td>CPMR</td>
<td>Coat protein-mediated resistance</td>
</tr>
<tr>
<td>CMV</td>
<td>Cucumber mosaic virus</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl triethyl methyl ammonium bromide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HPR</td>
<td>Host plant resistance</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole acetic acid</td>
</tr>
<tr>
<td>ICMV</td>
<td>Indian cassava mosaic virus</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>IPM</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>miRNAs</td>
<td>Micro RNAs</td>
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<tr>
<td>M.Ab</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MLCV</td>
<td>Melon yellow leaf curl virus</td>
</tr>
<tr>
<td>MNSV</td>
<td>Melon necrotic spot virus</td>
</tr>
<tr>
<td>MP</td>
<td>Movement protein</td>
</tr>
<tr>
<td>MSV</td>
<td>Maize streak virus</td>
</tr>
<tr>
<td>NBS-LRR</td>
<td>Nucleotide binding site/leucine rich repeat</td>
</tr>
<tr>
<td>NIBGE</td>
<td>National Institute for Biotechnology and Genetic Engineering</td>
</tr>
<tr>
<td>NSP</td>
<td>Nuclear shuttle protein</td>
</tr>
<tr>
<td>NWFP</td>
<td>North-Western Frontier Province</td>
</tr>
<tr>
<td>ORFs</td>
<td>Open reading frames</td>
</tr>
<tr>
<td>PaLCuV</td>
<td>Papaya leaf curl virus</td>
</tr>
<tr>
<td>PLRV</td>
<td>Potato leaf roll virus</td>
</tr>
<tr>
<td>PLV</td>
<td>Parsley latent virus</td>
</tr>
<tr>
<td>PPV</td>
<td>Porcine parvo virus</td>
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<tr>
<td>PRSV</td>
<td>Papaya ring spot virus</td>
</tr>
<tr>
<td>PTGS</td>
<td>Post-transcriptional gene silencing</td>
</tr>
<tr>
<td>PVX</td>
<td>Potato virus X</td>
</tr>
<tr>
<td>PVY</td>
<td>Potato virus Y</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RDR</td>
<td>Recombination dependent replication</td>
</tr>
<tr>
<td>REn</td>
<td>Replication enhancer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RMVR</td>
<td>RNA mediated virus resistance</td>
</tr>
<tr>
<td>RYMV</td>
<td><em>Rice yellow mottle virus</em></td>
</tr>
<tr>
<td>SCR</td>
<td>Satellite conserved region</td>
</tr>
<tr>
<td>SEL</td>
<td>Size exclusion limit</td>
</tr>
<tr>
<td>siRNAs</td>
<td>Short interfering RNAs</td>
</tr>
<tr>
<td>SLCCNV</td>
<td><em>Squash leaf curl China virus</em></td>
</tr>
<tr>
<td>SLCMV</td>
<td><em>Sri Lankan cassava mosaic virus</em></td>
</tr>
<tr>
<td>SqMV</td>
<td><em>Squash mosaic virus</em></td>
</tr>
<tr>
<td>SVT</td>
<td>Small vein thickening</td>
</tr>
<tr>
<td>TEV</td>
<td><em>Tomato etch virus</em></td>
</tr>
<tr>
<td>TGMV</td>
<td><em>Tomato golden mosaic virus</em></td>
</tr>
<tr>
<td>ToLCV</td>
<td><em>Tomato leaf curl virus</em></td>
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<tr>
<td>TMV</td>
<td><em>Tobacco mosaic virus</em></td>
</tr>
<tr>
<td>ToLCTWV</td>
<td><em>Tomato leaf curl Taiwan virus</em></td>
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<tr>
<td>ToLCBV</td>
<td><em>Tomato leaf curl Bangalore virus</em></td>
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<tr>
<td>ToLCNDV</td>
<td><em>Tomato leaf curl New Delhi virus</em></td>
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<tr>
<td>ToLCGV</td>
<td><em>Tomato leaf curl Gujarat virus</em></td>
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<tr>
<td>ToMoV</td>
<td><em>Tomato mottle virus</em></td>
</tr>
<tr>
<td>TPCTV</td>
<td><em>Tomato pseudo-curly top virus</em></td>
</tr>
<tr>
<td>TrAP</td>
<td>Transcriptional activator protein</td>
</tr>
<tr>
<td>TSWV</td>
<td><em>Tomato spotted wilt virus</em></td>
</tr>
<tr>
<td>TuMV</td>
<td><em>Turnip mosaic virus</em></td>
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<tr>
<td>TYLCV</td>
<td><em>Tomato yellow leaf curl virus</em></td>
</tr>
<tr>
<td>TYLCSV</td>
<td><em>Tomato yellow leaf curl Sardinia virus</em></td>
</tr>
<tr>
<td>VIGS</td>
<td>Virus induced gene silencing</td>
</tr>
<tr>
<td>WMV1</td>
<td><em>Water melon mosaic virus 1</em></td>
</tr>
<tr>
<td>WMV2</td>
<td><em>Water melon mosaic virus 2</em></td>
</tr>
<tr>
<td>WmCSV</td>
<td><em>Water melon chlorotic stunt virus</em></td>
</tr>
<tr>
<td>ZYMV</td>
<td><em>Zucchini yellow mosaic virus</em></td>
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Abstract

Begomoviruses as well as potyviruses infect cucurbits in different parts of the world but are shown to be independent pathogens able to cause severe disease on their own. A devastating disease suspected to be of viral origin was found in traditional muskmelon growing-areas of Punjab in the districts of Sahiwal, Pakpatten and Vehari during 2003. Characteristic symptoms of the disease in Punjab province were mosaic and chlorotic spots, leaf distortion and deformation, vein thickening, enations on the upper side of the leaves, leaf yellowing and stunting of affected plants. Initial screening of diseased samples revealed the presence of a bipartite begomovirus and the potyvirus *Zucchini yellow mosaic virus* (ZYMV). For comparison with Punjab, surveys were also conducted in Mardan district of North Western Frontier Province (NWFP) where plants showed leaf curling, mosaic and enations on the upper side of the leaf. Results showed that the disease found on muskmelon in Mardan district was caused by multiple infections of two viruses, ZYMV and *Cucumber mosaic virus* (CMV, family: *Cucumoviridae*). Dual infection of a potyvirus and a bipartite begomovirus associated with a severe yellow leaf curl disease of muskmelon in Punjab was a novel phenomenon and was investigated in detail. Analysis of the complete nucleotide sequence of the DNA A and DNA B of the begomovirus showed it to be a new species that is closely related to *Tomato leaf curl new Delhi virus* (ToLCNDV), another bipartite begomovirus widely distributed across the Indian subcontinent. The intergenic region of DNA A showed a high level of nucleotide identity with DNA A of ToLCNDV and was infectious when inoculated with DNA B of ToLCNDV to *Nicotiana benthamiana*. The DNA B of this new species named as *Muskmelon yellow leaf curl virus* (MYLCV) shows unusual features such as an intergenic region that did not match with ToLCNDV or any other begomovirus but retained some of features required for trans-replication, including the hairpin structure with nonanucleotide sequence conserved in all geminiviruses, and the rep-binding domains identical to those found on ToLCNDV. The movement protein (MP) shows high level (87%) of identity to ToLCNDV but the nuclear shuttle protein gene was truncated. The DNA B of MYLCV was unable to move systemically when inoculated with DNA A of either viruses but was maintained when co-inoculated with DNA A and DNA B of ToLCNDV. Inoculation of ToLCNDV DNA A and DNA B on muskmelons resulted in localized cell death, shown previously in tobacco and tomato
to be induced by the NSP. The expression of NSP under the control of the 35S promoter also caused cell death on muskmelons. To understand the possible role of ZYMV in the movement of MYLCV, the helper component protein (HC-Pro) of ZYMV was cloned in a PVX expression vector because HC-Pro of potyviruses is a multifunctional protein mainly involved in viral synergistic activities and movement. Inoculation of DNA A with PVX-HC-Pro resulted in severe leaf curling in *N. benthamiana* suggesting a synergistic interaction between potyvirus and MYLCV where the movement of DNA A of MYLCV was supported by HC-Pro of ZYMV. The data suggests that the defective DNA B of MYLCV is complemented by the potyvirus in cucurbits and that this dual infection results in the severe disease phenotype. The synergistic interaction between an RNA virus and a DNA virus suggests a novel mechanism to avoid host defense and complementation of movement by an unrelated viral protein.

The strategy to engineer a broad based resistance against the disease complex was based on breaking the synergism between these viruses by targeting ZYMV using RNA interference (RNAi) technology. The novel RNAi approach targeted HC-Pro and coat protein (CP) genes of ZYMV with a single construct. Both transient assays in wild type and transgenic *N. benthamiana* showed successful resistance and blocking of synergism between viruses. Application of the technology in melons may provide a durable solution to this important disease complex that is devastating melon crops in Pakistan.
Chapter 1

1.1.1. Introduction

Pakistan is the western-most country of south Asia. With a population of about 160 million and a total area of 796,100 km² population density exceeds 160 people/km². Major crops grown in Pakistan include wheat, cotton, rice, sugarcane, fodder, maize, tobacco, vegetables, and annual fruits. Much of Pakistan is classified as arid to semi-arid with a tropical or subtropical climate. Pakistan experiences four distinct seasons: winter (December-February), spring (March-April), summer (May-September), and autumn (October-November). During the spring and autumn seasons, daily temperatures do not exceed 10-25°C, while winters are even colder, and temperatures fall to single digit figures with occasional frost. Summers are considerably warmer with day temperatures between 40 and 45°C. In the mountainous areas, subzero temperatures are common during the winter, while summer temperatures are mild, hardly exceeding 25°C. The coastal areas are characterized by a lack of extreme temperature variation. Most vegetables are usually grown either in autumn or spring to avoid winter and summer or early summer extremes. Deviations occur due to the varied and milder climates of various regions. Vegetable production in Pakistan is well diversified in terms of the range of vegetable species grown. More than 36 species are grown and consumed as summer or winter vegetables. The major vegetable species grown are potatoes, onions, chili, tomato, and cucurbits.

Cucurbit crops, including summer squash (Cucurbita pepo), bottle gourd (Lagenaria siceraria), cucumber (Cucumis sativus), sponge gourd (Luffia acutangula), bitter gourd (Momordica charantia) snake/serpent gourd (Cucumis melo var. flexuosus), ash gourd (Benincasa carifera), Indian squash (Citrullus vulgaris var. fistulosis), long melon (Cucumis melo var fleruosus), muskmelon (Cucumis melo), red gourd (Cucurbita moschata), watermelon (Citrullus lanatus) and wax gourd (Benincasa hispida) produced throughout Pakistan, are important summer and winter vegetables. The members of family Cucurbitaceae are mostly prostrate or climbing herbaceous annuals comprising about 90 genera and 700 species that are further characterized by commonly having 5-angled stems and coiled tendrils. The leaves are alternate and usually palmately 5-lobed or divided; stipules are absent (Judson, 1935).
Introduction

Muskmelon is so named because of the delightful odor of the ripe fruits. Musk is a Persian word for a kind of perfume; melon is French, from the Latin *melopepo*, meaning "apple-shaped melon" and derived from Greek words of similar meaning. Muskmelon is native to Persia (Iran) and adjacent areas to the west and the east. Persia and the trans-Caucasus are believed to be the main center of origin and development, with a secondary center including the northwest provinces of India, also Kashmir and Afghanistan. Although truly wild forms of *C. melo* have not been found, several related wild species have been noted in those regions.

Muskmelon collection spread westward over the Mediterranean area in the middle ages and was apparently common in Spain by the 15th century. Columbus carried seeds of it on his second voyage and had them planted on Isabela Island in 1494. This was doubtless its first collection in the New World. At about the same time Charles VIII of France reputedly introduced muskmelons into central and northern Europe from Rome.

1.1.2. Factors responsible for low muskmelon production

Diagnosis of plant diseases at the right time is the most important in crop management practices. Delay causes disaster thus hitting the producer. Viruses belonging to unrelated species are major threats for cucurbit production (Kassanis, 1963; Falk and Bruening, 1994). Many studies have been carried out to understand and solve the issues related to these viral diseases (Rochow, 1972; Wang et al., 2002). Almost 35 different viruses have been reported to infect plants of the *Cucurbitaceae* family (Provvidenti, 1996). Usually severe diseases of cucurbits are associated with multiple infections where two or more unrelated viruses cause very severe disease and the phenomenon is known as synergism. There have been more than several reports of combinations in which a potyvirus is one member of the double infection (Rochow and Ross, 1955; Damirdagh and Ross, 1967; Bourdin and Lecoq, 1994; Lathman and Jones, 2001; Fattouh, 2003). Besides RNA viruses, cucurbits are also affected by whitefly-transmitted geminiviruses that have emerged as major pathogens on food and fiber crops throughout the world.

Among cucurbit infecting viruses *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* 2 (WMV-2), *Watermelon mosaic virus* 1
(WMV-1), ZYMV, Tobacco ringspot virus (TRSV), Tomato ringspot virus (TmRSV), Clover yellow vein virus (CYVV). Among DNA viruses Squash leaf curl virus (SqLCV), Watermelon chlorotic stunt virus (WmCSV) and Tomato leaf curl New Delhi virus (ToLCNDV) are important.

1.2. Viruses

A virus can be defined as “any of various simple submicroscopic parasites of plants, animals, and bacteria that often cause disease and that consist essentially of a core of RNA or DNA surrounded by a protein coat”. Unable to replicate without a host cell, viruses are typically not considered living organisms.

Viruses, like bacteria, are found everywhere. They are, however, much smaller. Before the germ theory of disease was established, people thought that diseases were caused by poisons and, since the Latin term for poison is "virus," that was the name adopted. Pasteur often referred to bacteria as viruses. Then, as research showed that microorganisms were the actual cause of some infectious diseases, various pathogenic microbes were identified and removed from the category of poisons (or viruses). Since viruses do not propagate in an artificial culture medium, observing them did not take place until the "golden age of microbiology," beginning in the late 1800s. By this time, the term "virus" had become permanently associated with these agents, and the original meaning essentially lost. By the late 1940s, procedures had been developed that allowed identification of 35 viruses associated with human diseases. During the following 15-20 years, 500 more were added to the list. The list continues to grow.

Virus particles are not cells and cannot carry out functions of their own. Their genetic material can be either DNA or RNA, occurring as either single stranded (ss) or a double stranded (ds) molecules. Some simpler viruses contain only a single molecule of nucleic acid and a protein coat. Other viruses may possess an envelope over a protein coat, while still others may have internal proteins and/or small projections called peplomers (Hull, 2001).

Most viruses, regardless of the types of hosts they infect, have similar functions. The outermost part has a "code" by which the virus is able to recognize which cells to enter and infect. This explains why so few viruses cause disease in different species
and why viruses are organ specific (Khan and Dijkstra, 2006). For example, hepatitis viruses target liver cells, Human immuno deficiency virus (HIV) looks for particular binding sites or markers on white blood cells, viruses responsible for the common cold target cells in the respiratory tract (Alcabes *et al*., 1994), Herpes simplex virus I (HSV) attacks the skin and mucous membranes of the mouth and lips, and Herpes simplex virus II (HSV) infect similar cells of the genitalia (Smith *et al*., 2001).

1.3. Plant viruses
Plant viruses, like all other viruses, are obligate intracellular parasites that do not have the molecular machinery to replicate without the host. The plant viruses are defined as viruses pathogenic to higher plants. The first virus discovered was Tobacco mosaic virus (TMV). Plant viruses have been classified into 73 genera, of which 49 have been classed into families. On the basis of type of genome plant viruses can also be classified as DNA and RNA virus (Khan and Dijkstra, 2006).

1.3.1 History of plant viruses
Martinus Beijerinck discovered the first plant virus causing disease, in 1898. After this there was need to classify any other known viral diseases based on the mode of transmission even though microscopic observations proved fruitless. In 1939 Holmes published a classification list of 129 plant viruses. This was expanded and in 1999 there were 977 officially recognised and some provisional plant virus species. Wendell Stanley first performed the purification of TMV (Stanley 1935), for which he was awarded with the Nobel Prize in Chemistry in 1946. Purification of infectious RNA in the 1950s proved that RNA is a carrier of genetic information to code for the production of new infectious particles. These days there is focus on diversity of viruses on large scale, virus-host interaction, the manipulation and modification of plant virus genomes to define gene function and to provide novel forms of resistance.

1.4 Classification of viruses
According to the current classification viruses are divided into three orders, 73 families, 9 subfamilies, 287 genera, 1950 species. Plants viruses are divided into following families (8th report of international committee on texonomy of viruses).
1.4.1 Caulimoviridae
All the members of this family replicate by reverse transcription. These viruses have circular dsDNA genomes with gaps/discontinuities at specific sites; one in one strand and between one and three in other strand. These sites play an important role in DNA synthesis during replication. There are six genera in this family falling into two groups: caulimoviruses and badnaviruses that have bacilliform particles. The genera are distinguished on the basis of genome organisation (Brunt et al., 1996).

1.4.2 Geminiviridae
The family Geminiviridae includes viruses that infect a large variety of plant species, both monocots and dicots. They are characterized by the geminate morphology of the virus particle (18–30 nm) and genetically by a genome consisting of one or two circular, single-stranded DNA (ssDNA) molecules. This family of viruses will be dealt with in more detail in section 1.9

1.4.3 Nanoviridae
The family Nanoviridae consists of aphid transmitted viruses with isometric virions, approximately 18–20 nm in size, and is divided into two genera; Babavirus, of which Banana bunchy top virus (BBTV) is the type member (Burns et al., 1994), and Nanovirus which contains Subterranean clover stunt virus (SCSV), Faba bean necrotic yellows virus (FBNYV) and Milk vetch dwarf virus (MDV). The status of a virus described as Coconut foliar decay virus remains uncertain, since at this time only a single component of this virus has been described (Aronson et al., 2000). It has been suggested that the component may, in fact, be a satellite-like molecule associated with a geminivirus (Briddon and Stanley 2006), although no evidence to support this contention is available at this time.

1.4.4 Reoviridae
The family Reoviridae comprises the genera of viruses that infect vertebrates, invertebrates and plants. Some members are restricted to vertebrates. Those that infect plants also infect their invertebrate vector. The virus particles are complex made up of one, two or three distinct shells each with an icosahedral symmetry. The genome comprises 10, 11 or 12 segments of linear dsRNA depending upon the genus. There are three genera that infect plants which are distinguished based on their
Introduction

structure and the number of segments into which their genomes are divided (Boccardo and Milne, 1984).

1.4.5 Partitiviridae
Members of the family Partitiviridae have small isometric non-enveloped virions 30-40 nm in diameter with dsRNA genomes divided into two segments, the smaller encoding the coat protein and larger the RNA-dependent RNA-polymerase (RdRP). Viruses of two of the genera of this family infect fungi and two infect plants. The plant infecting viruses are known as cryptic viruses since they cause no or very few symptoms. They are not graft transmitted and have no biological vector but are transmitted efficiently through seeds (Dallwitz, 1980).

1.4.6 Rhabdoviridae
Rhabdoviruses are bullet shaped or bacilliform particles enveloped in a membrane. On the inner surface of the membrane is a layer of protein that contains helically arranged nucleocapsid which is made up of (-) strand RNA (White and Fenner, 1994). These viruses infect vertebrates, invertebrates and plants. The plant-infecting rhabdoviruses are transmitted by insects in a circulative, propagative manner. The most common vectors are leafhoppers, planthoppers and aphids.

1.4.7 Bunyaviridae
This is a large family of viruses which infect both vertebrates and invertebrates. None of the viruses of this genus infect plants or their invertebrate vectors. The virions are spherical or pleomorphic with surface glycoprotein spikes embedded in a lipid bilayer envelope. Within this envelope the genome, comprising of three RNA species, is associated with a nucleoprotein forming a nucleocapsid (Elliott, 1996). The RNAs are either negative-sense or have their coding regions in an ambisense arrangement. These viruses are transmitted by thrips in a circulative and propagative manner.

1.4.8 Bromoviridae
This is a family of plant RNA viruses with a wide host range in crops and horticultural species. All viruses are readily transmitted by mechanical means and some by insects or via pollen. There are four genera: Alfamovirus, Bromovirus, Cucumovirus, and Ilarvirus. This family is characterised by its isometric or quasi-
isometric particles and its single-stranded, positive-sense RNA in three genomic and one subgenomic segment (Palukaitis et al., 1992). For more detail see section 1.7

1.4.9 Comoviridae

Virions are isometric with T=1 icosahedral symmetry. The capsids are made up of one or two coat protein species and encapsidate genomes comprising two positive sense RNA species. The RNAs are expressed as a polyprotein that is processed to give functional proteins (Ritzenthaler et al., 1991). Members of this family are transmitted by beetles and aphids.

1.4.10 Potyviridae

The potyviruses comprise the largest group of viruses that collectively infect most crop plants and many other wild plant species. The viruses typically have an RNA genome of approximately 10 kb that encodes a single polyprotein. This is cleaved into functional polypeptides by self-encoded proteases. These viruses are mostly aphid-transmitted in a non-persistant manner. For more detail see section 1.6

1.4.11 Tombusviridae

Viruses in this family have isometric icosahedral particles, 32-35 nm in diameter, with well a defined structure. Members of this family are divided into eight genera. The particles contain a single species of positive-sense ssRNA with a size ranging from 3.7-4.7 kb. The genome organization differs between genera in this family. The unifying feature of this family is the presence of a highly conserved RNA-dependent RNA polymerase that is interrupted by an in-frame termination codon that is periodically suppressed. Individual members have a narrow host range and can infect monocotyledonous or dicotyledonous species, but not both. These viruses are stable and are found in the open environment.

1.4.12 Sequiviridae

Members of this family have isometric particles, 30 nm in diameter, that contain positive-sense ssRNA 9-12 kb in size. The virions are made up of three protein species of approximately 32, 26 and 23 kDa in equimolar amounts. The RNA encodes a polyprotein that is cleaved to give functional proteins. Members of the family are
Introduction

divided into two genera. The viruses are transmitted by leafhoppers and aphids (Reddick et al., 1997).

1.4.13 Closteroviridae
Members of the family Closteroviridae have flexuous filamentous particles about 12 nm in diameter, the length being characteristic of the genus. The family is divided into two genera. The genomic nucleic acid is linear, positive-sense ssRNA that may be monopartite or bipartite depending on the genus. The host range of individual species is usually narrow. The viruses are usually phloem-limited and usually cause yellowing symptoms or pitting or grooving of woody stems (Dolja et al., 2006). Different genera are transmitted by aphid, whitefly and mealybug.

1.4.14 Luteoviridae
The viruses in this family have isometric icosahedral (T=3) particles 25-30 nm in diameter that are hexagonal in outline and comprise 180 subunits of a single protein species of 21-23 kDa. The particles contain a single molecule of positive-sense ssRNA 5.7-5.9 kb in size. Genera are distinguished on the basis of genome organization. Many members of this family are phloem-limited, causing yellow type disease; hence the name luteoviruses form the Latin “luteus” meaning yellow. Transmission of the viruses is by aphids in a specific, circulative, non-propagative manner. Members of the family also assist the transmission of other viruses (Gray and Gildow, 2003).

1.5 Transmission of plant viruses
1.5.1 Sap transmission of viruses
Generally TMV, PVX and CMV and some geminiviruses are transmitted via sap. It implies direct transfer of sap by contact of a wounded plant with a healthy one. This may occur during agricultural practices by tools, hands, or by animals feeding on infected plants.

1.5.2 Virus transmission by insects
Most often insects are the vector for transmission of plant viruses. The insect vector of a plant virus will often be the determining factor in the host range of the virus: it can only infect plants that the insect vector feeds upon. Plant viruses are also classified as
persistent, semi-persistent and non-persistent. In non-persistent transmission, viruses become attached to the distal tip of the stylet of the insect and on the next plant it feeds, it inoculates it into the plant. In case of semi-persistent viral transmission virus enters the foregut of the insect. In the persistent mode of transmission viruses manage to pass through the gut into the haemolymph and then to the salivary glands.

1.5.3 Virus transmission by nematodes
Soil-borne nematodes acquire and transmit viruses by feeding on infected roots. There have been no examples of replication of viruses in nematodes. These can be transmitted persistently or non-persistently. Plant viruses transmitted by nematodes can be divided into two taxonomic groups and have a worldwide distribution. Thirty eight nepoviruses and three tobraviruses have been reported to be transmitted by nematodes (Harrison and Robinson, 1981; Williamson and Gleason, 2003).

1.5.4 Virus transmission by plasmodiophorids
Soil-borne zoosporic prototzoa are responsible for persistent or non-persistent transmission of a number of plant viruses. Plasmodiophorid-transmitted viruses are positive-strand RNA viruses belonging to five genera. An example is *Polymyxa graminis*, which has been shown to transmit plant viral diseases in cereal crops. *Beet necrotic yellow vein virus* (BNYVV) is the causal agent of rhizomania and in transmitted by *P. betae* (Lemaire, et al., 1988).

1.5.5 Seed and pollen transmission of viruses
About 20% of plant viruses are transmitted from generation to generation by infecting the seed or pollen. When viruses are transmitted by seeds, the seed is infected in the generative cells and the virus is maintained in the germ cells and sometimes, but less often, in the seed coat. There does not seem to be a correlation between the location of the seed on the plant and its chances of being infected. It is environmentally influenced and that seed transmission occurs because of a direct invasion of the embryo via the ovule or by an indirect route with an attack on the embryo mediated by infected gametes. These processes can occur concurrently or separately depending on the host plant (Mink, 1993). Many plants species can be infected by seed transmissible viruses including, but not limited to the families *Leguminosae, Roseaceae, Curcurbitaceae* and *Gramineae*. *Rice yellow mottle virus* (RYMV) in
soyabees, CMV in pulses and, *Parsley latent virus* (PLV) in parsley are transmitted by seed.

1.6 *Potyviridae*

The potyviruses (*Potyviridae*) comprise the largest group of viruses that collectively infect most crop plants and many other wild plant species. It contains 218 definite and tentative species (Burger *et al.*, 2005). The viruses typically have an RNA genome of approximately 10 kb that encodes a single polyprotein. This is cleaved into functional polypeptides by self-encoded proteases. The genomes consist of single-stranded, linear RNA which is 11-15 nm in diameter and 650-950 nm in length. The genome is monopartite. Potyviruses are aphid transmitted (non-persistent) and are rarely seed transmitted.

Cytopathologically all the members of the family form crystalline cytoplasmic inclusions (CI) within infected cells. Some members also induce the formation of crystalline nuclear inclusions (NI) that consist of two nuclear proteins, Nla and Nlb (Shukla *et al.*, 1998).

1.6.1 Classification of the family *potyviridae*

The family *Potyviridae* is divided into six genera according to genome composition and vector specificity (Table 1.1) (Burger *et al.*, 2005). Earlier, it was divided into four genera Potyvirus, Rymovirus, Bymovirus and Ipomovirus depending on vector transmission (aphids, mites, fungi amd whiteflies) (Barnett, 1992). The new genera are distinguished based on genome organization, transmission, and nucleotide sequence.
### Table 1.1: Classification of family *Potyviridae*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type species</th>
<th>Genome</th>
<th>Vector</th>
<th>Number of species</th>
<th>Definite</th>
<th>Tentative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potyvirus</td>
<td><em>Potato virus Y</em></td>
<td>M</td>
<td>Aphid (np)</td>
<td></td>
<td>111</td>
<td>86</td>
<td>197</td>
</tr>
<tr>
<td>Ipomovirus</td>
<td><em>Sweet potato mild mottle virus</em></td>
<td>M</td>
<td>Whitefly (np)</td>
<td></td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Macluravirus</td>
<td><em>Maclura mosaic virus</em></td>
<td>M</td>
<td>Aphid (np)</td>
<td></td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Rymovirus</td>
<td><em>Rye grass mosaic virus</em></td>
<td>M</td>
<td>Mite (pc)</td>
<td></td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Tritemovirus</td>
<td><em>Wheat streak mosaic virus</em></td>
<td>M</td>
<td>Mite (pc)</td>
<td></td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Bymovirus</td>
<td><em>Barley yellow mosaic virus</em></td>
<td>B</td>
<td>Fungus (z)</td>
<td></td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>129</td>
<td>89</td>
<td>218</td>
</tr>
</tbody>
</table>

* M: monopartite; np: non persistant; pc: persistant circulative; z: zoospore

**Fig 1.1** Genome organization of the RNA genome (9395nt) of ZYMV which is expressed as a ployprotein (3080aa) and is cleaved into ten putative functional proteins by three virus encoded proteases.
1.6.2 Zucchini yellow mosaic virus (ZYMV)

ZYMV was first described in Europe (Lecoq et al., 1981; Lisa et al., 1981), after the other cucurbit infecting potyvirus Papaya ringspot virus type W (PRSV-W). In a few years (1981-88) the virus was identified in most cucurbit-growing areas, possibly indicating that the late identification was due to limited laboratory tools and not due to the emergence of a new virus (Desbiez and Lecoq, 1997). ZYMV causes a severe disease in cucurbit crops including melon (Cucumis melo L.), cucumber (Cucumis sativus L.), watermelon (Citrullus lanatus Schad.), squash (Cucurbita pepo L.) and pumpkin (Cucurbita maxima L., C. moschata L.). Early infection of all the host species generally causes a total yield loss (Blue and Perring, 1989; Walkey et al., 1992). Recovery phenomena following infection have not been reported and few sources of resistance are available. Transgenic resistance and cross-protection are the current methods for the control of ZYMV (Dasgupta et al., 2003).

1.6.2.2 Genome organization of ZYMV

Members of family Potyviridae have a single-stranded, positive-sense RNA genome. Out of six genera, the first five contain viruses with monopartite genomes. Bymoviruses have bipartite genomes with components named RNA-1 and RNA-2 (Shukla et al., 1998). The genome organizations of viruses of monopartite genera are quite similar to one another. They have a genome ~10 kb in length characterized by a 5’ untranslated region (5’UTR), a single ORF and a 3’UTR region terminated by a polyA tail. The major ORF encodes a large poly-protein that is co-translationally processed into 10 functional proteins (Adams et al., 2005). In descending order (5’-3’) these proteins are P1, HC-Pro, P3, 6K1, cylindrical inclusion protein (CLl), 6 K2, VPg (viral protein genome linked), Nla, Nlb and CP (Shukla et al., 1998) (Fig. 1.1).

1.6.2.3 Functions of potyvirus genes

The P1 coding sequences are the most variable part of the genome with the exception of C-terminal region (Adams et al., 2005b). P1 is the serine proteinase that self-cleaves the HC-Pro junction at a conserved YS or FS motif (Adams et al., 2005a; Yang et al., 1998). The region responsible for this activity was identified at the C-terminus of P1 with the catalytic triad H-(X7-11)-D-(X30-36)-S. The D residue of this triad is replaced by E for potyviruses of the Bean common mosaic virus ( BCMV) subgroup (Adams et al., 2005a). P1 binds non-specifically to RNA and it has been
suggested that P1 may be involved in viral movement (Brantley and Hunt, 1993). This was supported by the finding that P1 is localized in association with C1 in the cytoplasm (Arbatova et al., 1998). The fusion of P1 and HC-Pro enhances viral pathogenicity by suppression of post-transcriptional gene silencing (PTGS) in the hosts (Kasschau and Carrington, 1998). Maki-Valkama et al. (2000) showed that the mechanism and strain specificity of the resistance in plants transformed with the PVY P1 gene was based on PTGS.

HC-Pro is a multifunctional protein required for acquisition of virus by the vector, systemic and cell-to-cell movement and suppression of PTGS. When testing the virus transmission efficiency of four aphid species, Wang et al., (1998) found that some aphid species transmitted virus more efficiently than others. They showed that the food canal of aphids species differ in their ability to interact with HC-Pro, which, therefore, affected the ability of aphids to retain virions in the stylets. By mutation analysis, Blanc et al., (1998) determined that the N-terminal region of the HC-Pro, which contains the highly conserved K(I/L)(T/S)C motif (known as KITC motif), is required for interaction of HC-Pro with aphid mouth parts. Similarly, a PKT motif in the core region of HC-Pro was identified as important for virion binding (Peng et al., 1998).

Cronin et al. (1995) showed that a mutant in the highly conserved CC/CS motif located in the core region of Tobacco etch virus (TEV) HC-Pro was not capable of systemic movement. Systemic movement was restored, however, in transgenic plants provided with the intact HC-Pro. HC-Pro was shown to pass from cell-to-cell, to increase the size exclusion limit (SEL) of plasmodesmata, and therefore facilitate the passage of viral RNA between the cells. The region responsible for this activity was identified in the C-terminal part of the HC-Pro (Rojas et al., 1997). Kasschau et al. (1997) used mutation analysis to show that the central region of the TEV HC-Pro, that contains an IGN motif, is important for viral amplification. This hypothesis has been supported by the result of Urcuqui-Inchima et al. (2000) who showed that the independent domains, designated A and B, which confer the binding of HC-Pro to RNA, are located in the central region of HC-Pro.
Mallory et al. (2001) demonstrated that expression of HC-Pro in transgenic plants suppressed PTGS at a step before accumulation of small RNAs. The central region of HC-Pro has cysteine proteinase-like activity required for auto cleavage between HC-Pro and P3 at its C-terminus. Carrington and Herdon (1992) demonstrated the cleavage site between HC-Pro and P3 in TEV is G763-G764 and four amino acids surrounding this site were important for recognition by HC-Pro. The cleavage site G-G was conserved among all members of the family, with the exception of bymoviruses (Adams et al., 2005b).

P3 and P1 are two important variable proteins in the family Potyviridae (Adams et al., 2005b). P3 is also the least characterized potyvirus protein (Urcuqui-Inchima et al., 2001). However, P3 has been shown to have a role in pathogenicity by interaction with other viral proteins. For instance, the C-terminal region of the P3-6K1 complex carries a pathogenicity determinant in Plum pox virus (PPV) (Saenz et al., 2000). Similarly, Suehiro et al. (2000) showed that Turnip mosaic virus (TuMV) contains an important determinant in the P3 C-terminal region, which confers the ability of virus to infect different hosts.

Cylindrical inclusion protein (CI) is a major component of the replication complex. The CI protein belongs to the “Super family 2” of helicase proteins that are characterized by seven conserved segments (Kadare and Haeni, 1997). These fragments occupy the N-terminal half of the protein and have NTP binding, NTPase, RNA binding and RNA helicase activities (Fernandez and Garcia, 1996; Fernandez et al., 1997; Fernandez et al., 1995). Because replication of potyviruses requires a polymerase, a primer and a helicase to separate dsRNA templates, CI was considered the major component of a multi-component, membrane-associated replication complex of CI, VPg/NLa, and Nib (Shukla et al., 1998). In this case CI can unwind RNA duplexes with 3’ overhang in the 3’ to 5’ direction (Fernandez et al., 1995; Fernandez et al., 1997).

Although CI is not a true movement protein like CP or HC-Pro (Rojas et al., 1997), the presence of ATPase activity at plasmodesmata of Maize dwarf mosaic virus (MDMV) infected cells suggested cell-to-cell movement requires energy released from ATP hydrolysis (Chen et al., 1994). Therefore, since CI is the only viru-
encoded protein that has ATPase activity, it may participate in this process. While 6K1, in conjunction with P3, carries a determinant for the pathogenicity as mentioned before it was proposed that 6k2 is required for genome replication because it anchors the replication apparatus to the endoplasmic reticulum (Schaad et al., 1997).

The genome linked viral protein (VPg) is the N-terminal part of NIa and, apart from the CP, is the only viral protein present on virions and covalently linked to the 5’ end of viral RNA via a tyrosine residue (Murphy et al., 1991). The role of VPg in viral replication was shown indirectly in Tobacco vein mottling virus (TVMV) by mutation of the tyrosine residue (Tyr1860) that link Vpg to the viral RNA. The mutant virus did not accumulate to detectable levels in infected plants and was not infectious in protoplasts (Murphy et al., 1996). In a recent study, Anindya et al., (2005) showed that the VPg tyrosine 66 of Pepper vein banding virus (PVBV) was uridylated by NIb, and the uridylated VPg might function as a primer for viral RNA synthesis. A study based on chimeric TEV genome (Schaad et al., 1997) suggested that VPg interacts either directly or indirectly with host components to facilitate long distance movement. Dunoyer et al. (2004) indicated a cellular factor, potyvirus VPg-interacting protein (PVIP) that interacts with VPg N-terminal region of a diverse range of potyviruses. The interaction affected systemic symptoms involving both cell-to-cell and systemic movement in infected plants. VPg was reported to interact with plant translational initiation factors, such as eIF4E and eIF(iso)4E (Leonard et al., 2000, Leonard et al., 2004; Wittmann et al., 1997). However, the direct role of this interaction in potyvirus translation remains unknown because the VPg was not required for efficient cap-independent translation of TuMV (Basso et al., 1994; Niepel and Gallie, 1999). Several recessive genes for resistance to potyvirus have been identified in plants including pvr1 (pepper), mol (lettuce), sbm1 (pea) and rym4/5 (barley). These genes (with different alleles) encode the translational initiation factor, eIF4E. This property of VPg was identified based on observations that the resistance genes, in the homozygous state, containing point mutations which interrupted the interaction of eIF4E and VPg, created resistance phenotypes at different levels (viral accumulation, cell-to-cell and long distance movement) (Kang et al., 2005).
The major N-terminal region of NIa harbors the VPg, whereas the C-terminal region is a major trypsin-like protease (NIa-Pro) that cleaves the junction of P3/6K1, 6KI/CI, CI/6K2, 6K2/VPg, VPg/NIa-Pro, Nla-Pro/Nlb and Nlb/CP. The cleavage motif for this protease is V-xx-Q(E)-(ASGE or V) (Shukla et al., 1998; Adams et al., 2005a). Large nuclear protein (Nlb) is an RNA-dependent RNA -polymerase (RdRp) and this function was determined in TVMV for which Nlb has poly(U) polymerase activity and was able to utilize full-length TVMV RNA as a template for RNA synthesis. In addition, the mutation of the highly conserved GDD motif, which is present in many other viral RdRps, significantly reduced the polymerase activity of the TVMV Nlb (Hong and Hunt, 1996). The uridylation activity of Nlb has also been demonstrated recently in PVMV (Anindya et al., 2005).

The coat protein is a well characterized potyviral protein and is roughly divided into three domains: the N-terminal domain is highly variable and contains the major virus specific epitopes, whereas the core and C-terminal domains are more conserved. The variation in the core domain is similar to that of whole genome and, therefore, is a reliable index for genetic relatedness (Shukla et al., 1998). The CP N-terminal region is exposed on the virion surface and contains the highly conserved DAG motif located near the N-terminus. Site-directed mutagenesis analysis showed that the motif is essential for aphid transmission (Atreya et al., 1995). However, the context in which the DAG or equivalent motif is found is also important for efficient transmission (Lopez-Moya et al., 1999). A specific interaction between CP and HC-Pro with the involvement of the DAG and KITC motif in each component, respectively, was essential for aphid transmission (Blanc et al., 1997; Flasinski and Cassidy, 1998).

Dolja et al. (1994, 1995) used mutation analysis to show that the N-and C-terminal regions of the CP of TEV were indispensable for systemic viral movement, while the core region was essential for cell-to-cell movement. In contrast, Arazi et al. (2001) showed that the deletion or substitution with foreign peptides encoding up to 33 amino acids of the N-terminal region of the coat protein did not alter systemic infectivity of ZYMV. This finding was later supported by Kimalov et al. (2004) who showed that the maintenance of coat protein N-terminal neutralized net charge, but not primary sequence, was essential for ZYMV systemic infectivity. It was shown that CP (and HC-Pro as well) are two movement proteins that are able to increase the size
exclusion limit (SEL) of plasmodesmata and, therefore, facilitate cell-to-cell virus movement (Rojas et al., 1997). Apparently, CP and HC-Pro co-ordinate accumulation and movement (Andrejeva et al., 1999). The mechanism of assembly of flexuous viruses, such as potyviruses, is not completely understood. The CP of PVY subunits, in the absence of the viral RNA and under suitable conditions, self assemble to form 16 S disk or ring-like intermediates made up of 7-8 subunits, which then form non-helical virus-like particles (McDonald et al., 1976). The role of the CP N-and C-terminal regions in particle assembly are not defined. Two lines of evidence suggest that these two regions are not necessary for assembly. Firstly, the N-and C-termini are known to be surface exposed and could be removed by trypsin treatment without affecting reassembly of CP subunits (Shukla et al., 1998). Secondly, mutation analysis showed that the core region of CP is indispensable for this function, but not the N-and C-termini (Dolja et al., 1995; Dolja et al., 1994; Voloudakis et al., 2004).

However, recent studies showed both regions are required for assembly (Anindya and Savithri, 2003; Kang et al., 2006). The interaction between the CP and the NIb through the GDD motif of NIb (Hong et al., 1995) suggested that the CP may be involved in regulation of RNA synthesis. Based on mutation analysis, Mahajan et al. (1996) identified that the CP coding sequences appeared to stimulate genome amplification through two distinct mechanisms; (i) translation continues until 138 and 189 of the TEV CP-coding sequence and (ii) one or more signals (at the RNA level) located between codons 211-246 of the TEV CP might control viral RNA replication in a cis-acting manner. These signals appeared to be involved in a series of stem loop structures in this region, as confirmed later by Haldeman-Cahill et al., (1998).
### Table 1.2. Classification of family *Bromoviridae*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type species</th>
<th>Genome</th>
<th>Vector</th>
<th>Number of species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bromovirus</strong></td>
<td><em>Brome mosaic virus</em></td>
<td>T*</td>
<td>Beetles</td>
<td>6</td>
</tr>
<tr>
<td><strong>Alfamovirus</strong></td>
<td><em>Alfa alfa mosaic virus</em></td>
<td>T</td>
<td>Aphids</td>
<td>1</td>
</tr>
<tr>
<td><strong>Ilarvirus</strong></td>
<td><em>Tobacco streak virus</em></td>
<td>T</td>
<td>Pollen/seed</td>
<td>17</td>
</tr>
<tr>
<td><strong>Cucumovirus</strong></td>
<td><em>Cucumber mosaic virus</em></td>
<td>T</td>
<td>Aphids</td>
<td>3</td>
</tr>
<tr>
<td><strong>Oleavirus</strong></td>
<td><em>Olive latent virus 2</em></td>
<td>T</td>
<td>unknown</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>28</strong></td>
</tr>
</tbody>
</table>

* T = Tripartite
1.7 Family **Bromoviridae**

Members of this family have isometric particles with T=3 icosahedral symmetry, 25-35 nm in diameter, or bacilliform particles whose symmetry is based upon the icosahedron. The genomes of linear, positive-sense ssRNA are divided between three molecules. The subgenomic RNA for coat protein is often also encapsidated. Several members also encapsidate satellite or defective interfering RNAs. The family is divided into five genera as detailed below.

1.7.1 **Cucumoviruses**

The genus *Cucumovirus* contains three species, *Tomato aspermy virus* (TAV), *Peanut stunt virus* (PSV) and *Cucumber mosaic virus* (CMV). Based on serological and nucleic acid hybridization analyses, strains of CMV are further classified into two major subgroups (I and II) (Piazzolla *et al.*, 1979). Recent data showed that viruses from the same subgroup share 92±98% sequence similarity and those from different subgroups share 71±79% sequence similarity (Palukaitis *et al.*, 1992).

1.7.1.1 **Cucumber mosaic virus** (CMV)

*Cucumber mosaic virus* (CMV), type species of the genus *Cucumovirus* is recognized as a threat to the health of many crops. In the last decade, CMV has been identified as the causal agent of several disease epidemics. CMV is a multi-component virus for which the inoculation of 3 types of particles is required to infect plants. The three genomic segments of CMV are linear plus-sense, ssRNA molecules that are encapsidated in isometric particles. The numerous strains of CMV have been classified into two major subgroups (I and II) by serological relationships and nucleotide sequence similarity. Subgroup I has further been divided into two groups (IA and IB) by phylogenetic analyses. CMV is readily transmitted in a stylet-borne, non-persistent manner by more than 80 species of aphids. It has a host range of more than 1000 species of plants. Both serological and molecular techniques have successfully been applied to CMV detection and diagnosis. Cucumber mosaic, first described in 1916 (Doolittle, 1916), was one of the earliest plant diseases attributed to a virus (Jagger, 1916). Reports of the disease soon came from elsewhere in the USA, and later from Europe and Africa (Price, 1934) and other parts of the world. In the early days the tools for determining the presence of specific viruses were limited, and
as many as 40 different plant diseases were later shown to be caused by CMV (Kaper and Waterworth, 1981).

**1.7.1.2 Physical properties of CMV**
The virus particles are about 29 nm in diameter, and are composed of 180 subunits (T=3 icosahedral symmetry). The particles sediments with an S value of approximately 98. The virions contain 18% RNA, and are highly labile, relying on RNA-protein interactions for their integrity. The three genomic RNAs, designated RNA 1 (3.3 kb in length), RNA 2 (3.0 kb) and RNA 3 (2.2 kb) are packaged in individual particles. A subgenomic RNA, RNA 4 (1.0 kb), is packaged with the genomic RNA 3, making all the particles roughly equivalent in composition. In some strains an additional subgenomic RNA, RNA 4A is also encapsidated at low levels. The genomic RNAs are single-stranded, plus-sense RNAs with 5’ cap structures, and 3’ conserved regions that can be folded into tRNA-like structures.

CMV encodes five proteins that are distributed on the three genomic RNAs. RNA 1 is the only monocistronic RNA, encoding the 1a protein which is required for replication of virus and it also contains methyl transferase and helicase motifs (Kadare and Haenni, 1997; Rozanov *et al.*, 1992). RNA 2 encodes the 2a protein which is the viral polymerase (Ishihama and Barbier, 1994; O’Reilly and Kao, 1998), and the 2b protein, expressed from a low abundance sub-genomic RNA, RNA 4A (Ding *et al.*, 1994). The 2b ORF is overprinted on the carboxyl terminal portion of the 2a ORF. This ORF is only found in cucumoviruses, although an ORF in a similar position is found in *Tobacco streak virus*, a member of the Ilarvirus genus. The 2b protein of subgroup II CMV strains was shown to inhibit hosts PTGS (Beclin *et al.*, 1998; Brigneti *et al.*, 1998). RNA 3 is reported to encode the movement protein (MP) expressed from the 5’ ORF and the coat protein (CP) expressed from the subgenomic RNA 4. Both are required for virus movement (Canto *et al.*, 1997). CMV can support satellite RNAs (satRNAs) that in many cases dramatically alter the symptom phenotype induced by the virus. The CMV satRNAs do not encode proteins but rely on the RNA for their biological activity.
1.7.1.3 Host range of CMV

CMV has the widest host range of any known plant virus and is distributed worldwide. The host range includes a large number of horticultural crops, temperate pulses pasture and forage legumes and weed species. CMV causes severe disease in lentils, chickpeas and lupins. In areas where large aphid populations occur, crop losses can be high due to reduced herbage production and seed yield. The virus is seed transmitted in many pulse species.

1.8 Synergistic properties of plant viruses

In plants, viral synergisms occur when one virus enhances infection by a distinct or unrelated virus. Such synergisms may be unidirectional or mutualistic but, in either case, synergism implies that protein(s) from one virus can enhance infection by another. When synergisms are asymmetric, the two viruses are often referred to as the ‘helper’ and the ‘dependent’ viruses (Malyschenko et al., 1989). Viruses belonging to unrelated species are major threats for cucurbit production (Kassanis, 1963; Falk and Bruening, 1994). Many studies have been carried out to understand and solve the issues related to these viral diseases (Rochow, 1972; Wang et al., 2002). Thirty five different viruses have been reported to infect plants of the Cucurbitaceae family (Provvidenti, 1996). Usually severe diseases on cucurbits are associated with multiple infections where two or more unrelated viruses cause very severe disease and the phenomenon is known as synergism. There have been several reports of combinations in which potyviruses were one member of the double infection (Rochow and Ross, 1955; Damirdagh and Ross, 1967; Bourdin and Lecoq, 1994; Lathman and Jones, 2001, Fattouh, 2003). As described earlier, potyviruses such as ZYMV and WMV are positive-sense RNA viruses and contain a monopartite genome that encodes a single polypeptide that is cleaved into 9 or 10 mature proteins (Revers et al., 1999). This cleavage is performed by the HC-Pro. HC-Pro was recognized as an indispensable helper factor for virus host-to-host transmission, activity to release it from the precursor polypeptide, enhancer of infectivity, genome amplification and is indispensable for systemic and cell-to-cell movement in plants (Thornbury et al., 1985, Yang et al., 1998, German-Retana et al., 2000). The HC-Pro is a suppressor of PTGS and also mediates accumulation of unrelated viruses by suppression of RNA silencing (Anandalakshmi et al., 1998). The central domain of HC-Pro is required for both movement and replication of the potyvirus (Cronin et al., 1995) and the same
domain is also required for the synergistic interaction with PVX. Mutations within the coding region of the central domain of TEV HC-Pro eliminate the ability of the sequence to mediate PVX-potyviral synergistic disease in transgenic plants (Shi et al., 1997). Furthermore, mutations within the central region result in a premature shutdown of TEV RNA amplification, suggesting that HC-Pro may function to prolong potyviral RNA replication (Kasschau et al., 1997).

The best characterized example of plant viral synergism is the interaction between the potyvirus PVY and PVX in tobacco (Rochow and Ross, 1955; Goodman and Ross, 1974a, 1974b; Vance, 1991). Plants mechanically inoculated with both viruses developed synergistic disease, which was characterized initially by severe vein clearing and then necrosis of the first systemically infected leaf, with a three- to 10-fold increase in the level of PVX compared with singly infected plants. A similar synergistic response was induced in coinfections of PVX with several other potyviruses, including the TVMV and TEV (Vance et al., 1995). Vance et al., (1995) showed that PVX-potyviral synergistic disease did not require replication of the potyviral genomic RNA and that the response was mediated by expression of potyviral sequences in the 5' proximal one-third of the potyviral genome. This region of the potyviral genome includes the viral genomic 5' untranslated region (UTR) as well as the coding region for the N-terminal portion of the viral polyprotein, including P1, the helper component proteinase (HC-Pro), and approximately one-quarter of P3 (termed P1/HC-Pro sequence). Expression of the TEV P1/HC-Pro sequence also enhanced the pathogenicity and accumulation of both TMV and CMV, two unrelated plant viruses (Pruss et al., 1997), that cause synergistic disease in conjunction with a potyvirus (Pio-Ribeiro et al., 1978; Clark et al., 1980; Poolpol and Inouye, 1986). Pruss et al., (1997) also showed that in protoplasts, the potyviral sequence prolonged both the accumulation of PVX negative strand RNA and the expression of a reporter gene from a PVX subgenomic promoter, suggesting that it acted, at least in part, by transactivation of viral RNA replication. Synergism at the level of replication between Cymbidium mosaic potexvirus and Odontoglossum ringspot toboamovirus have been studied using protoplasts as tools (Hu et al., 1998). Synergistic reactions were observed by Elena et al., (2006) in mixed infections in tomato plants doubly infected with the positive-sense ssRNA crinivirus (Family Clostroviridae) Tomato chlorosis virus (ToCV) and the negative-sense ssRNA tospovirus Tomato spotted wilt virus.
Introduction

(TSWV). Synergism in a tomato cultivar susceptible to both viruses resulted in a rapid death of plants. More remarkable was the synergism observed in tomato cultivars which carry the Sw-5 resistance gene, which are resistant to TSWV. Pre-infection with ToCV resulted in susceptibility to TSWV, whereas co-inoculation did not. Co-infection of Sweet potato chlorotic stunt virus (SPCSV, genus Crinivirus) with Sweet potato feathery mottle virus (SPFMV, genus Potyvirus) results in sweet potato virus disease (SPVD), a synergistic disease that is widely distributed in the sweet potato (Ipomoea batatas) growing regions of the world (Milton et al., 2007). Data from this study showed that SPCSV, but not SPFMV, can cause synergistic diseases in sweet potato with all viruses tested, including members of the genus Potyvirus (Sweet potato latent virus, Sweet potato mild speckling virus), Ipomovirus (Sweet potato mild mottle virus), CMV, and putative members of the genus Carlavirus (Sweet potato chlorotic fleck virus and C-6 virus). Three distinct aphid-transmitted viruses associated with a yellowing disease of sugarbeet were examined in single and mixed infections and severe stunting, as measured by fresh plant biomass, was observed with mixed infections with Beet yellows virus (BYV) and Beet mosaic virus (BtMV), compared to single infections of these viruses (Wintermantel, 2005). Infection of maize with Wheat streak mosaic virus (WSMV) and the machlomovirus Maïze chlorotic mottle virus (MCMV) produces corn lethal necrosis disease (CLND). More recently, Stinger et al., (2007) reported that complete deletion of the WSMV helper component-proteinase (HC-Pro) coding region had no effect on induction of CLND symptoms following co-inoculation of maize with WSMV and MCMV. In this infection elevation of virus titers in double infections, relative to single infections, also was independent of WSMV HC-Pro. Thus, unlike potyvirus HC-Pro, WSMV HC-Pro was dispensable for disease synergism and may not be a suppressor of PTGS because WSMV HC-Pro did not suppress PTGS of a green fluorescent protein (GFP) transgene in an Agrobacterium-mediated coinfiltration assay in which potyvirus HC-Pro acted as a strong suppressor.

The first example in which synergism was reported between two DNA viruses was between two begomoviruses that infect cassava in Africa (Harrison et al., 1997). Molecular evidence for synergism between two geminiviruses ACMV and EACMV, in which cassava plants co-infected by the two viruses developed more severe symptoms in the field and experimentally, compared with plants infected by either
virus alone was reported by Fondong et al. (2000). Similarly, Vanitharani et al., (2004) reported that the severe cassava mosaic disease associated with synergism between the Cameroon strain of ACMV and EACMCV is a two-way process involving the DNA A components of both viruses in a double infection. In transient expression of ACMV-(CM) AC4 driven by the CaMV 35S promoter enhanced EACMCV DNA accumulation by 8-fold in protoplasts, while 35S driven TrAP of EACMCV enhanced ACMV-(CM) DNA accumulation, also by 8-fold.

Synergism between ACMV and EACMV results in enhanced accumulation of both viruses. This is in contrast to potyvirus-mediated synergism in which only the level of non-potyviral member increases (Rochow and Ross, 1955; Vance, 1991). More recently, there have been experimental examples of synergism between viruses across viral families enhancing tissue infiltration of the begomovirus *Abutilon mosaic virus* (AbMV) mediated by CMV (Wege and Siegmund, 2007).

### 1.9 Geminiviruses

Geminiviruses are plant viruses first described by Goodman in 1977 (Goodman, 1977a; Goodman, 1977b). They are characterized by the unique geminate shape of the fused icosahedral viral particle. They may be monopartite or bipartite (having one or two circular single-stranded DNA molecules) (Stanley et al., 2005). Geminiviruses are a major constraint to agricultural productivity in all tropical and subtropical regions of the world. Recently, they have spread into more temperate regions because of changes in agricultural practices and ecological conditions, as well as the global trade in agricultural products, which all encourage dissemination of the insect vectors. In many areas, geminiviruses are serious pathogens of economically important crops. The greatest constraint is on production of important crops such as cassava, beans, mungbeans, peppers, tomatoes and cotton (Mansoor et al., 1993; Hameed et al., 1994; Brown, 1994; Brown and Bird, 1992; Green and Kallo, 1994). The infection can produce leaf mottling that interferes with photosynthesis, decreasing yield of starchy foods such as cassava and disrupts flower and fruit formation in crops such as tomato, pepper and cotton (Moffat, 1999).
1.9.1 Structure and taxonomy of geminiviruses

Geminiviruses have a unique geminate (twinned) virion morphology and are 18x20 nm and have circular ssDNA genomes that replicate through dsDNA intermediates in infected cells (Lazarowitz and Lazdins, 1991; Noris et al., 1996). The genome size is approximately 2.5-5.6 kb.

The geminivirus group was established by the International Committee on the Taxonomy of Viruses (ICTV) in 1978 and upgraded to the family Geminiviridae in 1995. Geminiviruses are widely distributed plant viruses infecting everything from monocots, such as maize, or dicots, such as cassava and tomato (Hanley-Bowdoin et al., 1999). The Geminiviridae family consists of four genera that differ with respect to insect vector, host range, and genome structure (Rybicki, 1994; Briddon and Markham, 1995; Padidam et al., 1995a). The four genera of geminiviruses are Mastrevirus, Curtovirus, Begomovirus, and Topocuvirus.

1.9.1.1 Mastrevirus

In the genus Mastrevirus, Maize streak virus (MSV) and Wheat dwarf virus (WDV) are two well-studied members. Genome size is 2.6-2.8 kb. The genus Mastrevirus includes leafhopper-transmitted viruses with monopartite genomes that infect either monocot or dicot plants (Buck, 1999). The monopartite genome of mastrevirus contains a long and a short intergenic region (LIR and SIR, respectively) located opposite to each other on the genome. The LIR contains the origin of replication (ori) for virion-strand DNA synthesis similar to that of begomoviruses. The SIR contains the ori for the complementary strand synthesis and a short ssDNA sequence (70-80 nts). This primer-like sequence is annealed to the encapsidated genomic ssDNA and is thought to prime the minus-strand synthesis. The genome of mastreviruses encodes four proteins, Rep and RepA on the complementary-strands, MP and CP on virion sense strand. While RepA is produced from the unspliced transcripts containing the C1 ORF, Rep is expressed from the spliced transcripts with fused C1 and C2 ORFs. The spliced sequence contains signals typical of plant introns (Boulton, 2000; Gutierrez, 2002; Gutierrez et al., 2004; Hanley-Bowdoin et al., 2000; Palmer and Rybicki, 1998).
1.9.1.2 Curtovirus
Curtoviruses are leafhopper-transmitted viruses with monopartite genomes that infect dicots. The genomes of curtoviruses consist of one circular single-stranded DNA molecule of 2.9-3.0 kb (Buck, 1999). The genome organization is similar to that of monopartite begomovirus except for one extra protein (V2 protein) on the virion sense strand which is involved in the regulation of the level of ss/dsDNA (Stanley et al., 2005). Curtovirus infections are considered to be phloem-limited and viral replication, gene expression and virion formation occur in the nucleus (Esau, 1977; Latham et al., 1997). In the genus Curtovirus, Beet curly top virus (BCTV) is a well-studied example. (Fig. 1.2)

1.9.1.3 Begomovirus
These are whitefly-transmitted viruses and infect dicotyledonous plants. The genomes of most begomoviruses consists of two circular single-stranded DNAs, DNA A and DNA B, each 2.5-2.8 in size (Buck, 1999). *Bean golden yellow mosaic virus* (BGYMV) is a well studied example of this genus and from this the name of the genus was coined. Begomoviruses can be further divided into two subsets according to their geographical distribution (Howarth and Vandemark, 1989). Thus the viruses occurring in the New World (American and the Caribbean), such as BGMV and TGMV are distinct from those occurring from rest of the world such as ACMV and TYLCV (Briddon et al., 2001). Recently another entirely new group of begomoviruses, that are associated with both DNA β and DNA 1 (as described in section 1.13.1), has been identified. Such virus complexes are widespread and are associated with monopartite begomoviruses infections of large number of plants in the Old World (Mansoor et al., 1999; Mansoor et al., 2000; Mansoor et al., 2001; Briddon et al., 2001).

1.9.1.4 Topocuvirus
Topocuviruses are dicot infecting, treehopper-transmitted with monopartite genomes. *Tomato pseudo-curly top virus* is the only known member (Briddon et al., 1996). The genome organization of topocoviruses is similar to that of monopartite begomoviruses.
**Fig.1.2:** Genome organization of geminiviruses. Arrows indicate the direction of ORFs in relation to hairpin structure. The complementary-sense genes are designated C1-C4 while the virion-sense genes are named from V1-V3. The sequences common to DNA A and DNA B in begomoviruses called the common region (CR) is shown by a dark box. Begomoviruses are transmitted by the whitefly *Bemisia tabaci* and can be monopartite or bipartite. Monopartite begomoviruses lack DNA B component and originate only from the Old World. New World begomoviruses lack the AV2 gene. Mastreviruses and curtoviruses are transmitted by leafhoppers and are monopartite. Topocuviruses are monopartite and are transmitted by treehoppers. The photo on the right shows (from top to bottom) *B. tabaci*, *Cicadulina mbila*, *Micratalis malleifera* and *Circulliter tenellus* vectors of the begomovirus, the *African streak virus* TPCTV and *curtoviruses* respectively. (Courtesy by Dr. Peter Markham and Dr. Ian Bedford, John Inns Center, Norwich, UK)
Introduction

Begomovirus DNA A

Begomovirus DNA B

Mastreviruses

Topocovirus

curtoviruses
Table 1.3: Classification of *geminiviridae*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type member</th>
<th>Host</th>
<th>Insect vector</th>
<th>Genome</th>
<th>No. of species</th>
<th>Definite</th>
<th>Tentative</th>
<th>Total</th>
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<tr>
<td><em>Mastrevirus</em></td>
<td><em>Maize streak virus</em> (MSV)</td>
<td>Monocots/</td>
<td>Leafhopper</td>
<td>Monopartite</td>
<td>11</td>
<td>6</td>
<td>17</td>
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<td><em>Curtovirus</em></td>
<td><em>Beet curly top virus</em> (BCTV)</td>
<td>Dicots</td>
<td>Leafhopper</td>
<td>Monopartite</td>
<td>5</td>
<td>1</td>
<td>6</td>
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<tr>
<td><em>Begomovirus</em></td>
<td><em>Bean golden yellow mosaic virus</em> (BGMV)</td>
<td>Dicots</td>
<td>Whitefly</td>
<td>Bipartite/monopartite</td>
<td>132</td>
<td>53</td>
<td>183</td>
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<tr>
<td><em>Topocuvirus</em></td>
<td><em>Tomato pseudo curly top virus</em> (TPCTV)</td>
<td>Dicots</td>
<td>Treehopper</td>
<td>Monopartite</td>
<td>1</td>
<td>0</td>
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1.9.2. Genome arrangement of begomoviruses

Most begomoviruses have bipartite genomes, the components of which are designated DNA A and B (Stanley and Gay, 1983; Howarth et al., 1985; Frischmuth et al., 1990). DNA A encodes the capsid protein, proteins required for viral DNA replication and the control of viral gene expression (Townsend et al., 1986; Etessami et al., 1989). DNA B encodes proteins required for nuclear trafficking, cell-to-cell movement of viral DNA and symptom development (Von Arnim and Stanley, 1992; Sanderfoot et al., 1996; Bisaro, 1996). The DNA A contains six open reading frames (ORFs), four (replication associated protein [Rep], transcriptional activator [TrAP], replication enhancer protein [REn], and AC4) on the complementary-sense strand and two, coat protein (CP) and AV2, on the virion-sense strand (Fig 1.2). Rep and REn are involved in replication since mutations of Rep blocked viral replication, whereas a REn protein mutant greatly reduced DNA levels and resulted in severely delayed and attenuated symptoms (Elmer et al., 1988; Etessami et al., 1988; Etessami et al., 1989; Morris et al., 1991; Sung and Coutts, 1995). The AV2 is absent in New World begomoviruses.

The DNA B encodes two genes, the movement protein (MP) on the complementary-strand has a role in cell-to-cell movement (Noueiry et al., 1994; Ward et al., 1997) and NSP on the virion-strand binds and transports ssDNA across the nuclear envelope (Pascal et al., 1994; Sanderfoot et al., 1996).

The genomes of some begomoviruses from the Old World consist of a single DNA component (Dry et al., 1993; Kheyr-Pour et al., 1991; Mansoor et al., 1999; Navot et al., 1991; Noris et al., 1994). For these viruses, the arrangement of complementary and viron sense genes are identical to those on the DNA A component of bipartite begomoviruses.

There are several examples of monopartite begomoviruses that are experimentally infectious to host plants but are unable to develop disease symptoms in the hosts from which they were isolated. These include Ageratum yellow vein virus from Singapore and Cotton leaf curl Multan virus from Pakistan (Tan et al., 1995; Briddon et al., 2000). This group of begomoviruses has been shown to be associated with circular, ssDNA satellite and satellite-like molecules that are half the length of a begomovirus component. The first of these is a satellite-like molecule, termed DNA 1, which
resembles components encoding Rep of nanoviruses, a second group of plant-infected viruses with circular ssDNA genomes (Mansoor et al., 1999; Aronson et al., 2002). This molecule plays no part in symptom induction. The second molecule, the satellite DNA β, is required for the development of symptoms of both AYVV and CLCuV in the hosts from which they were isolated, Ageratum conyzoides and cotton respectively (Saunders et al., 2000; Briddon et al., 2001).

1.9.3 Functions of geminivirus proteins

1.9.3.1 Replication associated protein (Rep)

Rep is present in nuclei of infected plant cells (Nagar et al., 1995; Nagar et al., 2002), where it plays a key role in geminivirus DNA replication and transcription (Laufs et al., 1995a). Rep confers origin of replication recognition (Choi and Stenger, 1995; Jupin et al., 1995; Gladfelter et al., 1997) and initiates plus-strand DNA replication (Heyraud-Nitschke et al., 1995; Laufs et al., 1995a; Orozco and Hanley-Bowdoin, 1996; Chatterji, et al., 2001). Several biochemical activities have been demonstrated for Rep. It cleaves and ligates viral plus-strand DNA within the loop of the hairpin motif (Laufs et al., 1995a). Rep recognizes its cognate DNA ori in a sequence and site-specific manner, and this process involves iteron sequences downstream of stem loop structure (Fontes et al., 1994). Although the natural substrate for Rep binding in vivo is dsDNA, Rep binds to ssDNA in vitro (Fontes et al., 1994). The rep domain responsible for cleavage activity mapped to the first 211 amino acids of TYLCV Rep (Heyraud-Nitschke et al., 1995) and the first 120 amino acids in TGMV (Orozco et al., 1997). This sequence contains three motifs conserved among the Reps of all geminiviruses (Laufs et al., 1995). Motif L (FLTY) has an unknown function, Motif II (HLH) is a putative metal ion binding site and motif III contains a highly conserved Y residue that is essential for cleavage and joining activities (Laufs et al., 1995, Orozco et al., 1997). The protein binds with retinoblastoma-like plant proteins involved in cell cycle regulation and thus has essential functions in virus replication and gene expression (Collin et al., 1996). Rep binds with retinoblastoma-related proteins to prevent cell entry into S phase by sequestering transcription factors (Collin et al., 1996). Rep directs the replication complex to the origin of replication (Fontes et al., 1992; Thommes et al., 1993). It is also reported to be responsible for separation of replicated genomes into unit-length circular monomers for the production of progeny viruses by nuclease and ligase activity (Koonin and Ilyana, 1992; Heyraud-Nitschke
et al., 1995). Settlage et al., (1996) showed that the Rep of TGMV and BGMV formed oligomers. They demonstrated that this oligomerization occurred in a virus non-specific manner since Rep of two viruses complexed with each other and the addition of heterologous Rep had no effect on the efficiency of replication. The Rep domain responsible for oligomerization was mapped between amino acid 120 and 181 in TGMV.

1.9.3.2 Transcriptional activator protein (TrAP)
TrAP is a nuclear protein (Sanderfoot and Lazarowitz, 1995) that transactivates virion-sense gene expression (Sunter and Bisaro, 1991; Sunter et al., 1994). This function of TrAP is in the case of bipartite begomoviruses and curtoviruses while in the case of mastreviruses this function is provided by Rep (Collin et al., 1996). Various experiments established that activation is at the level of transcription (Sunter and Bisaro, 1992). For example, in transgenic plants containing CP promoter-reporter fusions, TrAP activated the promoter in mesophyll cells and de-repressed it in phloem tissue (Sunter and Bisaro, 1997). Similarly, ACMV infection activated a transgene that was under the control of the coat protein promoter (Hong et al., 1997). Noris et al. (1996) studied in vitro binding activity of TrAP of TYLCV. The protein binds both ssDNA and dsDNA but preferably binds ssDNA. Binding activity of TrAP was sequence independent and might be unrelated to its transactivation activity. It has been suggested that geminivirus TrAP may use a similar mechanism to activate virion-sense transcription to that used by Herpes simplex virus vp16 and Adenovirus EIA protein. These are potent transcriptional activators and do not bind DNA. Instead, they are recruited through protein interaction to promoters where they interact with basal transcriptional apparatus to activate RNA synthesis (Liu et al., 1997). TrAP also has been shown to regulate transcription of host genes (Trinks, et al., 2005). These results suggested that silencing suppression and transcription activation by TrAP are functionally connected and that some of the TrAP-inducible host genes discovered may code for components of an endogenous network that controls silencing. Wang et al. (2005) showed TrAP to be an effective silencing suppressor and showed that TGMV and BCTV TrAP can suppress RNA silencing directed against a GFP reporter gene when silencing is induced by a construct expressing an inverted repeat of GFP RNA (dsGFP). More recently, TrAP was shown to suppress silencing indirectly by activating the expression of a cellular protein that may function
as an endogenous negative regulator of the system. Also TrAP participates in an indirect suppression mechanism involving metabolic inhibition of siRNA-directed transmethylation, which could interfere with epigenetic modification of the viral genome (Bisaro, 2006). Hussain et al. (2007) demonstrated the role of TrAP in inhibiting HR, the first such activity identified for a plant-infecting virus. Analysis of all ToLCNDV-encoded genes pinpointed the transcriptional activator protein (TrAP) as the factor mediating the anti-HR effect. Deletion mutagenesis showed the central region of TrAP, containing a zinc finger domain and nuclear localization signal, to be important in inhibiting the HR. More recently, Gopal et al. (2007) showed that TrAP of Bhendi yellow vein mosaic virus (BYVMV) is involved in transactivation and only mildly in suppression of gene silencing of monopartite begomoviruses viruses and not in transmission.

1.9.3.3 Replication enhancer protein (REn)
The REn protein greatly enhances viral DNA accumulation of curtoviruses and begomoviruses (Elmer et al., 1988a; Sunter et al., 1990) by interacting with Rep (Settlage et al., 1996). Earlier, it was observed that REn protein is located in nuclei of infected plant cells at levels similar to Rep (Nagar et al., 1995) suggesting that it might act with Rep during initiation of viral DNA replication. Experimental observations suggested that REn protein might increase the affinity of Rep for the origin. For mastreviruses it is possible that their unique Rep A protein translated from the unspliced C1 ORF might serve a similar function, since mastreviruses do not encode REn (Laufs et al., 1995b; Orozco and Hanley-Bowdoin, 1996). Later on Settlage et al. (2001) showed the interaction of REn and Rep with pRBR, a plant homolog of the retinoblastoma tumor suppressor protein (pRb). REn protein of TLCV interacts with SINAC1, a member of the NAC domain protein family from tomato and enhances virus replication by inducing SINAC1 expression specifically in infected cells. SINAC1 is co-localized with REn to the nucleus and activated transcription of a reporter gene in yeast, suggesting that in healthy cells it function as a transcription factor. In a transient ToLCV replication system, over-expression of SINAC1 resulted in a substantial increase in viral DNA accumulation (Selth, et al., 2005).
1.9.3.4 AC4/C4 protein

AC4/C4 is encoded by dicot infecting geminiviruses. Mutation analysis of C4 of BCTV has shown that this protein is involved in symptom development. Stanley and Latham, (1992a) introduced stop codons at two different locations in the C4 gene without affecting the amino acid sequence of the overlapping Rep. When inoculated onto N. benthamiana, the mutant produced stunting and yellowing of leaves and downward leaf curling but not vein swelling and upward curling, which are characteristic symptoms produced by the wild type virus. The level of viral DNA of mutant virus was similar to the wild type virus. The mutant caused symptomless infection in Beta vulgaris, although the levels of viral DNA often reached those of wild type virus. These results suggested that C4 is the major determinant of pathogenesis of the virus. The expression of C4 protein in transgenic N. benthamiana produced virus-like symptoms and further confirmed its role in symptom development (Latham et al., 1997).

Mutation analysis of this ORF has shown that it is involved in symptom development in monopartite begomoviruses (Rigden et al., 1994; Jupin et al., 1994). Agro-inoculation of a mutant of ToLCV produced drastically reduced symptoms, although the level of viral DNA was similar to the wild-type virus and suggests that C4 is not required by ToLCV to replicate or to spread through the host plant but is involved in symptom development (Rigden et al., 1994). Mutation of AC4 in the bipartite geminiviruses, ACMV and TGMV, resulted in wild type symptoms and no role could be ascribed to this ORF (Etessami et al., 1989; Elmer et al., 1988b).

Now a measure of clarification has been achieved by the discovery that AC4 can suppress RNA silencing, allowing it to enhance disease and promote viral invasiveness. The suppression activity of AC4 from four different cassava-infecting geminiviruses was tested in the Agrobacterium-based transient assay in N. benthamiana 16c plants (Vanitharani et al., 2004). Two of the AC4 proteins, from viruses associated with recovery-type symptoms in cassava, showed suppressor activity with increased accumulation of GFP mRNA and inhibition of GFP-specific siRNAs. Two other AC4 proteins, from non-recovery-type viruses, showed little or no activity in this assay. Thus, the TrAP proteins of the non-recovery viruses were effective silencing suppressors, while those from recovery-type viruses were less
effective. Chellappan, et al., 2005 provided direct evidence that AC4 is a unique virus-encoded, PTGS suppressor protein that binds to and presumably inactivates mature miRNAs and thus blocks the normal miRNA-mediated regulation of target mRNAs, resulting in developmental defects in Arabidopsis. More recently, the results of Gopal et al. (2007) showed that C4 of Bhendi yellow mosaic virus (BYVMV), is also a strong suppressor of PTGS.

1.9.3.5 Coat protein (AV1)

In contrast to bipartite begomoviruses, monopartite geminiviruses (Boulton et al., 1989; Briddon et al., 1989; Liu et al., 1998; Rigden et al., 1993) need the coat protein (CP) and the product of a second gene preceding the CP gene for viral spread (Kotlizky et al., 2000; Rojas et al., 2001). Mutation of the CP gene lead to a decreased level of viral DNA in the infected plants while the level of viral DNA was not affected in protoplast, suggesting impairment of movement function (Boulton et al., 1991). The localization of the product of this ORF in secondary plasmodesmata with the onset of viral lesions is consistent with its role in the movement of monopartite geminiviruses (Dickinson et al., 1996). More recently, a protein closely related to a family of plant reversibly glycosylated peptides, designated SIUPTG1 and CP protein of ToLCV were found interacting in yeast and in vitro. Expression of SIUPTG1 in a transient ToLCV replication assay increased the accumulation of viral DNA. This data provided new insights into the role of CP in ToLCV infection and reveal another host pathway that geminiviruses may manipulate to achieve an efficient infection (Selth, et al., 2006). Work on mutants of ACMV which do not produce CP indicated that encapcidation is not required for infection of plants following mechanical inoculation (Stanley and Townsend, 1986; Ward et al., 1988). While whiteflies are unable to acquire such mutants. CP of BCTV has been shown to be essential for infectivity (Briddon et al., 1989) as have the CP of other leafhopper-transmitted geminiviruses, MSV (Boulton et al., 1989; Lazarowitz et al., 1989) and Wheat dwarf virus (WDV) (Woolston et al., 1989). Briddon et al. (1990) replaced the CP of DNA A of ACMV with that of BCTV and when co-inoculated with DNA B of ACMV on N. benthamiana produced the typical symptoms of virus infection. Moreover, when expressed under ACMV CP promoter, synthesized protein encapsidated both genomic components and the BCTV leafhopper vector (Circulifer tenelus) transmitted both components of the chimeric virus which showed that the
specificity for the vector resides on the coat protein. The most important function of CP is to form the shell in which genomic ssDNA is encapsidated. A study based upon MSV, using cryo-electron microscopy and three dimensional reconstruction (Zhang et al., 2001), revealed that geminivirus particles are assembled from 110 protein subunits, organized as 22 pentameric capsomers forming two abutting incomplete T=1 icosahedra joined together. Assembly and stability of the geminivirus particles relies on interaction between CP molecules.

Because geminiviruses replicate in the nucleus of the infected host cells, following their inoculation into cytoplasm by the vector, the virus need to be transported into the nucleus for replication. Although it is not clear if geminivirus enter the nucleus in the form of intact virions or as nucleoprotein complexes, the presence of only the viral CP in the nucleus following initial cellular entry suggest it may be involved in the nuclear import of viral DNA. The trafficking of the viral DNA-protein complex between the nucleoplasm and protoplasm occurs through a complex structure called nuclear pore complex (NPC) and is mediated by host transport receptors known as karyopherins that link to virus associated proteins and then become associated with the NPC. To be recognized by host receptors, these virus associated proteins must contain nuclear localization signals (NLS) (Gafni and Epel, 2002). Such signals have been determined for both monopartite and bipartite geminivirus and are mainly located in the N-terminal region of the CP: 63 amino acids for TYLCV (Kunik et al., 1998), 5-22 amini acids for MSV (Liu et al., 1999) and 54 amino acids for ACMV (Unseld et al., 2001). For ACMV, two other domains containing NLS, which are located in the central (100-127 amino acids) and C-terminal (201-258 amino acids) regions were also determined (Unseld et al., 2001).

The CP of geminivirus also participates in exporting the viral genome from the nucleus to the cytoplasm. In this case the nuclear export signals (NES) is required for recognition by a host receptor. A NES signal located in the C-terminal half of the TYLCV CP has been identified (Rhee et al., 2000).

1.9.3.6 Precoat protein (AV2/V2)

In contrast to New World begomoviruses, Old World begomoviruses have AV2/V2 and this is involved in the movement of monopartite viruses but its function for
bipartite begomoviruses is not fully understood (Rothenstein et al., 2006). Padidam et al. (1996) showed by mutation analysis that AV2 is also involved in the movement of bipartite geminiviruses. Rothenstein et al. (2007) analyzed viral intercellular transport in further detail. GFP was fused to AV2 and expressed from replicating viruses or from plasmids, AV2:GFP became associated with the cell periphery in punctate spots, formed cytoplasmic as well as nuclear inclusion bodies, the later as conspicuous paired globules. Upon particle bombardment of expression plasmids, AV2:GFP was transported into neighboring cells of epidermal tissues showing that the intercellular transport of the AV2 protein is not restricted to the phloem. The absence of this gene in begomoviruses from the New World has been believed to consistent with the fact that all begomoviruses from the New World are bipartite in nature and suggest an evolutionary divergence of these viruses from begomoviruses of the Old World. But the analysis of the \textit{Corchorus yellow vein virus} (CoYVV), a bipartite begomovirus infecting Jute mallow (\textit{Corchorus capsularis}, \textit{Tilliaceae}) in Vietnam showed the absence of AV2 ORF. Evidence is provided that CoYVV is probably indigenous to the region and may be the remnant of a previous population of New World begomoviruses in the Old World (Ha et al., 2006). Due to the overlapping nature of ORFs AV1 and AV2, the possibility that the mutation in mutV2 could affect CP expression resulted in no down regulation of CP (Bull et al., 2007). Rojas et al. (2001) investigated the properties of proteins (CP, V1, and C4) potentially involved with movement of the monopartite begomovirus. The \textit{TYLCV} V1 localized around the nucleus, at the cell periphery and colocalized with the endoplasmic reticulum. Thus, the V1 may be analogs of the bipartite begomovirus BC1 that have evolved to mediate \textit{TYLCV} movement within phloem tissue.

1.9.3.7. \textbf{Nuclear shuttle protein (NSP)}

Pascal et al. (1994) provided direct evidence that the NSP binds strongly to single-stranded nucleic acid with high affinity and localized to the cell nucleus. These results suggest that NSP is most likely to be involved in the nuclear transport of the viral genome. Affinity of NSP for ssDNA and specifically localization to the nucleus was also described by Sanderfoot and Lazarowitz (1995). NSP is a very basic protein and contains two nuclear localization signals (NLSs). A mutation in either of the two potential NLSs severely impaired or eliminated viral infectivity (Sanderfoot et al.,
1996). Mutational analysis suggests that the N-terminal sequences of the protein are involved in nuclear localization while C-terminal sequences are required for interaction with movement protein (Sanderfoot et al., 1996). More recently, expression of ToLCNDV NSP from a PVX vector in N. benthamiana and Lycopersicon esculentum resulted in hypersensitive response (HR), demonstrating that NSP of ToLCNDV NSP is a pathogenicity determinant and is a target of host defense responses in these hosts (Hussain, et al., 2005).

1.9.3.8 Movement protein (MP)
Movement protein (MP) is involved in virus movement from cell-to-cell through plasmodesmata as well as symptom development. Noueiry et al., (1994) used a technique of injecting of fluorescently-labeled movement protein into cells to determine the function of the protein and showed that MP binds dsDNA and moves extensively through plasmodesmata. These results showed that MP is a plasmodesmatal movement protein and mediates the movement of dsDNA (Sudarshana et al., 1998).

In a related study, Pascal et al., (1993) expressed both MP and NSP of Squash leaf curl virus (SqLCV) in transgenic plants. Their results showed that the expression of MP alone is sufficient to cause mosaic and leaf curl symptoms, typical of SqLCV infection. These results suggest a role of MP in symptom development for some bipartite begomoviruses. MP is also a determinant of pathogenicity and host range in bipartite begomoviruses (Sanderfoot et al., 1995; Duan et al., 1997; Hou et al., 2000; Ingham et al., 1995). MP may also have a role in transmission of bipartite geminiviruses (Liu et al., 1997). In a recent study, it was revealed that MP promotes redirection of the NSP of AbMV to the plasma membrane in fission yeast (Frischmuth et al., 2007).

1.10 Transmission of geminiviruses
Begomoviruses are transmitted by the sweet potato whitefly, Bemisia tabaci (Gennadius). B. tabaci was first described in 1889 (Gennadius, 1889), and was first reported as a pest in 1919 in India (Husain and Trehan, 1933). It has caused millions of dollars worth of crop losses in tropical and subtropical regions of the world (Brown, 1994). B. tabaci has a very wide host range, consisting of 500 species in 74
plant families (Greathead, 1986). This whitefly species is a vector of viruses in the Geminiviridae, Potyviridae and Comoviridae families and the genera Carlavirus and Closterovirus.

Over 100 begomoviruses are transmitted by at least two biotypes of whitefly to more than 20 cultivated species of socioeconomic importance. The main food crops affected by whitefly transmitted geminiviruses are cotton, common bean, mungbean, blackgram, lima bean, soybean, cowpea, tomato, potato, eggplant, pepper, chili peppers, melon, watermelon, squash, okra and cassava (Muniypapa, 1980; Brown 1994; Seal et al., 2006). Geminivirus transmission by B. tabaci is circulative and non-propagative (Duffus, 1987). Whiteflies can acquire and inoculate bipartite begomoviruses in short periods of time (10 min), but the efficiency of acquisition increases when the feeding period increases up to 24 h. Latent periods of four to 21 h between virus acquisition and the ability of the whitefly to transmit have been observed (Duffus, 1995). Studies of the transmission of TYLCV, a monopartite begomovirus, showed that whitefly feeding periods of 4hrs or longer were necessary to achieve TYLCV transmission rates near to 90% (Zeidan and Czescnek, 1991).

Hunter et al. (1998) established the location of Tomato mottle virus (ToMoV) and Cabbage leaf curl virus (CaLCuV) in various tissues of B. tabaci B biotype by immunofluorescent labeling of viral coat protein in freshly dissected whiteflies. They proposed a model for the movement of begomoviruses in the whitefly vector and described virus particles are ingested along with plant fluids into the whitefly oesophagus and foregut, after which nutrients and begomoviruses are concentrated in the filter chamber. Begomovirus particles adsorb to specific sites on the alimentary membrane or to sites along the anterior region of the midgut. Begomovirus particles move out of these tissues into the hemolymph, eventually invading the salivary glands. Curtoviruses are transmitted by the beetle leafhopper, Circulifer tenellus (Baker). The region associated with vector transmission was identified with in positions 124-174 (Hofer et al., 1997). The mutation in this region altered the virus transmission by the vector by either preventing particle assembly or inhibiting the passage of virus from gut to haemocoel or from haemocoel to the silivery gland of the vector (Harrison et al., 2002) Soto et al., (2005) described the N-terminus of Beet mild curly top virus (BMCTV) involved in virus movement through the leafhopper,
possibly receptor-mediated endocytosis in the gut or salivary glands. Another region of the BCTV CP, residues 179–191, was postulated to be an adaptation for leafhopper transmission (Bottcher et al., 2004). Whereas for TYLCV, residues for virus transmission were in the central region and correlated with virion formation (Noris et al., 1998). More detail of the role of the CP in virus transmission is given in section 1.9.3.5.

1.11 Movement of geminiviruses

Plant viruses have to overcome the barrier of the plant cell wall to move locally (cell-to-cell) within an infected leaf and enter the phloem, through which they will move to invade the entire plant. They accomplish this feat by encoding a unique class of proteins termed “movement proteins”. Movement proteins determine the host range and disease potential of the virus.

It has been suggested that MPs may participate in the establishment of membrane associated replication complexes, affect the intracellular distribution and modification of the properties of plasmodesmal pores to allow cell-to-cell spread of infection in case of plant viruses other than geminiviruses (Aaziz et al., 2001; Tzfira et al., 2000; Reichel and Beachy, 1998). Recent results suggest that movement proteins may also block a gene silencing signal to avoid host defenses (Voinnet, 2005). Recognized MPs fall into four superfamilies: the products of the triple gene block of potexviruses and related viruses, the tymoviral MPs, a series of small polypeptides (less than 10 kDa) encoded by carmo-like viruses and some geminiviruses and the ‘30K’ superfamily related to the 30 kDa TMV MP (Melcher, 2000).

The establishment of a virus infection depends upon the spread of the virus throughout the host plant. The movement of the virus in the plant occurs at two different levels. Firstly short distance cell-to-cell movement, secondly long distance movement that involves delivery of the virus to distal parts of the plant by the vascular system (Lazarowitz, 1992). The bipartite begomoviruses encode two movement proteins, MP and NSP.

Unlike the bipartite geminiviruses (Lazarowitz, 1992; Sanderfoot and Lazarowitz, 1995), the monopartite geminiviruses including MSV, encodes only one MP (Lazarowitz et al., 1988; Mullineaux et al., 1988) and the CP functions as the nuclear
localization protein. Although MSV MP is thought to be associated with plasmodesmata (Dickinson et al., 1996), and has been shown to move from cell-to-cell as an MP–GFP fusion (Kotlitzky et al., 2000), the role of MSV MP in the cell-to-cell movement of virus or viral DNA is unclear (Huanting et al., 2001).

Sanderfoot and Lazarowitz, (1995) proposed for bipartite begomoviruses that the NSP of SqLCV binds the replicated viral DNA in the nucleus, shuttles the complex out to the cytoplasm where MP specifically binds the NSP-DNA complexes and directs this to the cell periphery, where they are transported into the adjoining cells. The requirement of both proteins for intercellular movement was also demonstrated for BDMV, where mutation of the NSP and MP restricted the cell-to-cell movement of viral DNA (Sudarshana et al., 1998).

The MP of BDMV increases the size exclusion limit (SEL) of plasmodesmata into which it is injected, and the protein mediates viral DNA transport from cell-to-cell (Noueiry et al., 1994; Rojas et al., 1998). In contrast, the MP of SqLCV does not bind DNA but is present in virus-induced tubules that cross the walls of meristematic phloem tissues (Ward et al., 1997). The NSP is nuclear localized, binds to viral DNA (Noueiry et al., 1994; Pascal et al., 1994) and acts as a nuclear shuttle protein. Interaction of the two proteins regulates the directionality of intracellular viral DNA transport (Sanderfoot et al., 1996). Briddon and Markham (2001) demonstrated that geminiviruses of the genera Curtovirus and Topocuvirus are able to trans-complement efficient movement of DNA A for two bipartite begomoviruses in the absence of their corresponding DNA B. The spread of DNA A independent of DNA B, following Agrobacterium-mediated inoculation showed to require coat protein whereas trans-complemented spread of DNA A could occur independent of the coat protein encoded on DNA A.

1.12 Replication of geminiviruses

Geminivirus DNA replication follows a rolling circle mechanism. The rolling circle replication (RCR) of geminivirus can be divided into two phases (Gutierrez, 2000):
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1. conversion of viral ssDNA into dsDNA forms on entering the nucleus of the initially infected cells. This step of synthesis of viral minus strand is carried out by cellular enzymes and is still poorly understood.

2. Rolling circle phase to replicate viral ssDNA on dsDNA templates. This step requires the participation of Rep. Rep is the only viral protein absolutely required for RCR, as it is responsible for initiating DNA replication. Laufs et al. (1995a) described in detail the role of Rep in initiation and termination of RCR of geminiviruses.

Recently an additional method of replication of geminivirus and their satellite has been proposed (Alberter et al., 2005; Jeske et al., 2001; Preiss and Jeske, 2003). This model, recombination-dependent replication (RDR), was based on analysis of replication intermediates of AbMV, BCTV, TGMV, ACMV, ToLCV and one satellite molecule, DNA β, using two dimensional gel electrophoresis and electron microscopy. Apart from previously identified RCR intermediates (Saunders et al., 1991), a range of intermediates suggested an additional pathway. This is an analogous to RDR pathway of T4 bacteriophage (Kreuzer, 2000) that has also been named the “join-copy” pathway (Mosing, 1998), “break-induced replication” (George and Kreuzer, 1996) and “bubble-migration synthesis” (Formosa and Alberts, 1986). The RDR model three steps (Kreuzer, 2000; Mosing, 1998):

1. Processing of the broken dsDNA to produce the 3’ end ssDNA required for DNA strand invasion.
2. Invasion of a homologous duplex by 3’ end ssDNA to form a structure known as the ‘displacement loop’ (D-loop or bubble loop). DNA strand invasion by the third end of ssDNA allows it to serve as potential primer for DNA replication.
3. DNA heteroduplex extension (branch migration). At this step the protein directed branch migration occurs at rear of the loop as DNA polymerase extends the leading strand product at the front of the loop. Because both reactions occur at similar rate, the size of the loop is roughly unchanged.
This type of RDR does not need topoisomerase, even when the circular DNA templates are supercoiled, and the two parent strands do not need to separate from each other (Kreuzer, 2000).

RDR of geminiviruses apparently does not require participation of Rep in terms of its cognate virus recognition and nicking of ssDNA at the nonanucleotide sequence for initiation of replication. This possibility is also supported by a recent study, (Lin et al., 2003) in which mutants of ToLCV and its sat-DNA molecule that were impaired in their ability to bind Rep in vitro, were still infectious to tomato.

1.13.1 Satellite and satellite-like components associated with monopartite begomoviruses

Satellites are a common feature of many RNA viruses. They are defined as viruses or nucleic acids that depend on a helper virus for their replication but lack extensive nucleotide sequence homology to the helper virus and are dispensable for its proliferation (Murant et al., 1982). Recently, satellite molecules named DNA β were found to be required by certain monopartite begomoviruses. These molecules are approximately 1350 nt in length (approximately half that of the genomes of their helper viruses) and are unrelated in sequence to their helper viruses. They encode a single ORF called βC1 in complementary-sense that is a determinant of pathogenicity and a suppressor of gene silencing (Cui et al., 2004; Saeed et al., 2005; Saunders et al., 2004, Mansoor et al., 2006). DNA β was first identified for Ageratum yellow vein disease, a disease complex similar to CLCuD, which affects the weed Ageratum conyzoides in south-east Asia, and subsequently for CLCuD (Briddon et al., 2001; Saunders et al., 2000). In these two diseases, this component is essential for symptom induction by begomovirus.

These satellite molecules depend upon helper viruses for their proliferation and, in turn, modulate helper virus accumulation and symptom expression (Briddon et al., 2003). ToLCV-sat represents the first identified example of a satellite associated with a DNA virus. This satellite component is also dependent on ToLCV for replication, systemic movement and insect transmission, but is unrelated to the helper virus and only approximately a quarter of its size (Dry et al., 1997).
Tobacco plants transformed with \( \beta \)C1 protein under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter or with dimeric DNA \( \beta \) exhibited a severe disease-like phenotype while plants transformed with mutated version of \( \beta \)C1 appeared normal (Saeed *et al*., 2005). More recently they have reported that DNA \( \beta \) can replace the movement function of the DNA B of a bipartite begomovirus, thus showing that it has a role in virus movement. Inoculation of tomato plants with ToLCNDV DNA A alone induced local but not systemic infection, whereas co-inoculation with DNA A and the DNA \( \beta \) associated with CLCuD resulted in systemic infection (Saeed *et al*., 2007).

It has been proposed that DNA \( \beta \) might effect the replication of helper virus by either facilitating their spread in the host or by suppressing the host gene silencing (Mansoor *et al*., 2003; Saunders *et al*., 2000). The association of DNA \( \beta \) with geminiviruses appears to be common throughout Old World. Assessment of the geographical and sequence diversity of the DNA \( \beta \) satellites associated with begomoviruses showed DNA \( \beta \) molecules were widespread in the Old World but apparently absent from the New World and the nucleotide sequences of DNA \( \beta \) molecules examined clustered as two major groups based on the plant host from which they were isolated. Within these two groups the DNA \( \beta \) molecules segregate both by host and geographic origin (Briddon *et al*., 2003). DNA \( \beta \) has also found to be associated with *Malvastrum yellow vein virus* infecting *Malvastrum coromandelianum* and *Ageratum yellow vein China virus* in China (Zhou *et al*., 2003).

The first nanovirus-like DNA molecule associated with geminivirus was isolated from cotton infected with CLCuD in Pakistan (Mansoor *et al*., 1999). Subsequently, similar molecules were found in many other plants infected with many monopartite begomoviruses from the Old World (Briddon *et al*., 2004). These molecules were named DNA1, comprised approximately 1375 nts and have a conserved genome organization. They contain a predicted stem loop structure containing the loop sequence TAGTAATAT typical to that of nanoviruses, a single large ORF in the positive-sense that encodes a homolog of the nanovirus Rep and an adenine rich (A-rich) region immediately downstream of the coding region (typically 100-200nts). This is the only feature different from nanovirus Rep components (Briddon *et al*., 2004). It has been suggested the nanovirus-like DNA molecules were possibly
“captured” by geminiviruses during mixed infections by component exchange. This allowed them to be transmitted by geminivirus vectors and therefore increased their host range (Mansoor et al., 1999; Saunders et al., 2002). Wu and Zhou, (2005) reported that the interaction between DNA1 and the TbCSV/satellite complex can modulate viral symptoms and reduce viral DNA accumulation. They demonstrated that in leaf disc assay TbCSV-Y35 DNA1 replicated autonomously. Southern blot analysis revealed that TbCSV-Y35 DNA1 reduced viral DNA accumulation. Viral DNA accumulation was not reduced when plants were co-inoculated with TbCSV-Y35 DNAβ, but the TbCSV-Y35 DNAβ level was dramatically reduced in the presence of TbCSV-Y35 DNA1.

1.14 Natural plant defense against viruses

In their natural environments, plants are routinely challenged by parasites including viruses, bacteria, fungi, nematodes and insects, all of which have the molecular capability to manipulate host plants for their own sustenance. Plants have evolved an innate disease resistance involving a complex array of constitutively expressed R genes to detect foreign invaders and defend themselves (Dangl and Jones, 2001; Holub, 2001). Physiological studies have been complemented by the molecular characterization of several R genes (see below). Some of the downstream signalling components, including kinases, putative lipases, phosphatases and transcription factors have been identified. These local defence responses are amplified throughout the plant with the help of well known signal molecules such as salicylic and jasmonic acid, ethylene and MAP kinases (Dong, 1988; Glazebrook, 2001; Zhang and Klessig, 2001). Production of reactive oxygen species (ROS), including H2O2, is one of the earliest known responses in incompatible interactions between pathogens and plants. The ROS induce the accumulation of SA and trigger PR protein expression (Wu et al., 1997; Chamnongpol et al., 1998). SA is an essential signaling hormone for activation of local and systemic defenses against pathogens in many plant species (Cao et al., 1998; Dempsey et al., 1999; Zhang et al., 1999). SA, however, is not the essential mobile signal transmitted through plants to initiate systemic defense (Alvarez et al., 1998). Recent identification of a lipid transfer protein implies the nature of the long distant signaling molecule in Arabidopsis may be lipid-derived (Maldonado et al., 2002). Post-transcriptional gene silencing (PTGS) also known as RNA interference (RNAi) is an intrinsic plant defense mechanism can be efficiently triggered by double
stranded (ds)RNA-producing transgenes and can provide high levels of virus resistance by specific targeting of cognate viral RNA. PTGS is a homology-dependent RNA degradation process that targets RNA exclusively in the cytoplasm.

1.15 Control of plant diseases

Plant diseases caused by infectious viruses, bacteria, phytoplasmas, fungi, and nematodes result in problems in agriculture. These problems include reduced yields, lower product quality or shelf-life, decreased aesthetic or nutritional value, and, sometimes, food and feed contaminated with toxic compounds. Control of a plant disease is essential for providing an adequate supply of food, feed, and fiber. Growers currently spend large sums to achieve partial control of pathogens that attack crops and other plants. Even then, crop and commodity losses because of diseases cost billions of dollars each year. Reducing such losses has long been a high priority objective for agriculture. Knowledge and management of plant diseases of quarantine significance are vital, not only for protecting our domestic crops from foreign disease, but also for maintaining and expanding export markets for plants and plant products. Strategies for the control of plant diseases include planting resistant crop varieties, changing cultural practices or storage conditions to those less favorable for disease development, planting dates, employing biological controls, applying chemical pesticides, and using integrated disease management (combining two or more of the above approaches). The ability to develop any of these strategies depends first on identifying the pathogen causing the disease, then learning how to interrupt the disease cycle.

1.15.1 Approaches for disease resistance

After the “Green Revolution”, intensive agricultural practices reduced the varietal diversity and hence the natural virus resistance of crop species. Practices used to control diseases include the control of insect vectors by intensive pesticide spray, virus-free propagation, use of proper cultural practices and the use of resistant cultivars. However, each of these has their drawbacks.

Tremendous advances have taken place in our understanding of plant-virus interactions in the process of pathogenesis and resistance. These, along with associated advances in the genetic transformation of a number of crop plants, have
opened up the possibility of an entirely new genetic engineering approach towards controlling plant virus diseases (Dasgupta et al., 2003).

There are two main approaches for developing genetically-engineered resistance depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from other sources. The idea of pathogen-derived resistance (PDR) was put forward by Sanford and Johnson in 1985. They proposed that resistance could be engineered by transforming a susceptible genotype with gene sequences derived from the pathogen itself. For PDR, a part, or a complete viral gene is introduced into the plant, which, subsequently, interferes with one or more essential steps in the infection cycle of the virus. This was first illustrated in tobacco by the group of Roger Beachy (Beachy et al., 1990), who introduced the coat protein (CP) of TMV into tobacco and observed TMV resistance in transgenic plants. The concept of PDR has generated lot of interest and today there are numerous host-virus systems in which it has been amply demonstrated. Non pathogen-derived resistance, on the other hand, is based on utilizing host resistance genes and other genes responsible for adaptive host processes, elicited in response to pathogen attack, to obtain transgenic plants resistant to the virus. The use of non-PDR type of resistance, even though reported much less in the literature in comparison to PDR-based approaches, holds a better promise to achieve durable resistance (Lomonossoff, 1995).

In a number of crops, transgenic resistance to an infective virus has been developed by introducing a sequence of the viral genome in the target crop by genetic transformation. Virus resistant transgenics have been developed in many crops by introducing either viral CP or replicase gene coding sequences. Resistance obtained by using CP is conventionally called coat protein-mediated resistance (CPMR). Replicase-mediated resistance has been pursued in a number of laboratories and in most of these cases, resistance has been shown to be due to an inherent plant response, known as PTGS, Because of the essential nature of the viral movement protein for intercellular movement of plant viruses, movement protein sequences have also been used to achieve resistance. Other pathogen-derived approaches described in the literature, include the use of satellite RNA and defective interfering viral genomic components.
1.15.2 Coat protein mediated resistance

The use of viral CP as a transgene for producing virus resistant plants is one of the most spectacular successes achieved in plant biotechnology. Numerous crops have been transformed to express viral CP and have been reported to show high levels of resistance in comparison to untransformed plants. Abel et al., (1986) first reported resistance against TMV in transgenic tobacco expressing the TMV CP gene, as described in the previous section. The resistance was manifested as delayed appearance of symptoms as well as a reduced titer of virus in the infected transgenic plants, as compared to the controls. Transgenic potato, expressing the CP of potato virus X (PVX) also showed resistance against PVX (Hemenway et al., 1988). The story of CPMR will not be complete if one does not describe the economic benefits harvested in Hawaii after the introduction of transgenic papaya plants. When the transgenic papaya was crossed with another non transgenic papaya after 25 months of successful cultivation the yield increased from 5,600 kg/ha to 112,000 kg/ha as compared with non transgenic plants (Dasgupta 2003).

1.15.3 Replication associated protein-mediated resistance

Replicase protein-mediated resistance against a virus in transgenic plants was first shown in tobacco against TMV in plants containing the 54 kDa putative Rep gene (Golemboski et al., 1990). Similar resistances have been developed for several other viruses (MacFarlane and Davis, 1992). Gene constructs of Rep genes that have been used for resistance include full length, truncated or mutated genes. A truncated form of this gene, capable of expressing the N-terminal 210 amino acids (aa) of the Rep protein, have been utilized for developing resistance against TYLCV in N. benthamiana plants. The results obtained using both transient and stable gene expression systems show that the expression of the N-terminal 210 aa of the TYLCV Rep protein efficiently interferes with virus infection (Noris et al., 1996). Expression of the oligomerization domain of the Rep of ToLCNDV interferes with DNA accumulation of heterologous geminiviruses (Chatterji et al., 2001).

Many of the above resistance responses have now been shown not to require protein expression and to be mediated at the RNA level. To make the resistance broad based, it may be necessary to pyramid such genes from several dissimilar virus sources into
the plant genome. However, the resistance generated by the use of Rep sequences is very tight; a high dosage of input virus can be resisted by the transgenic plant (Dasgupta et al., 2003).

Long double-stranded RNAs induce an interferon like response in mammalian cells so this problem was solved by delivering short interfering RNA (siRNA) specific to the genes under consideration. Recently this approach has been exploited in plants to target ACMV replication associated protein gene in BY2 protoplasts. Synthetic siRNA targeted to the Rep gene of ACMV specifically interfered with ACMV DNA accumulation in protoplasts and dramatically reduced the accumulation of the corresponding mRNA. Codelivery of an siRNA designed to target the mRNA encoding the Rep protein of the geminivirus ACMV from Cameroon blocked Rep mRNA accumulation by ~91% and inhibited accumulation of the ACMV genomic DNA by ~66% at 36 and 48 h after transfection. As with siRNA-induced reporter gene silencing, the siRNA targeting ACMV Rep was specific and did not affect the replication of East African cassava mosaic Cameroon virus (EACMCV). This report demonstrated the occurrence of siRNA-mediated suppression of gene expression in cultured plant cells and that siRNA can interfere with and suppress accumulation of a nuclear-replicated DNA virus (Vanitharani et al., 2003)

1.15.4 Movement protein-mediated resistance

Movement proteins are essential for cell-to-cell movement of plant viruses. These proteins have been shown to modify the gating function of plasmodesmata, thereby allowing the virus particles or their nucleoprotein derivatives to spread to adjacent cells. This phenomenon was first used to engineer resistance against TMV in tobacco by producing modified MP, which is partially active as a transgene. The conferred resistance is believed to be based on the competition between wild type virus encoded movement proteins and the preformed dysfunctional movement proteins to bind to the plasmodesmatal sites (Lapidot et al., 1993). This resistance was moreover seem to be effective against distantly related and unrelated viruses. For example, resistance against TMV could be achieved in tobacco using the MP derived from brome mosaic virus, suggesting functional conservation of this protein among several viruses (Cooper et al., 1995).
1.15.5 Satellite RNA mediated resistance
Transgenic tobacco plants expressing multiple or partial copies of CMV satellite RNA showed attenuated symptoms when challenged with CMV (Baulcombe et al., 1986). In addition, tobacco plants transformed with antisense satellite RNA also showed delayed symptom development with the cognate virus. Tomato plants, containing non-necrogenic satellite RNA sequences, developed only faint symptoms following CMV infection. The timing of fruit set and fruit yield in transgenic plants was comparable with healthy plants. Thus, a high-level of tolerance to CMV was conferred by satellite RNA in tomato (Baulcombe et al., 1986).

1.15.6 Defective interfering viral nucleic acids
For several viruses, truncated genomic components are often detectable in infected tissues, which interfere with the replication of the genomic components. These species of DNA are also called defective interfering (DI) DNA and expression of delayed disease symptoms and recovery, coupled with increased resistance upon repeated inoculation have been observed in plants engineered with DI DNA (Kunik et al., 1994). For example, incorporation of subgenomic DNA B that interferes with the replication of full-length DNA A and B confers resistance to ACMV in N. benthamiana (Frischmuth et al., 1993).

1.15.7 Non-pathogen derived resistance
Genetically engineered resistance can be produced without involving pathogen genes. This includes incorporation of plant disease resistance genes from various hosts, ribosome inactivating proteins, plant proteinase inhibitors, antiviral antibodies expressed in plants, engineering for secondary metabolites conferring resistance, and systemic acquired resistance.

1.15.8 Other approaches for disease resistance
Incorporation of plant resistance genes is more durable than any other system. Resistance (R) genes encode proteins specific to the avirulence (avr) genes found in pathogen, and initiate a cascade of signaling reactions after sensing the avr gene products that results in local cell death known as the hypersensitive response (HR). Actually, this strategy is based on the classical gene-for-gene hypothesis (Flor, 1971).
A number of structural features are conserved across several R gene products. These include a leucine-rich repeat (LRR), nucleotide-binding site (NBS), serine-threonine kinase, leucine zipper and toll-interleukin region (TIR) (Hammond and Jones, 1997). The N gene of *N. tabacum* is well-characterized and the earliest known R gene which confers resistance against TMV. This gene was expressed in a heterologus host and a HR was increased (Whitham *et al*., 1994).

*Turnip crinkle virus* (TCV) resistance in *A. thaliana* is mediated by an altogether different mechanism. The *RTM* gene, present in ecotype Columbia-O, brings about a HR independent resistance against TCV by affecting its long distance movement and is present as two alleles, *RTM1* and *RTM2*. The RTM protein is believed to interfere directly with an essential component of the long-distance movement of the virus. Thus, model plants such as *Arabidopsis* can help us in looking for related R genes in crop plants (Whitham *et al*., 1994).

Several plants have been found to contain antiviral proteins known as ribosome inactivating proteins (RIPs). RIPs inhibit the translocation step of translation by catalytically removing a specific adenine base from 28S ribosomal RNA. They are synthesized either as pre- or pre-pro-proteins. The antiviral activity of several types of RIPs has been well-documented (Carzaniga, 1994). When purified RIPs are mixed with viruses and applied on plants, virus multiplication and symptom development are dramatically suppressed. The development of systemic resistance was reported following studies on induction of a 34 kDa basic protein from the RIP (CA-SRI) treated *Cyamopsis tetragonoloba* plants (Carzaniga, 1994).

In another experiment, the toxin gene, Dianthin was placed downstream of a transactivatable geminivirus promoter from ACMV. When transgenic *N. benthamiana* plants were inoculated with ACMV, dianthin was synthesized only in the virus-infected tissues where it inhibited virus multiplication (Hong and Stanley, 1996).

Another approach to control plant viruses is to express specific anti-viral antibodies, commonly known as plantibodies in plants. Single chain antibodies against the CP of *Beet necrotic yellow vein virus* have been expressed in *N. benthamiana* and a
significant delay in symptom development was reported, following mechanical inoculation (Fecker et al., 1997).

Following viral infection, plants develop an active resistance, which is at first localized only at the site of infection, but spreads systemically in due course. This resistance, called systemic acquired resistance (SAR), is characterized by the coordinate activation of several genes in uninfected, distal parts of the inoculated plants. Tobacco plants were transformed with two bacterial genes coding for enzymes that convert chorismate into salicylic acid (SA) by a two-step process. When the two enzymes were targeted to the chloroplast, the transgenic plants showed a 500- to 1000 fold increased accumulation of SA and SA-glucoside, compared to control plants. The level of PR-proteins was enhanced and these plants showed resistance to viral and fungal infection, in a mode similar to SAR in nontransgenic plants (Verbeme et al., 2000).

Metabolic pathways which are important in viral pathogenesis are key targets for intervention against viral infection. Another novel approach of interference with viral pathogenesis is to inhibit tetrapyrole biosynthesis by expressing antisense RNA of uroporphyrinogen decarboxylase or coporphyrinogen oxidase in N. tabacum. The plants were characterized by accumulation of photosensitizing tetrapyrrrole intermediates, accumulation of highly fluorescent Coumarin scopolin, PR proteins and reduced levels of infecting viral RNA (Mock et al., 1999).

RNA interference (RNAi) is a mechanism of suppressing gene expression by degrading specific messenger RNAs (mRNA). Introduction of double-stranded RNA (dsRNA) into the cytosol initiates the phenomenon of RNAi, in turn activating a pathway culminating in the degradation of the targeted gene transcript (Agrawal et al., 2003; Kuznetsov et al., 2003; Arenz and Schepers, 2003). In addition to RNA degradation, upon activation of the RNAi pathway, there are also cases where the promoter region of the gene is silenced through methylation (Mette et al., 2000; Wassenegger et al., 1994).
1.16.1 History of RNA silencing

In 1928, Wingard noticed tobacco plants in which only the initially infected leaves were necrotic and diseased owing to TMV. The upper leaves somehow became immune to the virus and consequently were asymptomatic and resistant to secondary infection (Wingard, 1928). At that time this recovery was a mystery as there was no obvious way to explain the specificity of the resistance to secondary infection but Wingard's paper was an appropriate starting point for the current interest in RNA silencing.

More than a decade ago, a surprising observation was made in petunias. While trying to deepen the purple color of these flowers, Richard Jorgensen and colleagues introduced a pigment-producing gene under the control of 35S promoter (Jorgensen, 1996). Instead of the expected deep purple color, many of the flowers appeared variegated or even white. Jorgensen named the observed phenomenon "co-suppression", since expression of both the introduced gene and the homologous endogenous gene was suppressed (Jorgensen et al., 1996). Nuclear run-on experiments showed that the homologous transcript is made, but that it is rapidly degraded in the cytoplasm and does not accumulate (Hammond et al., 2001). This finding lead to the discovery of RNAi. Since mRNA is formed normally in the plants but gene expression is suppressed, the phenomenon is also known as post-transcriptional gene silencing (PTGS). Co-suppression can also be the consequence of transcriptional mechanisms. Transcriptional gene silencing (TGS) involves changes at the DNA level that cause loss of transcription, by methylation and chromatin remodeling (Matzke and Matzke, 1991; Meyer et al., 1993).

Cosuppression has since been found to occur in many species of plants. It has also been observed in fungi, and has been particularly well characterized in Neurospora crassa, where it is known as "quelling" (Guru, 2000). RNA silencing also occurs in a wide variety of eukaryotic organisms. Initially, progress of research in this field was slow. Early research was mainly confined to plants and fungi until Fire et al. (1998) reported RNAi in C. elegans. The study of RNAi has become increasingly more rewarding and continually expanding following the completion of several recent genome sequencing projects such as the human and Arabidopsis projects.
The first evidence that dsRNA could lead to gene silencing came from work in the nematode *Caenorhabditis elegans*. In 1995, Guo and Kemphues attempted to use antisense RNA to shut-down expression of the *par-1* gene in order to assess its function. *Par-1* encodes a putative Ser/Thr kinase with similarity to kinases from yeasts and mammals. *PAR-1* protein is localized to the posterior periphery of the zygote and is distributed in a polar fashion preceding the asymmetric divisions of the germline lineage. *PAR-1* functions in germline development as well as in establishing embryonic polarity. As expected, antisense RNA disrupted expression of *par-1* (Guo and Kemphues, 1995).

Fire and Mello first injected dsRNA in *C. elegans* that resulted in much more efficient silencing than either the sense or the antisense strands alone (Fire et al., 1998). Over the last few years, these RNAi strategies have been used as reverse genetics tools in *Drosophila* to characterize various loss-of-function phenotypes (Kennerdell and Carthew, 2000; Dzitoyeva et al., 2001; Worby et al., 2001; Schmid et al., 2002).

### 1.16.2 Types of small RNA molecules

#### 1.16.2.1 Small interference RNA (siRNAs)

Small interfering RNAs (siRNAs) have an integral role in the phenomenon of RNAi. dsRNAs introduced into certain organisms or cells are degraded into ~22nt fragments. These 22nt siRNA molecules then bind to the complementary portion of their target mRNA and tag it for degradation. siRNAs are believed to have a role in conferring viral resistance and in preventing transposon transposition (Lippman et al., 2003).

#### 1.16.2.2 Micro RNAs (miRNAs)

Additional small 22nt RNA molecules known as microRNAs (miRNAs) were discovered in *Drosophila, C. elegans* and *HeLa* cells (Lau et al., 2001; Lee et al., 2002; Sharp and Zamore, 2000). These are formed from precursor RNA molecules that fold into a stem-loop secondary structure. miRNAs play a role in the regulation of gene expression (Grosshans and Slack, 2002). 100 new mRNAs were identified of which 15% were conserved (with 1-2 mismatches) across worm, fruitfly and mammalian genomes (Sharp and Zamore, 2000). All the identified miRNAs were located at either the 3’ or the 5’ side of a stem loop within a ~70 nt RNA precursor.
The expression pattern of the miRNAs varied. While some *C. elegans* and *Drosophila* miRNAs were expressed in all cells and at all developmental stages, others had a more restricted spatial and temporal expression pattern. This suggested that these miRNAs might be involved in post-transcriptional regulation of genes involved in development.

### 1.16.3 RNA silencing pathways in plants

Three silencing pathways have been reported in plants (Baulcombe, 2004). The first (cytoplasmic siRNA silencing) comprises PTGS mediated by 21 nucleotide siRNAs that are processed from dsRNAs. The source of dsRNAs includes replication intermediates of plant RNA viruses, transgenic inverted repeats, and products of RNA-dependent RNA polymerases (RdRps). This class of siRNA is possibly generated by DCL2 and DCL4. Duplex siRNA is unwound and incorporated into an RNA induced silencing complex (RISC) which has at least one Argonaute protein (AGO). The cleavage specificity is a consequence of complementary base pairing between the siRNA and the target mRNA. AGO protein in RISC is likely the “slicer” that carries out transcript cleavage. The second pathway involves a class of endogenous small RNAs, miRNAs. The miRNAs are generated by Dicer-like 1 (DCL1) from miRNA precursors that are transcribed from miRNA genes and down-regulate gene expression through base-pairing to target mRNAs, leading to either the degradation of mRNAs or the inhibition of translation or both. The miRNA directs RISC cleavage in the same manner as siRNA. The third pathway is transcriptional gene silencing (TGS) that is associated with siRNA-directed chromatin modifications including DNA and histone methylation.

### 1.16.4 Plant Dicer-like proteins and biogenesis of small RNAs

The Dicer-like 1 (SIN1/SUS1/CAF) was isolated through independent genetic screens for *Arabidopsis* mutants with abnormal embryo, ovule and flower development (Schauer *et al.*, 2004). SIN1/SUS1/CAF was shown to encode a conserved multidomain protein containing two RNase III domains (Jacobsen *et al.*, 1999). In the same year, small RNAs were detected in various plant PTGS systems (Hamilton and Baulcombe, 1999). However, the connection between DCL1 and small RNA production remained unknown until the discovery that its homologue in Drosophila, Dicer-1, processes dsRNA substrates into 22 nt small RNAs (Bernstein *et al.*, 2001).
Plants also have evolved a divergence of Dicer-like (DCL) RNase-III type enzymes that cleave dsRNA or ssRNA with ds features (Brodersen and Voinnet, 2006). Virus infections trigger PTGS against viral RNAs, resulting in a virus specific siRNA population in the cytoplasm of plant cells (Baulcombe, 2004). These viral siRNAs are generated in hotspots that may be selected by DCL on ssRNA substrates where fold back structures are formed (Molnar et al., 2005, Ho et al., 2007). The reason for generation of siRNA hotspots by DCL while processing dsRNA substrate is not clear. All DCLs have been recorded as processing viral derived RNAs in coordinated hierarchical actions in Arabidopsis (Moissiard and Voinnet, 2006; Deleris et al., 2006) and DCL2 dependent 22-nt siRNAs were produced redundantly to DCL4 dependent 21-nt siRNAs that mediate anti-viral silencing activities (Blevins et al., 2006; Fusaro et al., 2006).

In Drosophila, besides their involvement in small RNA biogenesis, Dicers are also required for RISC formation (Xie et al., 2004). Size exclusion chromatography suggested that DCL1 and DCL3 reside in >660 and 440 kDa complexes, respectively (Qi et al., 2005). Identification of the components of the complexes may reveal if plant DCLs also have dual functions in both the initiation and execution stages of RNAi.

1.16.5 Argonaute protein in plants

The AGO proteins in plants, animals and fungi have been implicated in all three pathways of RNA silencing. AGO proteins play a central role because they are components of the silencing effector complexes that bind to siRNAs and miRNAs. In Arabidopsis at least 10 AGO proteins have been found as candidates for Slicer but studies shown AGO1 as an excellent Slicer candidate. AGO1 was isolated through the genetic screening for mutants with aberrant leaf morphology and named after the small squid-like appearance of the mutant plant (Bohmert et al., 1998). AGO1 is required for PTGS (Fagard et al., 2000). miRNA accumulation is decreased in AGO1 null mutants with increased levels of miRNA target genes, whereas in hypomorphic mutants, miRNA accumulation is not substantially changed but target mRNA cleavage is decreased (Vaucheret et al., 2004). Recently, it has been shown that in Arabidopsis, one trans-acting siRNA (ta-siRNA) and three miRNAs could be detected in AGO1 immuno-precipitates, indicating that these ta-siRNA and miRNAs and
AGO1 associate in vivo. Such complexes are helpful for cleaving the target mRNAs of the ta-siRNA and miRNAs in vitro (Qi et al., 2005). Similar results were also obtained using an Arabidopsis line expressing a tagged AGO1 (Baumberger et al., 2005). Data from genetic studies and biochemical analyses strongly suggest that AGO1 is a key component of Arabidopsis RISC and is at least one of the Arabidopsis Slicers (Baumberger et al., 2005; Qi et al., 2005). AGOs in effector complexes are unable to accept siRNAs of inappropriate sizes. Attempts to assemble RISC with exogenous siRNA duplexes were unsuccessful (Tang et al., 2003; Qi et al., 2005), maybe due to the lack of a siRNA unwinding activity in plant extracts (Matranga and Zamore, 2004).

1.16.6 Initiation and amplification of silencing signals

According to the model, the RNA-dependent RNA polymerases (RdRp) proteins could mediate primer-dependent and primer-independent mechanisms of RNA silencing. In primer-independent process dsRNA is produced from a single-stranded template, so that silencing can be initiated in virus-infected plants or with transgene RNAs. In vitro assays with N. crassa (Makeyev and Bamford et al., 2002) and tomato enzymes (Schiebel et al., 1993) demonstrated that RdRp catalyse primer-independent synthesis of dsRNA on a ssRNA template. Similarly, in wheat germ extracts, ssRNA can be copied into complementary RNA by an unidentified enzyme that, presumably, is an RNA-dependent RNA polymerase. The mechanism of differentiating viral or transgene RNAs targeted for silencing from non silenced endogenous RNA by RdRp is not understood. Perhaps the presence ‘aberrant’ features in silenced RNA play a role in this selection. Alternatively, the aberrant RNA might lack features that are present in normal RNA.

The second RdRp mechanism requires that primary siRNAs from a virus, transposon or transgene are primers in RdRp-directed synthesis of dsRNA. Makeyev and Bamford (2002) showed that QDE1 RdRp protein from N. crassa incorporated a labeled 20-nucleotide antisense RNA into the complementary-strand of an ssRNA in vitro. In C. elegans and plant systems the initiator of silencing comes from part of a target gene. In these systems, the secondary siRNAs that accumulate in the silenced tissue are dependent on RdRp proteins. In A. thaliana and N. benthamiana the secondary siRNAs are from both the 5’ and the 3’ side of the initiator (Makeyev and
Bamford, 2002) on the ssRNA, and so cannot be produced from a simple priming mechanism on a single RNA species. This can be explained by the fact that the silencing target is transcribed from both strands. The 3’ secondary siRNAs would then result from extension of a siRNA primer on an antisense RNA template. As a result of the RdRp-mediated mechanisms, a single aberrant RNA species or primary siRNA molecule could generate many dsRNAs which would then silence even more target molecules. This amplification process is essential for virus defense because it can ensure the silencing of viral RNAs with the replication and accumulation of viral RNA. Similarly, in genome defense, the amplification steps can ensure that a few molecules of transposon RNA could activate the chromatin-silencing pathway.

1.16.7 Mobile silencing signals
RNA silencing in plants involves an as-yet-unknown signal that moves out from the cells undergoing RISC-mediated RNA degradation, spreading the silencing effect over short distances (Himber et al., 2003), as well as to other parts of the plant (Palaqui et al., 1997; Voinnet and Baulcombe, 1997). A mobile silencing signal could move either with or ahead of the virus to silence the viral RNA before, or at the same time, as the virus moves into a cell. The signal is either RNA or it has an RNA component because this systemic effect has nucleotide-sequence specificity corresponding to the initiator dsRNA. In plants the systemic silencing mechanism is same as that in C. elegans. The signal does not have to cross any membranes because most of the cells in a plant, including the phloem cells of the vascular system, are connected by plasmodesmatal channels. None of the host proteins involved in movement of this silencing signal has been identified. Analysis of systemic signalling from GFP transgene coupled to a phloem-specific promoter indicated that the signaling mechanism in plants can be resolved into short (up to 15 cells) and longer range phases extending up to several centimeters (Himber et al., 2003). However, systemic silencing is transmitted from grafted plants in which both the 21 and 24 nucleotide siRNAs are suppressed by the viral HCPro suppressor of silencing. It is therefore possible that other silencing RNAs, including long ssRNA, dsRNA or siRNAs, could be signal molecules because any of them can initiate silencing if they are introduced into a cell with a suitable target (Mallory et al., 2001).
1.16.8 Applications of RNAi in plants

Gene knockout mutants, antisense RNA, ribozyme and co-suppression have been used for the down-regulation of gene expression, but RNAi, dsRNA-based degradation of a target mRNA has shown much higher potential for such a purpose (Tuschl 2003; Hannon and Rossi, 2004; Mello and Conte, 2004). Technologies have emerged for making use of RNAi for discovering or validating gene functions and for improving crop traits. In most cases a crop trait results from expression of a number of genes interacting with each other in gene expression networks. By using RNAi technologies, the expression of any gene involved in a trait can be down-regulated in a highly specific way without affecting the expression of other genes. The selective modulation of biochemical pathways underlying crop traits has thus become feasible.

RNAi technology is used to produce sufficient amounts of dsRNA within plants which has homology with endogenous messenger RNAs and can trigger the initiation of the silencing mechanism. The most efficient delivery methods for dsRNA in plants is the inoculation of plants with engineered plant viruses which produce dsRNA intermediates in their life cycles (Robertson, 2004) or transformation of plants with transgene constructs from which the RNA transcripts are folded into dsRNA structures (Waterhouse et al., 1998). In the case of a transient virus-based approach, it permits efficient gene function discovery and validation in research programs carried out in high-throughput mode. Whereas a transgene based approach permits the specific down-regulation of genes for trait development in different crops. The degree of degradation of the targeted plant RNAs can vary from partial to complete degradation and depends on exogenous as well as endogenous factors, including temperature and physiological status of the plant respectively. A number of RNA viruses which are not pathogenic to plants have been converted into vectors in which fragments of plant genes can be integrated to form “chimeric viruses”. As the chimeric viruses also carry small fragments of a plant gene, not only the viruses but also the corresponding plant gene is targeted for silencing. Within approximately 10-12 days the expression of the targeted gene becomes down-regulated to a level which causes physiological changes which become manifested in a visible change in phenotype. The plants with phenotypic changes are subjected to biochemical changes. To overcome the stability problems in RNAi the idea of uncoupling the function of virus replication and induction of gene silencing in a two-component system was called satellite-virus-induced silencing system (SVISS) (Gossele et al., 2002). They
integrated small fragments of plant genes into satellite RNA virus vectors and coinoculated them together with an appropriate helper virus into young tobacco plants. Within 10-12 days changes were observed in plants phenotypes according to the function of the targeted gene and the level of satellite virus RNA exceeded by far the level of RNA of the accompanying helper virus.

To help identify the functions of genes in rice a Gateway vector, pANDA, for RNA silencing of rice genes has been developed (Miki and Shimamoto, 2004). This vector suppressed mRNA expression in more than 90% of the transgenic plants examined, indicating that RNAi clearly functions in monocot cells. A similar vector, pANDA-mini, was also successfully used for the characterization of a flowering-time control gene and disease-resistance genes in rice (Hayama et al., 2003; Lieberherr et al., 2005). The tissue-specific and quantitative regulation RNAi has not yet been characterized in detail. In proof of concept experiments, numerous genes involved in leaf and flower pigmentation, cell wall synthesis and flower development have been efficiently down-regulated with SVISS constructs.

Higher plants produce a wide variety of secondary metabolites, including more than 25,000 terpenoids, about 8,000 phenolic compounds, and about 12,000 alkaloids (Croteau et al. 2000). RNAi can be used to increase the production of useful secondary metabolites in plant. Transgenic California poppy cells transformed with BBE RNAi vector (BBEir cells) showed a marked reduction of BBE expression and accumulation of the pathway intermediate reticuline. This is the first report of target-gene silencing to produce an important precursor in secondary metabolism. On the other hand, Allen et al. (2004) reported reticuline accumulation in transgenic opium poppy with a codeine reductase RNAi vector, although reticuline is not a precursor of codeine reductase.

RNAi vector systems which can be used for stable transformation of model and crop plants have been developed and tested in depth. These vectors contain T-DNA elements for stable integration of the RNAi construct into the genomes of plants and promoter regions which facilitate the expression of high levels of dsRNA (Helliwell and Waterhouse, 2004).
1.17 Counter defense strategies adopted by plant viruses
Viruses have evolved a wide range of mechanisms to overcome RNA-silencing, providing yet another example of the continuing evolutionary arms race between hosts and parasites (Voinnet, 2005). It is now well established that plant viruses encode suppressors of RNA silencing to specifically counteract the RNA silencing-based defense mechanism in order to ensure successful systemic invasion of the host plant.

1.17.1 Suppressors of RNA silencing encoded by plant infecting RNA viruses
The strongest evidence for the now widely accepted idea that RNA silencing acts as an adaptive defense is the existence of viral suppressors (Roth et al., 2004). Viruses from different families have acquired a variety of unrelated suppressors that affect different, and perhaps multiple, steps in the silencing pathway. HC-Pro encoded by TEV and other potyviruses is able to reverse established silencing in plants and block local silencing in transient assays (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Llave et al., 2000). In addition, it interacts with the cellular protein rgsCaM that is itself a silencing suppressor, suggesting that HC-Pro stimulates an endogenous mechanism that negatively regulates RNA silencing (Anandalakshmi et al., 2000). HC-Pro also partially inhibits dsRNA processing by Dicer and interferes with the unwinding of duplex siRNA and miRNA, thereby preventing the incorporation of targeting information into RISC (Chapman et al., 2004; Dunoyer et al., 2004). In contrast, the p19 protein of Cymbidium ring spot virus and other tombusviruses cannot reverse established silencing, although it can suppress local silencing and block production of the systemic silencing signal. The suppression activity of p19 is attributable to its ability to bind and sequester, preventing their incorporation into RISC (Lakatos et al., 2004; Silhavy et al., 2002; Vargason et al., 2003). That p19 and HC-Pro impact both siRNA and miRNA metabolism underscores the similar and overlapping nature of these pathways (Chapman et al., 2004; Dunoyer et al., 2004). On the other hand Turnip crinkle virus coat protein (TCV-CP) does not significantly affect the miRNA pathway. This protein blocks local RNA silencing and prevents systemic spread by interfering with the activity of DCL-2, which does not play a major role in processing miRNA precursors (Xie et al., 2004). The 2b protein of CMV, cannot inhibit the initiation of silencing but effectively prevents its systemic spread to native tissues (Brigneti et al., 1998; Guo and Ding, 2002). Thus, RNA
viruses have adopted many different counter-defense strategies aimed at different aspects of RNA silencing. From this perspective, the molecular basis for synergistic diseases that can result from mixed infections with viruses carrying unrelated suppressors becomes clear (Pruss et al., 1997). It is also clear that viral suppressors can be powerful tools for the analysis of RNA silencing mechanisms and the relationships between different silencing pathways.

1.17.2 RNA silencing suppression by geminiviral protein

TrAP is a transcription factor that was initially found to be required for the expression of late viral genes (Sunter and Bisaro, 1992, Sunter and Bisaro 1997, Sunter and Bisaro 2003). This function is not virus-specific among the begomoviruses, and the proteins from several other New and Old World begomoviruses have been shown to complement the transcriptional activation defect of a TGMV TrAP mutant (Sunter et al., 1994). Consistent with its ability to activate transcription, studies with GFP fusion proteins have shown that TrAP localizes to the nucleus (van Wezel et al., 2001). Four consecutive arginine residues located in the N-terminus comprise part of the nuclear localization signal (NLS), which appears to be bipartite (Dong et al., 2003; Trinks et al., 2005). The less studied BCTV TrAP protein does not appear to be a transcription factor. As noted above, it cannot complement a begomovirus TrAP mutant in this regard, and unlike TrAP, it is not required for the expression of late viral genes (Hormuzdi and Bisaro, 1993; Stanley et al., 1992; Sunter et al., 1994). In addition, it lacks a recognizable activation domain and is at best a weak and inconsistent self-activator in the yeast two-hybrid system. However, both TGMV TrAP and BCTV TrAP condition an enhanced susceptibility phenotype when expressed in transgenic N. benthamiana or tobacco, indicating that they share functions in viral pathogenesis (Sunter et al., 2001). Neither the TGMV nor the ACMV protein binds siRNA or miRNA, ruling out the possibility that they might act by a mechanism similar to P19 (Chellappan et al., 2005; Wang et al., 2005). Studies with TrAP from Mungbean yellow mosaic virus (MYMV) have confirmed the requirement for an intact zinc finger and NLS, and further demonstrated a requirement for the activation domain (Trinks et al., 2005). Taken together, these observations suggest that TrAP from Old World viruses (ACMV, MYMV, and TYLCV) acts in the nucleus by a mechanism that depends on interaction with DNA and transcriptional activation activity. Transcriptional profiling in Arabidopsis protoplasts following transient expression of
ACMV and MYMV TrAP showed that these proteins induced the expression of about 30 genes, including WEL1 (Werner exonuclease-like 1). Subsequent analysis of WEL1 indicated that it is capable of suppressing RNA silencing in *N. benthamiana* line 16c (Trinks *et al.*, 2005). These remarkable findings suggest that TrAP suppresses silencing indirectly by activating the expression of a cellular protein that may function as an endogenous negative regulator of the system.

The C4 of ACMV can suppress RNA silencing, allowing it to enhance disease and promote viral invasiveness. The suppression activity of AC4 from four different cassava-infecting geminiviruses was tested in the *Agrobacterium*-based transient assay in *N. benthamiana* 16c plants (Vanitharani *et al.*, 2004). Two of the AC4 proteins, from viruses associated with recovery-type symptoms in cassava, showed suppressor activity with increased accumulation of GFP mRNA and inhibition of GFP-specific siRNAs. Two other AC4 proteins from non-recovery-type viruses showed little or no activity in this assay. Conversely, the TrAP of the non-recovery viruses was an effective silencing suppressor, while those from recovery-type viruses were less effective. Besides revealing a new function for AC4, these experiments provide some insight into the molecular basis for synergistic disease that can result from mixed infection. Specifically, mixed infection of cassava by ACMV (recovery-type, with a relatively strong AC4 suppressor) and EACMV (non-recovery-type) with a relatively strong TrAP suppressor) cause an unusually severe disease in the field (Vanitharani *et al.*, 2004). It is important to note that synergy is made possible by the fact that not all TrAP and AC4 proteins are alike with respect to their ability to suppress silencing. The different phenotypes of these viruses further suggest that TrAP and AC4 act at discrete steps in the silencing pathway and that the effect of AC4 is more transient and can be overcome by some hosts. Transgenic expression of AC4/C4 leads to severe developmental defects which might be explained by effects on the miRNA pathway (Chellappan *et al.*, 2005; Latham *et al.*, 1997). AC4 from ACMV, but not EACMV, causes developmental defects when expressed as a transgene in *Arabidopsis*. The defects are associated with reduced accumulation of specific miRNAs and a parallel over-accumulation of their target mRNAs. Surprisingly, ACMV AC4, but not EACMV AC4, bind single-stranded miRNA and siRNA *in vitro* but does not bind the corresponding duplex forms. Thus, AC4 appears to block cytoplasmic RNA silencing, and coincidentally the miRNA pathway, by a
novel mechanism that involves binding single-stranded siRNA and miRNA. This suggests that silencing-active AC4 proteins interfere with RISC loading by acting downstream of small RNA biogenesis and duplex unwinding, possibly by facilitating the degradation of single stranded miRNAs and siRNAs.

1.17.3 Silencing suppression by βC1 protein encoded by begomovirus associated satellite

Mutagenesis has shown that single open reading frame of DNA β encodes the essential pathogenicity determinant βC1, and transgenic expression of the 14 kDa βC1, or expression from a PVX vector, results in severe developmental abnormalities (Cui et al., 2004; Saeed et al., 2004; Zhou et al., 2003; Qazi et al., 2007). The molecular basis of βC1 pathogenicity can be explained by silencing suppression activity. The βC1 protein of Tomato yellow leaf curl China virus (TYLCCV) has been shown to behave as a silencing suppressor in N. benthamiana 16c plants (Cui et al., 2005). Infection of plants silenced for GFP expression showed that TYLCCV plus DNAβ, but not TYLCCV alone, could prevent silencing in newly emerging leaves of infected plants. Expression of βC1 also interfered with local silencing in transient Agrobacterium-based assays. The recombinant protein binds ssDNA and dsDNA in vitro in a sequence-nonspecific fashion, and βC1 fusion proteins are primarily localized in the nucleus in insect and plant cells. The putative NLS is required for silencing suppression activity (Cui et al., 2005). However βC1 of CLCuMV, BYMDV, ToLCJV localize to cell periphery (Saeed et al., 2007). In addition, TrAP and BCTV TrAP do not generate developmental defects when expressed in transgenic plants (Chellappan et al., 2005; Sunter et al., 2001). Thus, the developmental defects observed with βC1 expression suggest that it targets a different step in the silencing process and most likely one that overlaps the miRNA pathway. However, there is insufficient information at present to allow the separation of AC4 and βC1 activities in this regard. Again, since related monopartite begomoviruses, including TYLCV and even a different strain of TYLCCV (Dong et al., 2003), can cause disease on their own and encode functional silencing suppressors, it is logical to assume that the requirement of βC1 for pathogenicity reflects attenuated function of other suppressors in viruses associated with DNAβ.
1.18 Objectives of the study

Aphid and whitefly transmitted cucurbit infecting viruses are important limiting factors that can cause up to 100% crop loss. A severe disease with virus-like symptoms appeared in 2003 on muskmelon crop in commercial muskmelon-growing areas of Punjab and spread rapidly across the province. To develop the sustainable genetically modified resistance against viral diseases, data on the molecular mechanism of the disease is not available in Pakistan. To address this issue, a study commenced in 2004 with a major aim being to improve the understanding of molecular basis of the disease by development of molecular diagnostic methods and to develop genetically engineered resistance. The overall objectives of the study include:

1) Identification of viruses associated with the disease
2) Development of PCR based diagnostic methods for rapid and accurate identification of the viruses
3) Cloning of components of geminiviruses and RNA viruses associated with the disease
4) DNA sequencing and phylogenetic analysis of components for identification and relationship of these components
5) Infectivity analysis of the geminivirus components
6) Development of an RNAi construct for pathogen derived resistance, stable transformation of the construct in *Nicotiana benthamiana* and evaluation of transgenic plants.
Chapter 2

Materials and methods

2.1 Collection of samples and detection of viruses associated with the disease
A severe epidemic of melon, suspected to be of virus etiology was reported by farmers of melon-growing areas of Punjab. The epidemic was so severe that farmers had to abandon muskmelon cultivation in traditional melon growing areas. During a search for cucurbit viruses in May 2004, samples were collected from four different fields in Faisalabad, Vehari, Sahiwal and Khanewal districts of the Punjab province and from four different fields in Mardan, NWFP. At each location melon fields were visited and samples were collected, moving diagonally across the field, from plants showing infection. Each sample, consisting of three leaves per plant, was wrapped in polythene bags and placed in an ice box. Asymptomatic leaf samples were taken as controls. Samples were brought to Molecular Virology Laboratory of the National Institute for Biotechnology and Genetic Engineering, Faisalabad.

2.1.1 Serology
On the basis of symptoms cucurbit-infecting viruses were suspected. Samples were screened for the presence of *Zucchini yellow mosaic virus* (Desbiez and Lecoq, 1997) and *Cucumber mosaic virus* (CMV) by DAS-ELISA using virus specific polyclonal antisera according to manufacturer’s instructions (Loewe, Germany).

2.1.2 Total genomic DNA extraction
DNA was extracted from leaf samples by the method described by Doyle and Doyle (1990). The water bath was turned on and set at 65°C and 2X CTAB (cetyl triethyl methyl ammonium bromide) (appendix-1) without 1% 2-mercaptoethanol was preheated. The autoclaved pestles and mortars were pre-cooled with liquid nitrogen. Three young leaves weighing 2 grams were detached from each sample preserved, washed with distilled water, blotted dry and ground into a very fine powder with liquid nitrogen in a mortar and pestle. The powder was transferred to a 50ml tube and 15ml of hot (65°C) 2X CTAB was added to the tube before the frozen powder started thawing. The paste was mixed gently by inverting the tube several times and incubated at 65°C for 30 mins with occasional swirling. An equal volume (15ml) of chloroform-isoamylalcohol (24:1 v/v) was added and mixed gently by inverting the
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tube to form an emulsion. Then the tubes were centrifuged for 10 minutes at 9000rpm (Eppendorf centrifuge 5810: Germany). The the top aqueous phase was transferred to a new 50ml tube and the remaining chloroform phase was discarded. To precipitate the DNA, 0.6 volumes chilled isopropanol was added. The tubes were again centrifuged at 9000rpm (Minispin, Eppendorf: Germany) for 5 minutes and the supernatant solution was discarded. The pellet was air dried and re-suspended in 50 µl de-ionized double distilled (d$_3$H$_2$O). The suspensions were transferred into eppendorf tubes and then 5ul of RNase (100µg/ml) was added and incubated for one hour at 37°C. An equal volume of chloroform-isooamyl alcohol (24:1) was added and mixed gently. The eppendorfs were spun for 10 minutes at 13000rpm (Eppendorf centrifuge 5810: Germany) in the micro centrifuge and the supernatant was transferred into new autoclaved eppendorfs. To the mixture 1/10th volume of 3M sodium acetate (pH 5.2) was added and mixed gently. The DNA was precipitate with chilled absolute ethanol (2.5 volumes) and placed at -20 ºC for 30 minutes. The samples were spun at 13000 rpm for 10 minutes, the supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was finally air dried and re-suspended in d$_3$H2O and stored at -20°C.

2.1.3 DNA quantification

The concentration of DNA in each sample was determined using a SmartSpec Plus (Bio-RAD, USA) fluorimeter (Appendix-). Each DNA sample was diluted to a uniform concentration of 1µg by adding d$_3$H$_2$O. The DNA dilutions were stored at 4°C untill further use. The quality of DNA was checked on 0.8% (w/v) agarose gels.

2.1.4 Geminivirus detection by PCR

PCR was carried out using universal primers which have the ability to amplify any begomoviruses. Primers were designed in a highly conserved region of the C1 ORF (Briddon and Markham, 1995). The primers used in PCR were Begomo1 (CCGTGCTGCTGCCCCCATTGTCCGTCAC) and Begomo2 (CTGCCACAACCATGGGATTCCACGCACAGGG) that were expected to produce a PCR product of about 1.1 kb. The other set of primers used in PCR consisted of Begomo3 (GTTCCCCTGTCGTGATCCATGGTGTGG) and Begomo4 (TTTTGTGCAGCGACATGGGGGGGCAGCA) that were expected to produce a PCR product of about 1.7 kb. PCR was carried out using Taq DNA polymerase.
According to the manufacturer’s instruction (Fermentas, Germany). The PCR was done in a reaction volume of 50 μl containing 10 pmol of each primer. Viral DNA fragments were amplified for 35 cycles of 94°C for 60sec, 50°C for 60sec, and 72°C for 120sec after an initial denaturation of 94°C for 5min. A final extension of 72°C for 10 min was included. The optimum reagents used were template DNA 5μl (2μg), 5μl dNTPs (2mM), 5μl PCR Buffer (10X), 3μl MgCl₂ (1.5mM), 1μl Primer1 (5μM), 1μl Primer2 (5μM), 0.5μl Taq polymerase (1.5U), 29.5μl d³H₂O. thermal cycler (Thermo PxE 0.2) with heated lid was used for PCR.

2.1.5 Agarose gel electrophoresis of PCR products

Amplified products were analyzed by electrophoresis in 1.0% (w/v) agarose gels and detected by staining with ethidium bromide (100μg/ml). Before loading PCR products in the gel, 5μl 6X loading dye (appendix-10) was added to the reaction mixture. Only 10 μl of PCR product from each reaction was loaded on the gel submerged in 0.5X TBE buffer (appendix-2). Samples were electrophoresed for approximately two hours at 50 volts. After electrophoresis, the amplified products were viewed on an ultraviolet trans-illuminator and photographed using a Stratagene Eagle Eye still video system.

2.1.6 Designing of abutting primers for amplification of full-length components of geminiviruses

Primer design was based upon the sequence data of the partial sequences obtained by using universal primers. Primers were designed around a unique KpnI restriction site in the CP. Since the PCR product was to be cloned directly into the TA cloning vector, no extra bases were added before KpnI site. These primers were Melonfor (5’GGTACCTAAGGACCTGGGTTCTG3’) and Melonrev (5’GGTACCTGGATATGCTAGGTGTATAGG3’).

Similarly the primers for genomic length amplification of DNA B were designed on the basis of partially sequenced data obtained by using MP primers. HindIII restriction site in MP gene was used in the forward and reverse primers designed for full-length DNA B amplification. The primers were TLCVB F (GCTAAGCTTTCTGCTCGAACATGGATGGAA) and TLCVB R (CAGAAGCTTA GCCAGTTGAGGAATAGGTAG).
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2.1.7 PCR for full-length amplification of DNA A and DNA B

For amplification of full length DNA A of begomovirus the PCR was done in a reaction volume of 50 µl containing 10pmol of each set of primers. Viral DNA fragments were amplified using 40 cycles of 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes after an initial denaturation of 94°C for 5 minutes. A final extension of 72°C for 20 minutes was included. The reagents used were as described in section 2.1.4. A Thermo Electron Corporation thermal cycler (PxE 0.2) was used for PCR.

For amplification of genomic length DNA B both primers were used at a concentration of 10pmol and the reaction volume was 50µl. The viral component was amplified for 40 cycles. First 8 cycles of 94°C for 1 minute, 45°C for 2 minutes, and 72°C for 3 minutes were used. Then 32 cycles were amplified with the usual profile of 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes. The reagents used were in same conc. as described in section 2.1.4

2.1.8 Cloning of full length DNA A and DNA B in pTZ57R

Phenol-chloroform purification of each PCR product for both DNA A and DNA B was performed and then they were precipitated using 3M sodium acetate (Appendix-5) and ethanol. After that each PCR product was ligated to the pTZ57R vector. Amount of PCR product used was based on the intensity of band on agarose gels. The ratio of the reagents used in reaction mixture was vector 2µl pTZ57R/R (0.055µg/µl), 5µl PCR Product (0.165µg), 2µl ligase buffer (10X), 2µl PEG4000, 1µl T4 DNA ligase (5u) and 9µl d3H2O. The total volume of reaction mixture was 20µl. These ligations were kept at 16°C overnight and then transformed into heat shock competent *E. coli* (10B) (Appendix-5). The transformation mixtures were then spread on LB agar (appendix-8) ampicillin plates after one hour of incubation at 37°C. Plates were incubated overnight at 37°C and the next day colonies were cultured in LB broth (Appendix-8) containing ampicillin and placed overnight in shaking incubator at 37°C. Plasmid isolation from cultures was performed the next day (Appendix-6). Cloning was carried out by using *E. coli* strain 10B. TA cloning vector
pTZ57R (MBI Fermentas) was used for cloning purposes. It has ampicillin resistance and blue/white selection.

2.1.9 Confirmation of clones of DNA A and DNA B and sequencing
For confirmation of clones the isolated plasmids were digested with EcoR1 and PstI enzymes. Total size of the insert was determined by counting the sizes of bands on the gel excluding plasmid vector band. Then selected clones were restricted with specific restriction enzymes for which sites were inserted into the primers, used to amplify the components, to finally confirm the clone. The reaction mixtures were incubated at 37°C for 1 hr and after digestion, the insert was released from the vector, which was checked on 1% agarose gel using standard DNA markers (GeneRuler 1kb DNA ladder, Fermentas, Germany). Reagents and their ratio used in restriction analysis was: DNA 2 µl (4µg/20ml), KpnI (10u/µl, for DNA A) or HindIII (10u/µl, for DNA B) 0.5 µl, KpnI buffer (for DNA A) or red buffer (for DNA B) 2µl, RNase enzyme (0.01µg/µl) 1µl, d3H2O 14.5µl and the total volume of reaction mixture was 20µl. Putative full length clones were sequenced commercially (Macrogen Korea).

2.2 Detection of begomovirus in plants by southern blot hybridization
Total DNA of transgenic plants was isolated by CTAB method (Doyle and Doyle, 1998) and was electrophoresed on 1% agrose gel and transferred to membranes for further processing.

The gel was treated with solutions I, II and III (Appendix-3). After the treatment the gel was placed on the wedge of filter paper and on the gel by nylon membrane was placed, exactly the size of the gel. The edges of the gel were dipped into 20XSSC (Appendix-3). The 20XSSC soaked filter papers were also placed on the nylon membrane and these were placed under a weight overnight. Following day membrane was separated from the gel and was UV cross linked for 2 minutes in Stratalinker (Model C1-1000).

After UV cross-linking the blot was transferred in the pre-hybridization solution. The pre-hybridization solution was composed of 10ml 20XSSc, 5ml 50X denhart, 5ml 10% SDS, 10µl Salmon sperm DNA (5ng) and finally distilled water was added (25ml) to make the total volume of 50 ml. The blot in the hybridization bottle was
incubated at 65°C for 2 hours in prehybridization solutions in a Hybaid (Midi dual-14) hybridization oven.

The radioactively labeled probe was made from the PCR product of sequences of HC-Pro used in RNAi construct. 10 ng of DNA was used to prepare the probe. The reaction mixture contained 10µl DNA (10µg), 10µl buffer, 3µl Mix c, 3µl dCTP, 2µl Klenow enzyme (1.5u), 17µl nuclease free H₂O and the total volume of the reaction mixture was 45µl. The reaction was finally incubated at 37°C for 1hr. After this, the probe was denatured at 94°C in a heating block (Thermoplax, 16500 Dri-Bath) for 2mins. The reaction mixture was then transferred into hybridization bottle and incubated for 16hrs at 65°C. After 16 hours the blot was washed three times with 2XSSC and 0.5XSSC respectively keeping the SDS concentration constant (0.1%w/v). The blot was wrapped on clink film and then placed on the X-Ray film (Super RX: Fujifilm) for four hours and then developed.

2.3 Electron microscopy for virus particles
One gram (1g) of infected muskmelon tissue was homogenised in distilled water and centrifuged at 3000rpm for 5 mins in a microcentrifuge. A 10-25µl drop of the preparation was loaded on to a carbon coated grid (AE1) (38µm, 400 mesh) and left for 30 seconds. The excess liquid was removed with the help of a small piece of filter paper. Then 20µl of 2% uranyl acetate (pH 3.5) was loaded on the grid and left for one minute and the grid was drained using same technique. After this a second stain, 5% potassium permanganate, was applied and left for 30 sec and drained. The grid was washed twice by putting a 20 µl drop of water on the grid. After drying, the grid was left for 2 minutes to air dry and examined with a JEOL 100s electron microscope.

2.4 Production of constructs for Agrobacterium mediated transformation
A partial duplication of the DNA A genomic component was made in two cloning steps. First a 1680 bp fragment was released with KpnI and BamHI from the genomic length clone of DNA A in pTZ57/RT and cloned into pGreen0029. In the second cloning step the pGreen0029 vector having the 1680 bp fragment was restricted with KpnI. The full-length (2.8 kb) fragment was excised from the pTZ57RT clone and ligated into the KpnI site. The orientation of clone was confirmed by digesting with BamHI. The binary vector carrying the partial tandem repeat (PTR) of DNA A was
mobilized into Agrobacterium strain LBA4404 and coinoculated to N. benthamiana with a known infectious clone of DNA B of ToLCNDV kindly provided by C.M. Faquet (Padidam et al., 1996).

A partial repeat molecule of DNA B was also constructed in pGreen 0029 vector. The full-length clone of DNA B was restricted with HindIII and MluI and the 823bp band excised was ligated into pGreen0029. The full length DNA B clone was partially digested with HindIII and ligated into pGreen0029 having the partial segment of DNA B digested with HindIII.

2.4.1 Agroinoculation of clones for infectivity analysis
The infectivity of the partial repeat constructs of DNA A and DNA B components was determined by coinoculating with infectious PTRs of the components of ToLCNDV. The PTR constructs in pGreen0029 were transformed into A. tumefaciens (GV3101) by electroporation (Appendix-7). Agrobacterium cultures were grown at 28°C for 48 hrs in liquid LB medium containing 50µg/ml of kanamycin and 25µg/ml rifampicin. The bacterial cells were pelleted (5,000 × g for 10 mins at 20°C) and were re-suspended in 10 mM MgCl₂ and 150µg/ml acetosyringone. After three hours of incubation the cells were infiltrated using a 5ml sterile syringe into young fully expanded leaves of 4 week old N. benthamiana plants. For co-infiltration, cultures of different constructs were mixed in equal proportions.

2.5.1 Isolation of total RNA
Before RNA isolation the work bench was cleaned with bleach and ethanol. All the apparatus, including pestle, mortar, spatula, forceps, eppendorf tubes and tips were autoclaved. The eppendorf tubes and tips were treated with 0.01% DEPC (diethyl pyrocarbonate), autoclaved and baked in an oven at 80°C overnight before use. Gloves were worn at all the times during RNA preparation since RNases present on the fingers can degrade RNA quickly. The integrity of the total RNA isolated was checked by electrophoresis on 1% agarose gel. The concentration was measured spectrophotometrically and the total RNA was stored at -70°C after making appropriate aliquots.
2.5.2 Cloning of HC-Pro

First strand cDNA was synthesized from total RNA using reverse transcriptase (Fermentas). For synthesis of the first strand the primer HC-ProF (ATGATGGCCAGCGAAGTTGACCACTATTC) was used. The reaction mixture was prepared in a tube on ice and contained 10µl total RNA, primer (10pM) 1µl and d$_3$H$_2$O 12µl. Reagents were mixed gently and centrifuged for 3-5 seconds in a microcentrifuge. The reaction mixture was incubated at 70°C for 5mins, then chilled on ice for two minutes and collected at the bottom of the tube by brief centrifugation. To this 4µl reaction buffer (5x), 1µl ribonuclease inhibitor (1.5u), 2µl dNTPs (10mM) were added and centrifuged briefly. The tubes were incubated at 37°C and 1µl reverse transcriptase (5U) was added and incubated at 42°C for 60minutes. The reaction was stopped by heating at 70°C for 10 minutes and chilled on ice. The full-length HC-Pro gene of ZYMV was amplified from cDNA by using prime rs HC-Pro F (ATGATGGCCAGCGAAGTTGACCACTATTC) and HC-ProR (CCCGGGTTAGCCAACTCTGTAATGTT). The primers were designed using gene sequence data available in the database (GenBank, NCBI). The gene fragment was amplified using 40 cycles of 94°C for 1 minute, 52°C for 2 minutes, and 72°C for 3 minutes after an initial denaturation of 94°C for 5 minutes; a final extension was 72°C for 10 minutes was included. The reagents used were in the same concentration as described in section 2.1.4. A Mycycler (Fermentas) thermal cycler was used for PCR.

2.5.3 Production of deletion mutant of HC-Pro

In order to assess the role of HC-Pro in synergism a mutant was produced by deleting the central 208 amino acids. The N-terminal part of HC-Pro was amplified by PCR using primers HC-Pro F (ATCGATGGCCAGCGAAGTTGACC) and HC-mutR (AAATCATATGTTGACGTTTCCTTGAA). Similarly, the C-terminal part of HC-Pro was amplified by PCR using primers HC-mutF (TGTACATATGTTGACGTTTCCTTGAA). PCR conditions were as used in the amplification of the whole HC-Pro gene. Amplified products were ligated together, using Nde1 enzyme site introduced in both products, in pgR107.
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Figure 2.1 Diagram of the structure of PVX-HC-Pro (A) and HC-Pro-RNAi (B). (A) PVX-HC-Pro vector carries the coding region of ZYMV HC-Pro with appropriate translation start and stop codons. Smal and ClaI restriction sites were used for cloning HC-Pro in the PVX vector (pGR107). (B) Sense ZYMV HC-Pro and CP sequences were ligated together using introduced HindIII sites and cloned into pFGC5941 in sense orientation using XhoI and NcoI restriction sites. Similarly antisense fragments were cloned using BamHI and XbaI sites.
2.5.4 Cloning of HC-Pro and HC-Pro mutant in PVX expression vector

Intact and mutant HC-Pro genes were cloned in pgR107 (PVX vector, Appendix-10). A start codon at the 5’ end and a stop codon at the 3’ end were included so that mature HC-Pro product is produced. The primers (HC-Pro F and HC-Pro R) had introduced SmaI and ClaI restriction enzymes. HC-Pro, pgR107 and the mutant HC-Pro were restricted with SmaI and ClaI. After this, next step was to ligate the restricted products together in the ratio: insert 4 µl HC-Pro (2µg/20µl), vector (pgR107) 2µl (0.055µg/µl), ligase buffer (10X) 2µl, 1µl T4DNA ligase (1.5u), d.H2O 11 µl and 20 µl was the total volume of mixture. After ligation the products were transformed into E. coli as described earlier and then into A. tumefaciens GV3101. Transformed A. tumefaciens cultures were grown in LB medium as described in section 2.4.1

2.6 Cloning of the coat protein fragment of ZYMV

Seventeen sequences of ZYMV were retrieved from the data base (NCBI) and aligned using MegAlign (DNAstar). One set of primers CP F ( TAGTAAGCTTTTAGGCC GCCTACCTAGGTT) and CP R (GCCTCTCGAGCTTATTCGTGAGAGGCTCA) were designed to a highly conserved area of the C-terminus to amplify 178 bases. PCR profile described earlier for amplification of HC-Pro was used. The coat protein gene amplified was cloned into pTZ57RT and confirmed by restriction endonuclease digestion as described earlier.

2.7.1 Production of RNAi construct with ZYMV sequences

HC-Pro and CP sequences of ZYMV were cloned in sense and antisense orientation under 35S promoter in pFGC5941 RNAi plant expression vector. E. coli strain 10b and A. tumefaciens (strain LBA4404) were used for cloning and plant transformation experiments. XhoI, NcoI, BamHI, XbaI and HindIII (MBI Fermentas) were used. Certified seeds of N. tabacum (var. Samsun) and N. benthamiana were used.

2.7.2 PCR amplifications of HC-Pro and CP segments

HC-Pro was first amplified using primers HC-Pro F (AACACCATGGCTTAAGAGCC CCGACGAAG) with introduced NcoI restriction site and HC-Pro (RTCGTAAGCTT TCCTGGGTAATTCAGCGC) with introduced HindIII site using conditions described previously. Similarly, the CP was amplified using primers CP F
(TAGTAAGCTTTAGGCCGCTACCTAGGTT), with a HindIII restriction site, and CP R (GCCTCTCGAGCTTATTCGTGAGAGGCTCA), with XhoI restriction site. Then HC-Pro and CP PCR products were ligated together using the introduced site of HindIII site and cloned into pTZ57R/T pTZ57R/T. An antisense fragment was amplified from the sense clone using primers ATS F (AACAGGATCCCTTAAGAGCCCGACGAAG) and ATS R (GCCTTCTAGACTTATTCGTGAGAGGCTCA) and ligated into pTZ57R/T. The sense fragment was cloned into pFGC5941 using NcoI, XhoI after restriction from pTZ57R/T. The antisense cassette was cloned into pFGC5941 containing the sense cassette using BamHI and XbaI.

2.7.3 Transformation of RNAi constructs in Agrobacterium tumefaciens (LBA 4404) by electroporation

PFGC5941, containing the RNAi expression cassette was transformed into Agrobacterium tumefaciens (strain LBA 4404) by electroporation (Appendix-7). These transformations were confirmed by PCR using primers HC-Pro F and HC-Pro R.

2.7.4 Expression constructs and Agro-inoculation of cloned DNA components

PTR constructs of DNA A and DNA B in pGreen0029 were transformed into E.coli 10b and then into A. tumefaciens GV3101 (Hoekema et al., 1983). Similarly, complete HC-Pro and a mutant of HC-Pro with deleted central region were also cloned in PVX expression vector (pgR107) at ClaI and SmaI restriction. Similarly a mutant of HC-Pro was produced by deleting central 208 amino acids and transformed into E.Coli and then into A. tumefaciens. Agrobacterium cultures were prepared as described previously in section 2.4.1.

2.8 Plant transformation

2.8.1 Transient assays

For transient assay of virus movement seven types of expression constructs were used. DNA A and DNA B of ToLCNDV (dimeric clones) in pBin 20 vector, DNA A and DNA B of MYLCV (PTR) in pGreen0029 vector, HC-Pro and HC-Pro mutant in pgR107 vector, RNAi construct of HC-Pro in pFGC5941 vector. Cultures were grown in 250 ml of LB medium containing 100 µg/ml of kanamycin for 2-3
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days at 28°C in a shaking incubator. After three days the optical density (OD) of all the cultures were made uniform (1 at 600nm) and then centrifuged in 50 ml centrifuge tubes in an Avanti centrifuge at 4000rpm. The supernatant was discarded and the pellet was re-suspended in 10ml of freshly prepared 10 mM MgCl$_2$ containing 100µM acetosyringone. These cultures were kept at room temperature for 12 hrs.

2.8.2 Agroinfiltration

Three replicates of experiments were conducted. For each plant three leaves were infiltrated and approximately equal amount of inoculum was delivered to each leaf with the help of a syringe without needle. Viruses components were inoculated in different combinations to infect *N. benthamiana*. Ten plants were inoculated for each inoculum.

2.8.3 Agrobacterium mediated transformation of *Nicotiana benthamiana*

Seeds were surface sterilized by soaking them in 5% liquid bleach, 1% HgCl$_2$ and 1% SDS for 15mins followed by a dip in 70% ethanol for one minute. Ethanol was removed and seeds were thoroughly rinsed three times with double distilled water. Seeds were sown in seed germination medium (Appendix-9). After two weeks, plantlets were transferred to fresh MSO medium. After 3-4 weeks, fully expanded leaves were used for cutting leaf discs.

A single colony of *A. tumefaciens* harbouring the RNAi construct was picked and inoculated into 25ml of liquid LB medium (Appendix-8) containing having 100µg/ml rifampicin and 50µg/ml BASTA in a 100ml flask and incubated with shaking (150-250rpm) for 48 hrs in the dark. Leaf discs were cut from sterilized leaves under aseptic conditions and placed on MS0 medium (Appendix-9), in petridishes with 25-30 discs per plate. Plates were sealed with parafilm and incubated with 16/8hrs light and dark cycle at 25°C. Explants were left to preincubate for 24 hours.

The *Agrobacterium* culture was centrifuged at 3000 rpm for 10 mins and resuspended in MS0 medium (Appendix-9) to an OD of 0.4-0.5 at 590 nm. 30ml of bacterial suspension was placed in a sterile falcon tube and leaf discs were placed in the bacterial suspension. The tube was inverted gently for about 2 to 5 minutes. The leaf
discs were removed and blotted dry on sterile filter paper and transferred to Petri plates containing co-culture medium (Appendix-9). Plates were sealed with parafilm. Co-culture was performed for 48-72 hours at 25 °C (depending on the growth of bacteria) in an incubator with a 16/24 light-dark cycle.

Leaf discs were removed from the co-culture medium and placed on selection medium (Appendix-9) after washing with cefotaxime (250 mg/l). Leaf discs were placed up with the cut edge in contact with the agar. For each transformation 10-15 leaf discs were placed on each petri plate, plates were sealed with parafilm and incubated with a 16hr photoperiod at 25° C for two weeks. Regeneration started after 15-20 days. About 70% regeneration was observed in the beginning but some discs bleached out due to the presence of selection agent (BASTA). Regenerated leaf disc were transferred to fresh selection medium (MSO selection medium; Appendix-9). When shoots became too large for the petri dishes, they were transferred into magenta containers containing rooting media (Appendix-9).

Shoots were cut from the callus when at least 1 internode was formed. Shoots were transferred into rooting media with reduced antibiotic concentration (Appendix-10). Plants were transferred to pots containing sterile soil and kept at 25°C with a 16 hrs photoperiod to harden. The agar medium was removed by washing under running tap water. Plants were covered with polyethylene bags to retain humidity. After 7-10 days, bags were gradually opened to reduce humidity until plants were acclimatized to ambient humidity and temperature conditions.

Control experiments were also performed. Non-transformed leaf discs were placed on two different media, one herbicide and the other without antibiotic, to observe the effect of selection agent (BASTA). All leaf discs on the plates having antibiotic bleached out while the discs in the plates without antibiotic regenerated vigorously. This indicated that on selection medium only those discs would regenerate that had the gene for BASTA resistance expressed by pFGC5941.
Chapter 3

Results

3.1 Survey for melon leaf curl disease

A survey was conducted in cucurbit growing areas of the Punjab and NWFP provinces of Pakistan in the summer seasons of 2004-06. Five districts of Punjab province (Faisalabad, Toba Tek Singh, Sahiwal, Vehari and Khanewal) were surveyed (Fig. 3.1). Virus-like symptoms were observed in all fields visited in the Punjab in spring/early summer season in all three years. A destructive disease was observed on muskmelon (*Cucumis melo*, varieties Ravi and T96) in all melon growing districts. The field survey of muskmelon revealed a high level of disease incidence and greater disease severity in Vehari and Sahiwal districts, where there was almost 100% crop loss in some areas (Fig: 3.2). Aphids were observed early in the season while both aphids and whiteflies were present in the field later in the season. For comparison, muskmelon fields in Mardan district (NWFP) were surveyed for the presence of viruses in the same period. These areas are widely distributed from each other and have different cropping patterns due to differences in climatic conditions. Similar mosaic symptoms, leaf distortion and enations on the upper side of the leaves were observed but the fields were less yellow and the severity of the disease was low in comparison to the Punjab.

3.2 Symptoms of the disease

Symptoms of the disease were found on the vegetative portion of muskmelon but not on the fruit. In the case of Punjab province, leaf yellowing and stunting of plant growth was also observed and fields looked yellow from a distance (Fig. 3.2). Mosaic was a common symptom widely present in infected fields of both provinces. The disease started with leaf curling, mosaic and with enations on the upper sides of leaves followed by stunting. Symptoms were so severe that fields were yellow. In NWFP, leaf distortion, mosaic and enations on the upper side of the leaves were observed (Fig. 3.3). Similarly the symptoms were observed on leaves and stem but not on the fruit. Leaf curling, enations and chlorosis symptoms were present in the field but it did not look yellow. The severity of the disease was higher in Punjab as compared to that occurring in NWFP. Consequently, the yield was very low.
Fig.3.1: Map of Pakistan map showing muskmelon growing areas of the Punjab and NWFP (encircled in red). Map sourced from: http://www.pakconhk.com
Figure 3.2. Symptoms of disease in the Punjab. (A) Muskmelon field in Sahiwal district of Punjab showing widespread disease. The whole field shows devastation and thus crop loss. (B and C) Infected melon plants showing typical symptoms of the disease including leaf curling, enations on the upper side of the leaf, leaf distortion, yellowing and stunting in the plant. Severe leaf curling and stunting along with mosaic type of symptoms in Vehari district (D and E)
Results

A

B

C

D

E
Figure 3.3. Symptoms of disease on muskmelon in NWFP. (A) Muskmelon field in Mardan district of NWFP with less intensity of disease in comparison to the situation in the Punjab. (B and C) Infected muskmelon plants showing typical symptoms of the disease including enations on the upper side of the leaf, leaf distortion and mosaic. Leaves are less yellow in comparison to those observed in Punjab. Leaf curling and mosaic symptoms in field conditions (D and E)
Results

A

B

C

D

E
3.3.1 Melon yellow leaf curl disease is associated with ZYMV

Most of the cucurbit growing areas of the both provinces are multiple cropping areas. Crops belonging to several families, including Cucurbitaceae, Fabaceae and Malvaceae are hosts of many plant-infecting viruses. In view of the symptoms of the disease and prevalence of viruses in the area, both begmoviruses and RNA viruses were suspected. These viruses have been reported to affect cotton, tomato and chili crops in the area (Mansoor et al., 1993; Hussain et al., 2004; Shih et al., 2003).

Electron microscopy on sap extracts of symptomatic and asymptomatic leaves samples from Punjab was performed using negative staining. Only long flexuous rod shaped particles, typical of potyviruses (Daphne et al., 1984), could be observed (Fig. 3.4). This finding strongly suggested that a potyvirus could be associated with the disease.

Symptomatic samples were tested against commercially available polyclonal antisera to ZYMV and CMV by Enzyme-linked immunosorbent easy (ELISA). A total of sixty infected melon plants were screened from each province. The results of serological tests showed the presence of ZYMV in infected samples of both provinces. However, CMV was found only in samples from NWFP (Table 3.1). Specific primers were used for partial amplification of CP after synthesis of cDNA for identification of the strain of ZYMV present on muskmelon in the Punjab. Partial sequences of coat protein (CP) of ZYMV showed 98% sequence identity to an already reported ZYMV isolate from Pakistan (acc. no. AB127936; Ali et al., 2004) belonging to worldwide subgroup 1 isolates occurring in China and Veitnam (Ha, et al., 2007).

These results show that MYLCD infected plants in both the Punjab and NWFP are associated with ZYMV, a virus previously reported in muskmelon in Pakistan and that is of global importance for cucurbit production. In contrast CMV appears to occure only in muskmelon plants originating from NWFP.

3.3.2 Melon yellow leaf curl disease is associated with a begomovirus

Total DNA was used as template in PCR with a set of degenerate primers (begomo1 and begomo2; section 2.1.4) designed for partial amplification of begomovirus DNA
A. Fragments of the expected size were amplified from nine samples out of twelve (Fig. 3.5) and products were cloned in the TA cloning vector pTZ57RT (Fermentas). Confirmation of clones was done by EcoRI and PstI restrictions sites in plasmid vector and a fragment of 1.1 kb was released. Sequence analysis of the cloned product showed the highest sequence identity (86%) to ToLCNDV. To check the bipartite nature of the virus, specific primers for the MP (MPF and MPR) gene of ToLCNDV encoded on DNA B were used in PCR reactions. All nine muskmelon samples found positive for DNA A were also found positive for DNA B. Amplified MP products were also cloned in a pTZ57RT and were completely sequenced. The MP showed the highest level of sequence identity (89%) to the MP of ToLCNDV.

Samples were also tested for the presence of begomoviruses by Southern blot hybridization using the DNA A of ToLCNDV (Padidam et al., 1995) as a general probe for begomoviruses. Post hybridization washes were done at medium-stringency and should in theory detect all begomoviruses under these conditions. The probes hybridized with all nine samples collected from symptomatic plants found positive by PCR, whereas the probe did not hybridize with asymptomatic samples (Fig. 3.6). These results further confirmed the presence of a begomovirus in symptomatic muskmelon samples. All symptomatic samples originating from the Punjab were positive for begomovirus, whereas all samples from NWFP were negative (Fig. 3.6).

All the samples positive for begomoviruses in the Punjab were also positive for ZYMV. Thus the disease in Punjab was associated with dual infection of begomoviruses whereas samples from NWFP were positive for CMV and ZYMV. None of the sample positive for begomovirus was positive for CMV in the Punjab. In view of the severe disease in Punjab caused by begomovirus and ZYMV, further characterization.
Figure 3.4. Long flexuous particles detected in extracts of melon from the Punjab. Samples are negatively stained with 5% uranylacetate and viewed on a JEOL JEM 1010 electron microscope.
### Table 3.1. Results of ELISA screening for the presence of ZYMV and CMV and PCR for the detection of begomoviruses

<table>
<thead>
<tr>
<th>Origin of sample</th>
<th>No. of samples tested</th>
<th>CMV</th>
<th>ZYMV</th>
<th>Begomovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field 1 (Mardan)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>-</td>
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<td>10</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Sahiwal (Punjab)</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Faisalabad (Punjab)</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Toba Tek Singh (Punjab)</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Khanewal (Punjab)</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 3.5. PCR amplification of a 1.1 kb DNA A fragment of begomoviruses, using diagnostic primers begomo1 and begomo2, from infected muskmelon samples from fields in the Punjab. Lane 1 shows 1Kb ladder (MBI, Fermentas), lane 2 is a negative control (genomic DNA extracted from a healthy muskmelon plant), lane 3 is a positive control (genomic DNA extracted from an *N. benthamiana* plant experimentally infected with ToLCNDV clones by *Agrobacterium*-mediated inoculation) and lane 4-6, 8-11, 13-14 are muskmelon samples from Punjab province. Lane 7, 12 and 15 show samples negative for cucurbit viruses.
**Figure 3.6.** Southern blot of nucleic acids extracted from field collected melon samples originating from the Punjab. The blot was probed with radioactively-labeled PCR product spanning the Rep gene of ToLCNDV (U15015). The samples run in lanes 1-13 originate from the Punjab (Vehari [lanes 1-3], Sahiwal [lanes 4-6], Faisalabad [lanes 7 and 8], Toba Tek Singh [lanes 9 and 10] and Khanewal [lanes 11-13]). Samples in lanes 14-19 originate from NWFP (Mardan District). Total DNA extracted from a *N. benthamiana* plant experimentally infected with the clones of ToLCNDV (Padidam *et al.*, 1995) was run as a marker in lane 20. Approximately equal amounts of DNA (10µg) were run in each lane.
3.3 The satellite DNA β is not associated with MYLCD

The possibility that MYLCD affected muskmelon plants additionally contained the DNA β satellite was examined by diagnostic PCR with universal DNA β primers (Briddon et al., 2002). These primers have proven very reliable for the identification and subsequent cloning of DNA β satellites. Amplifications with these primers and nucleic acids extracted from MYLCD affected plants were universally negative (results not shown). In a few cases, amplification products larger than expected (~1.7kb) were produced. Cloning and sequence analysis showed these amplification products not to be related to DNA β (results not shown).

Southern blots of gels containing nucleic acids extracted from MYLCD affected muskmelon plants, probed for the presence of DNA β using a CLCuD DNA β (Briddon et al., 2001) as a probe, and washed at low stringency (which should detect the majority of DNA β) were similarly negative (results not shown). These findings indicate that DNA β is not associated with MYLCD.

3.4 Molecular characterization of the begomovirus associated with MYLCD

3.4.1 Cloning and sequence analysis of DNA A

On the basis of partial sequences obtained, back-to-back primers for amplification of full-length DNA A (MelF and MelR; section 2.1.6) were designed to the coat protein region on a naturally present, unique KpnI restriction site. A full-length product (approximately 2.7 kb) was obtained and cloned in pTZ57RT. Restriction of the clone with KpnI resulted in a single band on the gel whereas restriction of clone with SmaI linearized the clone and a 5.6 kb band appeared on the gel. The full-length clone was sequenced in both orientations. A sequence similarity search (Blast) was performed by comparing the sequence to other begomovirus sequences in the database. The results showed that the sequence has a high level of identity to begomoviruses from Asia. The DNA A comprised of 2756 nucleotides and showed the highest levels of nucleotide sequence identity (86.7%) to a ToLCNDV isolate from Rahim Yar Khan, Pakistan (accession no. DQ116885). On the basis of DNA A sequence identity, the begomovirus was proposed as a new species and was named Muskmelon yellow leaf curl virus (MYLCV). Sequence analysis showed that it is a typical Old World begomovirus and encodes four open reading frames (Rep, REn, TrAP and AC4) in the complementary-sense and two (precoat and CP) in the virion-sense (Table 3.2).
Results

The IR contains the conserved nonanucleotide motif (TAATATTAC) forming part of the loop of a predicted stem-loop structure found in all geminiviruses, a TATA motif (a sequence found in the promoter region and binding site for transcription factors (Lauf et al., 1995) (2664-2667 nt)) and an iteron sequence (GGTGTC) 5' of the TATA motif (Lauf et al., 1995; Chatterji et al., 1999). The predicted proteins were of the sizes expected for DNA A encoded proteins (Table 3.2) (Padidam et al., 1995). A gene-wise comparison of DNA A of MYLCV is given in table Table 3.3. genome organization of MYLCV DNA A is shown in Fig 3.7

3.4.2 Cloning and sequence analysis of DNA B

The partial sequences of DNA B observed were used to design back-to-back primers for amplification of full-length DNA B. These primers (TLCVBF and TLCVBR; section 2.1.6) were designed in the movement protein (MP) gene. Products of a range of sizes were detected on the gel (0.9, 1.1, 1.4 and 2.7 kb). The desired sized fragment (2.7 kb) of DNA B was cloned in pTZ57RT. The clone was fully sequenced and genomic length of DNA B sequence was 2724 bases. The DNA B showed 73.3% sequence identity to a Pakistani isolate of ToLCNDV (AY150305). However, maximum nucleotide identities in Blast search of ORFs were 73.3% (AY150305) and 85.7% (AY150304) for NSP and MP respectively. The second closest specie is a cucurbit infecting virus originating from Bangladesh known as Cucumber leaf curl virus (CuLCuV; EF450316).

NSP has a size of 555 nucleotides (184 amino acids) and it is truncated at the N-terminus since first 84 amino acids are missing in comparision to the ToLCNDV sequences available in the database (Fig. 3.8). The MP has a size of 884 bases (294 amino acids) (Table: 3.2). Non-coding IR sequences of DNA B are distinct from already published sequences (Fi. 3.8). The intergenic regions of DNA A and DNA B show only 38.2% identity but share a 34-bp potential stem-loop forming region (GGCCATTTCGTTATAATTACCAGAATGGCCGCGGT). This sequence has the conserved nonanucleotide sequence (TAATATTAC). The iterated elements (iterons) were close to a TATA box in the common region and were identified as GGTGTC being the same as those identified in DNA A (Lauf et al., 1995).
Results

Efforts were made to identify a full-length DNA B closely resembling to DNA B of ToLCNDV or having no truncation in the NSP. Three sets of primers were designed in the NSP and non-coding sequences of DNA B to explore the possibility of the presence of some other DNA B than that described above. None of the primer sets was able to amplify a product of approximately 2.8kb. The ability of these sets of primers to amplify full-length DNA B was confirmed by the use of these primers on tomato samples infected experimentally with ToLCNDV. All sets of primers amplified products of the expected size. To further rule out the possible presence of other DNA B, phi29 polymerase was used which has the ability to amplify circular molecules. The amplified product was restricted with PstI endonuclease (for which there are two restriction sites in DNA A) yielding two bands of the expected sizes (969 bases and 1786 bases). The DNA B of MYLCV has two PstI restriction sites that are separated by 62 bases. The restriction analysis suggested the presence of a single type of DNA B of MYLCV and the absence of DNA B of ToLCNDV (results not shown).
### Table 3.2 Positions and coding capacities of genes encoded by MYLCV DNA A and DNA B

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position of start codon</th>
<th>Position of stop codon</th>
<th>Size (number of nucleotides)</th>
<th>Predicted size of encoded product (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AV2</td>
<td>120</td>
<td>458</td>
<td>338</td>
<td>13.2</td>
</tr>
<tr>
<td>CP</td>
<td>280</td>
<td>1050</td>
<td>771</td>
<td>29.8</td>
</tr>
<tr>
<td>REn</td>
<td>1457</td>
<td>1047</td>
<td>411</td>
<td>16.0</td>
</tr>
<tr>
<td>TrAp</td>
<td>1596</td>
<td>1177</td>
<td>410</td>
<td>15.9</td>
</tr>
<tr>
<td>Rep</td>
<td>2602</td>
<td>1499</td>
<td>1104</td>
<td>41.6</td>
</tr>
<tr>
<td>AC4</td>
<td>2445</td>
<td>2269</td>
<td>177</td>
<td>6.7</td>
</tr>
<tr>
<td>DNA B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSP</td>
<td>681</td>
<td>1235</td>
<td>555</td>
<td>20.9</td>
</tr>
<tr>
<td>MP</td>
<td>2146</td>
<td>1301</td>
<td>884</td>
<td>32.0</td>
</tr>
</tbody>
</table>
Table 3.3. Virus isolate to which each component and encoded gene of MYLCV has the highest level of nucleotide sequence identity

<table>
<thead>
<tr>
<th>Component</th>
<th>Highest nucleotide identity (%)</th>
<th>Virus isolate (accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA A</td>
<td>86.6</td>
<td>ToLCNDV-IN[PK:Sol:04] (DQ16885)</td>
</tr>
<tr>
<td>Rep</td>
<td>89.5</td>
<td>ToLCNDV-IN[IN:ND:AVT1] (AY428769)</td>
</tr>
<tr>
<td>TrAp</td>
<td>88.9</td>
<td>ToLCNDV-IN[IN:ND:AVT1] (AY428769)</td>
</tr>
<tr>
<td>REn</td>
<td>88.9</td>
<td>ToLCNDV-IN[IN:ND:AVT1] (AY428769)</td>
</tr>
<tr>
<td>AC4</td>
<td>94.4</td>
<td>ToLCNDV-IN[IN:ND:AVT1] (AY428769)</td>
</tr>
<tr>
<td>AC5</td>
<td>83.7</td>
<td>ToLCNDV-IN[PK:Kha:Chi:04] (DQ116880)</td>
</tr>
<tr>
<td>CP</td>
<td>85.6</td>
<td>ToLCNDV-IN[PK:Kha:Chi:04] (DQ116880)</td>
</tr>
<tr>
<td>AV2</td>
<td>84.7</td>
<td>ToLCNDV-IN[PK:Kha:Chi:04] (DQ116880)</td>
</tr>
<tr>
<td>DNA B</td>
<td>76.7</td>
<td>ToLCNDV-IN[PK:Isl:T1/8:00] (AY150304)</td>
</tr>
<tr>
<td>MP</td>
<td>85.7</td>
<td>ToLCNDV-IN[PK:Isl:T1/8:00] (AY150304)</td>
</tr>
<tr>
<td>NSP</td>
<td>73.3</td>
<td>ToLCNDV-N[PK:Dar:T5/6:01] (AY150305)</td>
</tr>
</tbody>
</table>
Figure 3.7 Genome organization of MYLCV DNA A and DNA B. The positions and orientations of predicted ORFs are shown with arrows. The common region (CR) of each component of the virus is indicated by a grey box. The position of the stem-loop structure, containing the conserved nonanucleotide loop sequence (TAATATTAC), is indicated with a black circle.
Figure 3.8. Dotmatcher comparison of the DNA B sequences of MYLCV DNA B (Y-axis) and ToLCNDV DNA B (acc. no. AY150304, X-axis). The position of the MP and NSP genes, as well as the common region (CR) is shown in each case. For the MYLCV DNA B the position of the mutation that disrupts NSP is shown (line through the gene with preceding area in gray). The settings for the comparison were - threshold 85 and window size 25. Dotmatcher is part of EMBOSS (Rice et al.; 2000) and was run online (http://emboss.bioinformatics.nl/).
3.4.3 Phylogenetic analysis of MYLCV DNA A and DNA B

A phylogenetic analysis, based upon an alignment of the complete DNA A sequence of MYLCV with selected other begomoviruses in shown in Figure 3.9. This analysis shows MYLCV to be most similar to CuLCuV-[BD:06], a virus recently identified in Bangladesh (ref). These two viruses group with, and are basal to, the ToLCNDV isolates but distinct from SLCCNV isolates. This is well supported by bootstrapping. A phylogenetic analysis based upon an alignment only the IR of these same viruses (Figure 3.10) is much less certain of the position of both MYLCV and CuLCuV, with very low bootstrap values. These low bootstrap values, at the nodes linking MYLCV, CuLCuV and the ToLCNDV isolates, indicate that there is significant similarity between their IR sequences. However, the IR sequences of all these are very distinct from those of SLCCNV (with a bootstrap value of 100%).

A phylogenetic analysis, based upon an alignment of the complete DNA A sequence of MYLCV with selected other begomoviruses in shown in Figure 3.11. In this analysis MYLCV is again basal to the ToLCNDV isolates which form a clade distinct from the SLCCNV isolates. It is also noteworthy that the DNA B of ToLCGV-[IN:Var:01] is included in this analysis and shows it to have a ToLCNDV-like DNA B, as discussed later. The position of MYLCV in the tree is well supported by bootstrapping. When only the nucleotide sequence of the MP gene is considered (Figure 3.12), a similar relationship is seen, with high bootstrap values at the node linking MYLCV to the ToLCNDV isolates. A similar tree topology is obtained for a dendrogram based upon only the NSP gene sequence (Figure 3.13). However, in this case the node linking MYLCV to the ToLCNDV isolates is not well supported by bootstrapping (67.6%). This indicates that, although the NSP of MYLCV is more similar to ToLCNDV than to the other viruses examined, it is distinct from this. This confirms the analysis of the NSP sequence presented earlier which shows only low levels of sequence identity to the NSP of ToLCNDV isolates. Thus this analysis is in agreement with earlier comparisons that the DNA B of MYLCV is most similar to that of ToLCNDV, but that it is is recombinant with an NSP coding sequence originating from a source which has yet to be identified.
**Results**

![Phylogenetic tree diagram](image)

**Figure 3.9.** Neighbour joining phylogenetic dendrogram based upon an alignment of the complete nucleotide sequences of MYLCV DNA A and the DNA A components of selected other viruses. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). Vertical lines are arbitrary and horizontal lines are proportional to calculated mutation distances. The tree is rooted on the DNA A sequences of AbMV, a distantly related virus from New World.
Figure 3.11. Neighbour joining phylogenetic dendrogram based upon an alignment of the complete nucleotide sequences of MYLCV DNA B and the DNA B components of selected other viruses. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). Vertical lines are arbitrary and horizontal lines are proportional to calculated mutation distances. The tree is rooted on the DNA B sequences of AbMV, a distantly related virus from the New World.
Figure 3.10. Neighbour joining phylogenetic dendrogram based upon an alignment of the nucleotide sequences of intergenic region of MYLCV DNA A and the intergenic region sequences of DNA A components of selected other viruses. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). Vertical lines are arbitrary and horizontal lines are proportional to calculated mutation distances. The tree is rooted on the intergenic sequences of AbMV DNA A, a distantly related virus from New World.
Figure 3.12. Neighbour joining phylogenetic dendrogram based upon an alignment of the MP nucleotide sequences of MYLCV DNA B and the MP sequences of DNA B components of selected other viruses. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). Vertical lines are arbitrary and horizontal lines are proportional to calculated mutation distances. The tree is rooted on the MP sequence of DNA B of ToMoV, a distantly related virus from New World.
Results

Figure 3.13. Neighbour joining phylogenetic dendrogram based upon an alignment of the NSP nucleotide sequences of MYLCV DNA B and the NSP corresponding sequences of selected other viruses. Numbers at the nodes are percentage bootstrap confidence values (1000 replicates). Vertical lines are arbitrary and horizontal lines are proportional to calculated mutation distances. The tree is rooted on the NSP sequence of DNA B of ToMoV, a distantly related virus from New World.
3.5 Infectivity of MYLCD components

3.5.1 Infectivity of begomovirus clones from muskmelon

The infectivity of MYLCV clones was assessed by *Agrobacterium*-mediated inoculation to *N. benthamiana* (Fig. 3.14, Table 3.4). Plants inoculated with MYLCV DNA A and DNA B did not develop symptoms, even 2 months post-inoculation whereas PCR for the presence of MYLCV DNA B in systemic leaves was found positive. In constrast, plants inoculated with ToLCNDV DNA A and DNA B developed upward leaf curl and vein swelling within 14 days of inoculation. These symptoms are typical of ToLCNDV in *N. benthamiana* (Padidam et al., 1995). The poor infectivity of the MYLCV clones to *N. benthamiana* indicates that they are not competent for inducing a systemic infection. *N. benthamiana* has an unusually high susceptibility to virus infection. Of the dicot-infecting geminiviruses, only the legume infecting begomoviruses *Mungbean yellow mosaic virus* (Hussain et al., 2004) and *Mungbean yellow mosaic India virus* are reported not to be capable of systemic infection of this species. This has been attributed to *N. benthamiana* encoding a mutant RdRP1, an enzyme involved in PTGS (Yang et al., 2004).

The agroclones of begomoviruses may lose their infectivity due to deletions or mutations in clones (Shivaprasad et al., 2006). To rule out possible defects in clones arising from deletions, partial duplication clones of MYLCV DNA A and DNA B were inoculated by biolistic methods. Biolistic inoculation resulted in very mild symptoms. PCR with specific primers was used to confirm systemic infection. The results showed the presence of both DNA A and DNA B in systemically infected leaves. This result shows that DNA B of MYLCV is capable of movement with MYLCV DNA A although not inducing typical disease symptoms.

3.5.2 Pseudorecombination of MYLCV and ToLCNDV

To ascertain which of the components (or whether both components) was responsible for the lack infectivity, the MYLCV clones were inoculated as pseudo-recombinants with the components of ToLCNDV. Inoculation of ten *N. benthamiana* plants with ToLCNDV DNA A and MYLCV DNA B did not develop begomovirus symptoms but seven plants were found positive in PCR for the presence of DNA B. In contrast, plants inoculated with MYLCV DNA A and ToLCNDV DNA B developed typical of ToLCNDV upward leaf curl and vein swelling symptoms 14 days post-inoculation.
Results

These results demonstrate that the defect(s) preventing infection of the MYLCV clones reside on DNA B. The earlier sequence analyses (section 3.5.2) indicated that MYLCV DNA B contains a truncation of the NSP gene due to a premature stop codon. This is the likely cause of the lack of ability of MYLCV DNA B to support symptoms development. The ability of MYLCV to trans-replicate and establish a systemic infection with ToLCNDV also indicates that the Rep encoded by MYLCV DNA A is able to interact with the origin of replication of ToLCNDV. The sequence analysis showed that the predicted iteron sequences of MYLCV are the same as those of ToLCNDV (Padidam et al., 1995) and these elements are crucial in the interactions required for Rep to initiate rolling-circle replication (Fontes et al., 1992).

3.5.3 Infectivity of ZYMV on melon

When healthy muskmelon plants were agro-infiltrated with infectious clones of ToLCNDV, localized cell death was observed along the veins of newly emerging leaves ten days post inoculation (Fig: 3.15A) which is typical of a hypersensitive response. No necrosis was evident from control plants inoculated with only Agrobacterium. Similarly, transient expression of NSP under the control of 35S promoter by agro-infiltration resulted in localized cell death. Necrosis started from the point of inoculation and spread across the leaf with the passage of time (Fig: 3.16A). Plants inoculated with MP expressed from 35S promoter did not show any effect (Fig: 3.16B). Muskmelon plants remained asymptomatic and no hypersensitive response was observed on inoculation with DNA A and DNA B of MYLCV. All inoculations were repeated three time and results were consistent. Pathogenicity of ZYMV was also checked on muskmelon. Healthy muskmelon plants were mechanically inoculated with sap extracted from symptomatic leaves of muskmelon by the leaf abrasion method. Thirty healthy muskmelon plants exhibited typical symptoms of ZYMV 21 days post-inoculation (Fig 3.17A). This symptomatic phenotype included a characteristic mosaic pattern, enations on the upper side of the leaves and curling of the leaves. In none of the cases, were plants stunted and bushy. ELISA for these symptomatic plants was found positive for ZYMV only. Symptomatic muskmelon plants were found negative for MYLCV by PCR and Southern blot hybridization, ruling out possible sap transmission of MYLCV.
Results

Figure 3.14. *N. benthamiana* plants agroinoculated with MYLCV DNA A (A), MYLCV DNA A and DNA B (B) and MYLCV DNA A and ToLCNDV DNA B (C). (D) shows plant only inoculated with *A. tumefaciens* without vector construct. The plants were photographed 21 days post-inoculation.
Figure 3.15. Cell death along the veins of systemic leaves of a muskmelon plant agroinoculated with ToLCNDV DNA A and DNA B (A). Necrotic lesions along the veins of systemic leaf of a muskmelon plant micrografted with leaf discs of a *N. benthamiana* plant infected with ToLCNDV by agroinoculation (B).
**Figure 3.16.** A. Hypersensitive cell death around the inoculation site of a muskmelon leaf agroinfiltrated with ToLCNDV NSP expressed under control of 35S promoter (A). Inoculation site of a muskmelon leaf agroinfiltrated with ToLCNDV MP expressed from a PVX vector (B). The necrosis in this case does not extend to all inoculated cells and is due to damage during infiltration.
**Figure 3.17.** Muskmelon plant showing typical symptoms of ZYMV photographed 21 days after inoculation with sap extracted from leaves of field infected muskmelon plant by the leaf abrasion method (A). Control non-inoculated muskmelon plant (B).
Table 3.4. Infectivity analysis of pseudo-recombination between MYLCV and ToLCNDV partial duplication clones

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Infectivity (no. of plants infected with begomovirus/no. of plant inoculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. I</td>
</tr>
<tr>
<td>MYLCV DNA A + MYLCV DNA B</td>
<td>0/20</td>
</tr>
<tr>
<td>MYLCV DNA A + ToLCNDV DNA B</td>
<td>20/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A + MYLCV DNA B</td>
<td>0/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A + ToLCNDV DNA B</td>
<td>20/20</td>
</tr>
<tr>
<td>MYLCV DNA A</td>
<td>0/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A</td>
<td>0/20</td>
</tr>
</tbody>
</table>
3.6 ZYMV HC-Pro complements the movement of MYLCV DNA A

The MYLCV clones were shown to be infectious to *N. benthamaina* but were unable to develop disease symptoms due to a defect in the DNA B (section 3.5.1). This raised the question of how the virus is maintained in synergistic infections with ZYMV. Unfortunately an infectious clone of ZYMV was not available to investigate the interaction with MYLCV. Since synergistic interactions involving potyviruses have been shown to be mediated by the HC-Pro (Shi *et al*., 1997), the possibility of this being the case in the interaction between ZYMV and MYLCV was investigated by expressing the ZYMV HC-Pro from a PVX vector. The ZYMV HC-Pro coding sequence was obtained by RT-PCR from infected melon. In *N. benthamiana*, PVX infection induces only very mild symptoms; consisting of vein yellowing. The PVX vector expressing the HC-Pro of ZYMV was fully infectious in *N. benthamiana*, inducing systemic mild foliar mottling (Fig. 3.14E). Total number of inoculated, symptomatic and asymptomatic plants are shown in Table 3.5.

On single inoculation MYLCV DNA A, *N. benthamiana* plants did not produce any viral symptoms. Coinoculation of plants with PVX vector and MYLCV DNA A only produced PVX symptoms. Southern blot hybridization using Rep gene of ToLCNDV as a probe for detection of DNA A of begomovirus revealed that there was no signal in lane (lane-2) loaded with DNA isolated from the plant only inoculated with MYLCV DNA A. Similarly no signal was detected in lane loaded with DNA having combination MYLCV DNA A and PVX (lane-3). This indicated that the single component of MYLCV is unable to produce symptom alone or in combination with PVX. When the plants were co-inoculation with MYLCV DNA A and PVX-HC-Pro showed symptom phenotype similar to that induced by ToLCNDV DNA A and DNA B (Fig 3.18B). Plants exhibited upward leaf curling along with vein thickening and stunted plant growth. There was a marked reduction of leaf size in newly emerging leaves and shortening of internodes. Infected plants did not set the normal numbers of flowers and a majority of the seed pods were empty. This indicated that in the presence of PVX-HC-Pro MYLCV DNA A produced typical viral symptoms. The signals in Southern blot hybridization were very strong where the plant was inoculated with MYLCV DNA A and PVX-HC-Pro. These results show that ZYMV HC-Pro is able to complement the movement of ToLCNDV DNA in the absence of DNA B. A PVX vector expressing a mutant of HC-Pro (with the central 208 amino
acids deleted) induced only symptoms of PVX and not the foliar mottling typical of the vector expressing the intact HC-Pro.

The complementation of movement of MYLCV DNA A by HC-Pro was further confirmed when co-inoculated with an RNAi construct targeted against HC-Pro of ZYMV and only mild PVX symptoms appeared on inoculated plants (Fig 3.18C). Similarly absence of signal in lane 10-11 (Fig. 3.19DNA of plants inoculated with MYLCV DNA A, PVX-HC-Pro and PVX-HC-Pro-RNAi) confirms that the movement complementation of MYLCV DNA A by PVX-HC-Pro is blocked when translation of HC-Pro is hindered by siRNA produced by RNAi construct.

In the same experiment MYLCV DNA B and ToLCNDV DNA B were inoculated in different combinations. Inoculation of plants with MYLCV DNA A and ToLCNDV DNA B resulted in normal symptoms produced by ToLCNDV and strong signals were detected on the blot (lane6-7). Whereas inoculation of plant with MYLCV DNA A and MYLCV DNA B did not produce symptoms as described in section 3.5.1 The symptoms were more severe when N. benthamiana plants were coinfiltrated with ToLCNDV DNA A, DNA B and PVX-HC-Pro (Fig: 3.18D) and strong signals appeared on the blot. This show ToLCNDV DNA B and PVX-HC-Pro where inoculated simultaneously had additive pathogenic effect resulting in more severe symptoms. In the mean time presence of signals in plant DNA inoculated with MYLCV DNA A, ToLCNDV DNA B, PVX-HC-Pro and PVX-HC-Prp RNAi construct show that even if expression of HC-Pro is blocked by RNAi construct DNA A and DNA B are sufficient to produce symptoms because the virus has both functional components. Ability of DNA B to move with MYLCV DNA A in the presence of PVX-HC-Pro of ZYMV was checked by PCR with DNA isolated from combinations of inoculation, MYLCV DNA A, MYLCV DNA B and PVX-HC-Pro. Amplification of MP gene showed that DNA B of MYLCV is also maintained and replicates in the presence of PVX-HC-Pro.
Figure 3.18. *N. benthamiana* plants agroinoculated with MYLCV DNA A and ToLCNDV DNA B (A), MYLCV DNA A and PVX-HC-Pro (B), MYLCV DNA A, PVX-HC-Pro and the RNAi construct containing HC-Pro sequences (C), MYLCV DNA A, ToLCNDV DNA B and PVX-HC-Pro (D) and PVX-HC-Pro (E).
Results

A

B

C

D

E
### Table 3.5. Results of combinations of inoculations made to infect *N. benthamiana*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Infectivity (no. of plants infected with begomovirus/no. plant infected with PVX/no. of plant inoculated) #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. I</td>
</tr>
<tr>
<td>MYLCV DNA A+ToLCNDV DNA B</td>
<td>20/-/20</td>
</tr>
<tr>
<td>MYLCV DNA A+ MYLCV DNA B</td>
<td>0/-/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A+MYLCV DNA B</td>
<td>0/-/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A+ ToLCNDV DNA B</td>
<td>0/-/20</td>
</tr>
<tr>
<td>MYLCV DNA A</td>
<td>0/-/20</td>
</tr>
<tr>
<td>MYLCV DNA A+MYLCV DNA B+PVX-HC-Pro</td>
<td>13/13/20</td>
</tr>
<tr>
<td>MYLCV DNA A + PVX-HC-Pro</td>
<td>15/15/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A + PVX-HC-Pro</td>
<td>14/14/20</td>
</tr>
<tr>
<td>MYLCV DNA A+PVX-HC-Pro++ PVX-HC-ProRNAi</td>
<td>0/0/20</td>
</tr>
<tr>
<td>PVX-HC-Pro</td>
<td>0/20/20</td>
</tr>
<tr>
<td>MYLCV DNA A + PVX</td>
<td>0/20/20</td>
</tr>
<tr>
<td>ToLCNDV DNA A + PVX</td>
<td>0/20/20</td>
</tr>
<tr>
<td>Mock *</td>
<td>0/-/20</td>
</tr>
</tbody>
</table>

* Plants inoculated with *A. tumefaciens* without binary vector construct.

# Results of visual assessment for symptoms of infection by begomovirus (upward leaf curling) or PVX-HC-Pro (foliar mottling) or PVX (vein yellowing)
Figure 3.19. Southern blot analysis of total nucleic acid extracted from inoculated *N. benthamiana* plants probed for the presence of begomovirus DNA A using the radioactively labeled Rep gene of ToLCNDV (Padidam *et al.*, 1995; accession no. U15017). Nucleic acids were extracted from plants inoculated with MYLCV DNA A (lane 2), PVX-HC-Pro (lane 3), MYLCV DNA A and PVX-HC-Pro (lanes 4 and 5), MYLCV DNA A and ToLCNDV DNA B (lanes 6 and 7), MYLCV DNA A and ToLCNDV DNA B and PVX-HC-Pro (lanes 8 and 9), PVX-HC-Pro-RNAi construct with DNA A and PVX-HC-Pro (lanes 10- and 11), MYLCV DNA A and ToLCNDV DNA B and PVX-HC-Pro and PVX-HC-Pro-RNAi (lanes 12 and 13). The DNA run in lane 14 was extracted from a symptomatic tomato plant experimentally infected with ToLCNDV by agroinoculation with DNA A and DNA B. The positions of single-stranded (ss), supercoiled (sc) and open-circular (oc) virus DNA forms are indicated. Approximately equal amounts (10μg) of nucleic acid were loaded in each well. The blot was washed at low stringency (0.5xSSC) and exposed to film for 12 hours.
3.7 Dual infections of begomoviruses and ZYMV are common in cucurbits in the Punjab

A survey for incidence of dual infection of begomoviruses and ZYMV in cucurbit crops was conducted in Punjab in the same five districts (Faisalabad, Toba Tek Singh, Sahiwal, Vehari and Khanewal). Plants with virus-like symptoms were sampled for detection of described viruses in summer season of 2007 (Fig: 3.16). Some cucurbit species were found singly positive for begomovirus or ZYMV while others were doubly infected. Out of thirteen cucurbit species, Indian squash, muskmelon and watermelon were found positive for both ZYMV and begomovirus by PCR (Table: 3.5). Eight cucurbit species were positive for begomoviruses and six for ZYMV. This indicates that double infection of ZYMV and begomovirus is not specific to muskmelon and is present in other cucurbit hosts also.
Table 3.6. Detection ZYMV and CMV (by ELISA) and begomovirus (by PCR) infections of cucurbits collected in the Punjab.

<table>
<thead>
<tr>
<th>Crop (number of samples examined)</th>
<th>ZYMV</th>
<th>ToLCNDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indian Squash (10)</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Cucumber (10)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Sweet squash (10)</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Muskmelon (10)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Watermelon (10)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Squash (10)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Snake gourd (10)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pumpkin (10)</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Wild melon (10)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sponge gourd (10)</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Long melon (10)</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Bitter gourd (10)</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Red gourd (10)</td>
<td>-</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 3.20. Virus-like symptoms exhibited by cucurbits in the Punjab in 2006. Squash in a field in Vehari district with mild symptoms of disease (A). A wax gourd field in Sahiwal district showing severe symptoms consisting of leaf curling, vein thickening, mosaic, leaf deformation, yellowing, chlorosis and stunting of plants (B). Field of pumpkin in Khanewal district with with plants showing foliar enations and mosaic (C), Squash plant showing mosaic and yellowing (D). Sponge gourd leaf with upward leaf curling, vein thickening, vein clearing and chlorosis in Faisalabad (E).
Results

A

B

C

D

E
3.8.1 Stable transformation of *N. benthamiana* with RNAi construct

Confirmed cultures were used to stably transform *Nicotiana tabacum* with RNAi construct targeting HC-Pro and transformed explants of tobacco and regenerants were selected based on resistance to basta glufosinate. Transformed leaf disks started regeneration on selection media (Fig. 3.21A). After shoot development, transformed plants were shifted to root development media (Fig 3.21B). Finally, putative transgenic plants were shifted to soil after acclimatization in growth chamber. DNA of transformed plants (12 lines) was isolated by CTAB method. Further dilutions were made to check the amplification of the transgene by PCR and total DNA was resolved on 1% agarose gel to reveal the successful integration of transgene in the plant through Southern blot hybridization.

Total DNA was extracted at 5-6 leaf stage and success of transformation was first confirmed by PCR amplification (Fig: 3.22A) of the transgene using the primers that were used for partial gene amplification of the HC-Pro and CP of ZYMV. The amplification of desired size product confirmed the successful transformation of the transgene. For further confirmation through Southern hybridization total DNA was probed with HC-Pro sequences radioactively labeled with $^{32}$P dCTP and showed hybridization from lesser to higher extent of signals (Fig: 3.22B). No signal was detected in non-transformed tobacco plant used as negative control

Transgenic plants which were produced by the integration of the HC-Pro-CP RNAi construct were normal in their phenotype in every aspect. These plants grew and matured at a normal rate. Correlating with this normal pattern of growth and development, there was no decrease in fertility. Most lines set normal numbers of flowers and a majority of the seed pods had normal number of seeds and were indistinguishable in appearance from control tobacco plants.
3.8.2 Transgenic plants expressing the RNAi construct show resistance to PVX-HC-Pro ZYMV

In order to check effectiveness of RNAi construct of HC-Pro and CP in transgenic plant, five plants of each line were evaluated by inoculation of individual plants with PVX-HC-Pro construct. The manifestation of symptoms was monitored at regular interval after inoculation. All 12 transgenic lines showed 100% resistance as compared to the control plants (non-transformed *N.benthamiana*), which developed characteristic mosaic symptoms 12 day after inoculation. None of the inoculated transgenic plants became symptomatic (Fig 3.23A). This shows that siRNAs produced by the hairpin construct blocked the translation of HC-Pro product and plants remained asymptomatic (Fig 3.23B.). Inoculation of plants with DNA A and PVX-HC-Pro did not produce leaf curling symptoms indicating a lack of complementation of movement. Similarly, inoculation of plants with MYLCV DNA A, DNA B and PVX-HC-Pro resulted in no symptoms of begomovirus infection as compared to control plants. The inoculated plants were normal in development of vegetative parts and also produced seed normally. All the transgenic lines maintained resistance throughout the growing period. These viruses showed that the movement of DNA A of MYLCV is supported by HC-Pro. Transgenic plants where the HC-Pro was targeted by RNAi were unable to support movement and no phenotype was produced. Compared to this, non-transgenic plants when inoculated with MYLCV DNA A and PVX-HC-Pro produced symptoms typical of the virus. Thus, silencing of the HC-Pro not only protected transgenic plants from RNA virus but was also able to suppress the begomovirus due to the absence of complementation of movement of MYLCV by HC-Pro.
Figure 3.21. *N. benthamiana* transformation on selection medium (basta glufosinate) (A). Regenerated plantlet growing on rooting medium in a jar (B). Note the development of roots.
Figure 3.22. Analysis of *N. benthamiana* plants transformed with the RNAi construct (A). Primers for HC-Pro gene were used in PCR for amplification of HC-Pro. Lane 1 shows 1kb DNA ladder, lane 2 is negative control (*N. benthamiana*), lane 3, 4 and 7-10 plants show the amplification of transgene, lane 5 and 6 show plants with out transgene. Analysis of transgenic plants for the presence of transgene by Southern blot hybridization (B). Blot was probed with PCR product of HC-Pro and washed at low stringency. 10µg of DNA per lane was loaded on the gel. Lane 1-10 show different lines of plants and lane 11 shows positive control (HC-Pro clone).
Figure 3.23. *N. benthamiana* plant transformed with the RNAi construct (A) and a non-transformed *N. benthamiana* (B) agroinoculated with PVX-HC-Pro. Plants were photographed 21 days post-inoculation.
Chapter 4

Discussion

Phytopathogenic viruses are major constraints to agricultural productivity throughout the World. However, losses are the highest and cause the most hardship in developing countries of the tropics and sub-tropics. There are a number of reasons for this and they include the fact that the diversity and incidence of plant-infecting viruses of agricultural significance are higher, the environmental conditions are more favourable for the vectors of the viruses, the farmers can not afford the expensive chemical control agents as well as, in many cases, not having the educational standard and training to use them effectively.

It is an unfortunate fact that Pakistan, in common with all other countries of southern Asia, is home to members of virtually all taxonomic groups of plant-infecting viruses (Ali et al., 2004; Mali and Ragegore 1979; Naidu et al., 1989; Verma et al., 2004; Raikhy et al., 2003; Mandal et al., 2004; Amin et al., 2007; Lauren et al., 2006) which, at least in part, explains the low agricultural productivity in the country. Prime amongst these viruses are the whitefly-transmitted geminiviruses (begomoviruses).

The geminiviruses evolve/adapt by a number of mechanisms. Contrary to expectation the geminiviruses have nucleotide substitution rates comparable to those of RNA viruses (Duffy and Holmes, 2007). Such high substitution rates would not be expected from a virus that utilizes host-encoded DNA polymerases with error correction. Additionally, recombination and component exchange (known as pseudo-recombination) are the major processes of geminivirus evolution (Padidam et al., 1999; Roye et al., 2000; Pita et al., 2001; Harrison and Robinson 2005; Rothenstein et al., 2006). A prime example of this is the ongoing pandemic of cassava mosaic disease that originated in northern Uganda, spread across eastern Africa and continues to spread throughout central and western Africa. The severe cassava mosaic disease of the pandemic is attributed to a recombinant strain of East African cassava mosaic virus known as the “Uganda Variant” (Zhou et al., 1997). This virus contains a small fragment of the CP gene of African cassava mosaic virus; the significance of this for the severity of the disease is unclear. Interestingly the cassava mosaic pandemic also is an example of synergism. In addition to the “Uganda variant”, plants affected also
contain ACMV (Harrison et al., 1997). Vanitharani et al. (2004) have shown that the synergism in this case is at the level of post-transcriptional gene silencing. Each of the components of the complex, EACMV-UG and ACMV, provide a distinct suppressor of PTGS that is required to establish and maintain the severe disease in cassava; either virus alone does not have this ability. There are numerous examples of component exchange in the geminivirus literature. Probably the most striking is that associated with the epidemic of cotton leaf curl disease (CLCuD) that occurred in southern Asia in the late 1980s and 1990s. CLCuD is caused by monopartite begomoviruses that associate with a distinct DNA β satellite, known as the CLCuD DNA β (Briddon et al., 2001). It is generally believed that the epidemic started with a single begomovirus (probably Cotton leaf curl Multan virus or a close relative) and CLCuD DNA β and that, as the disease spread, further distinct monopartite begomoviruses were recruited into the disease complex. At this time 7 begomoviruses, but only a single DNA β, have been identified in CLCuD affected plants in the epidemic area (Amin, et al., 2006; Rojas et al., 2005; Radhakrishnan et al., 2004; Briddon et al., 2002; Padidam et al., 1999). The work presented in this thesis shows yet another twist in the begomovirus story, highlighting the ability of the begomoviruses to evolve and adapt and the ability of these viruses to form synergistic interactions with RNA viruses.

The limited surveys carried out in Punjab and NWFP provinces of Pakistan highlighted a difference in the presence of synergistic viruses in melon; consisting of ZYMV and CMV in NWFP and ZYMV and a begomovirus in the Punjab. The reason for this difference is unclear. The more temperate climatic conditions in NWFP may not be conducive for the whitefly vector of begomoviruses. However, begomoviruses and B. tabaci do occur in this region (personal observation). Thus, possibly the temporal spread of begomoviruses, which is likely to be later in NWFP due to the more temperate conditions, may not be conducive for establishment of a synergistic infection with ZYMV. ZYMV and CMV have some common aphid vectors and may thus be co-transmitted, desirable for establishment of a synergistic disease. Nevertheless, this does not explain why ZYMV-CMV synergistic infections were not detected in the Punjab. Possibly the synergism with a begomoviruses provides ZYMV with a selective advantage which out-competes a ZYMV-CMV infection. Further investigation will be required to elucidate the basis for this observation.
Co-infections, likely leading to synergistic disease, are widespread in cucurbits. This indicates that the situation is dynamic, rather than static, with a constantly changing complement of viruses causing disease. These newly identified interactions will be interesting subjects of future investigations.

DNA sequence data and, to lesser extent, biological characteristics are the currently accepted criteria for defining species of geminiviruses. 89% nucleotide sequence identity between the genomes (or DNA A genomic components) is the current threshold for defining a new species of begomovirus (Fauquet, et al. 2003). Since many begomoviruses do not associate with a DNA B component, this is not considered in defining species for these viruses. Based on this threshold value, the begomovirus identified in muskmelon is a new species in the genus (having a DNA A nucleotide sequence similarity of 86% to its closest relative ToLCNDV), for which the name Melon yellow leaf curl virus (MYLCV) has been proposed.

The high levels of nucleotide sequence similarity, of both DNAs A (86%) and B (73%), between ToLCNDV and MYLCV indicate that they have a recent common ancestor and that MYLCV represents a lineage that has diverged from ToLCNDV and possibly has adapted to infect cucurbits and co-exist with ZYMV in a synergistic interaction (as discussed later). ToLCNDV has frequently been identified in cucurbits (Maruthi et al., 2007; Tahir and Haider, 2005; Guzman et al., 2000; Maruthi et al., 2003; Revill et al., 2003; Morales and Jones, 2004). However, Koch’s postulates for ToLCNDV causing diseases of cucurbits has not been satisfied. Since, in most cases, the complete virus was not isolated and the presence of additional viruses was not investigated, it remains possible that many of the diseases of cucurbits reported to be caused by ToLCNDV are actually caused by synergistic interaction of this virus with another virus, such as ZYMV. This highlights the need for caution when attributing the etiology of a disease to a particular virus when Koch’s postulates have not been fulfilled.

ToLCNDV is a cosmopolitan species that occurs throughout southern Asia, having been reported from Pakistan, India and Thailand (Chatchawankanphanich et al., 1993; Usharani et al., 1993; Padidam et al. 1995; Srivastava et al., 1995; Mansoor et al., 1997; Mansoor et al., 2000; Samretwanich et al., 2000; Hussain et al., 2005; Tahir
and Haider, 2005; Haider et al., 2005). The genome organization of MYLCV is typical of that of Old World begomoviruses (having an AV2 gene) and phylogenetic analysis (Figure 3.9) shows it and ToLCNDV to be most similar to (to group with) other begomoviruses with their center of diversity and origins lying in southern Asia (Chatchawankanphanich et al., 1993; Padidam et al., 1995; Srivastava et al., 1995; Mansoor et al., 1997; Mansoor et al., 2000; Samretwanich et al., 2000; Hussain et al., 2005).

Recombination contributes to the genetic diversification of geminivirus populations and has been related to the emergence of some serious plant diseases, as discussed earlier (Zhou et al., 1997; Moffat, 1999; Padidam et al., 1999; Cui et al., 2004; Li et al., 2005). The prerequisite for recombination to occur is co-infection of the same cell of a host plant (Mansoor, et al., 2005). MYLCV has high levels of sequence identity to ToLCNDV-IN[PK:Kha:Chi:04] (accession number DQ116880) in the virion-sense (including the intergenic region) and to ToLCNDV-IN[IN:ND:AVT1] (AY428769) in the complementary-sense, indicating that this molecule is likely an intraspecific recombinant. Recombination is common among plant and animal RNA viruses (Simon and Bujarski, 1994) and DNA viruses (Roossinck, 1997). This phenomenon is among the major forces driving the evolution of viruses (Roossinck, 1997; McDonald 2002; Seal, et al., 2006). The most prominent examples of recombination among geminiviruses are the viruses involved in causing cotton leaf curl disease across Pakistan and India (Sanz et al., 1999; Sanz et al., 2000) and the viruses causing tomato yellow leaf curl disease across the Mediterranean basin (Garcia-Andres et al., 2007).

The integrity of the genomes of bipartite begomoviruses is maintained by them having compatible Rep binding sequences in the CR. Thus, the DNA A-encoded Rep is able to recognize and initiate the replication of both components (Heyraud-Nitschke et al., 1995; Laufs et al., 1995a; Orozco and Hanley-Bowdoin, 1996; Chatterji, et al., 2001). Iteron sequences are usually species specific, thus in most cases, the Rep of one virus would not be expected to recognize the iteron sequences of a distinct species (Choi and Stenger, 1995; Jupin et al., 1995; Gladfelter et al., 1997). The iteron sequences of ToLCNDV have been identified experimentally (Chattergi et al., 1999). ToLCNDV and MYLCV have the same Rep binding sites and this likely explains our
experimental finding that MYLCV DNA A is able to \textit{trans}-replicate ToLCNDV DNA B and initiate a productive, symptomatic systemic infection of plants.

ToLCNDV is a part of viral complex consisting of several DNA As that exchange DNA Bs readily. Here evidence is provided that, in addition to ToLCNDV and \textit{Tomato leaf curl Gujarat virus} (Figure 3.10; Chakraborty \textit{et al}., 2003), the complex also encompasses MYLCV. This ability to readily form genomic reassortants (pseudo-recombination) is probably an adaptation that gives the virus complex an evolutionary advantage. Since DNA B components are involved in movement in plants (Sandrfoot and Lazarowitz 1996; Noueiry \textit{et al}., 1994; Rojas \textit{et al}., 2005), and thus host range determination (Berrie \textit{et al}., 2001; Unseld \textit{et al}., 2000; Hofer, 1997), the ability to readily interact with a distinct DNA B will undoubtedly allow the viruses of the complex to alter/adapt their host ranges to take advantage of new niches.

For bipartite begomoviruses DNA B is considered as an integral part of the genome (a genomic component), rather than a satellite, due to the presence of the conserved region (CR) which is present in both components. Satellites are defined as molecules which share no significant sequence similarity with their helper viruses but require them for replication and movement in host plant (Briddon and Stanley 2006). Although clearly a DNA B, the lack of appreciable sequence similarity between the DNA A and DNA B components of MYLCV, with the exception of the iteron sequences and nonanucleotide sequence, means that the MYLCV DNA B component could be deemed a satellite. This contention is strengthened by the ability of the DNA A to readily exchange the component, a feature in common with satellites such as DNA $\beta$.

Like the DNA A component, the DNA B component of MYLCV is a recombinant. The virion-sense sequences (containing the MP gene) originate from ToLCNDV (showing the highest sequence identity with isolate ToLCNDV-IN[PK:Isl:T1/8:00]; accession no. AY150304) while the intergenic region and virion-sense sequences derive from an as yet undiscovered (or extinct) virus; having no high sequence similarity to any sequences in the databases. The DNA B of MYLCV is a mutant with a truncation of the NSP gene. It is unable to support symptoms development when
inoculated to *N. benthamiana* in the presence of MYLCV DNA A. The integrity of the DNA A was shown by inoculation with ToLCNDV DNA B to *N. benthamiana*, which supported a full, symptomatic infection with symptoms typical of ToLCNDV in this host. Mutational analysis suggests that the N-terminal sequences of NSP are involved in nuclear localization while C-terminal sequences are required for interaction with the MP (Sanderfoot *et al.*, 1996). Thus, truncation of MYLCV NSP at the N-terminus likely abolished the nuclear localization of the protein resulting in a defective molecule which can not support infection or the protein is not produced at all since it is prematurely truncated. PCR amplification with universal and specific DNA B primers failed to show the presence of an intact DNA B in melon samples, indicating that this defective molecule is the only DNA B present in melon.

Recently Hussain *et al.* (2005) have shown that the NSP of ToLCNDV is a target of host defence in *N. benthamiana*, *N. tabacum* and tomato. Expression of NSP in these plants leads to a hypersensitive response (HR) characterised by cell death. They also showed, for the first time for a plant-infecting virus, that the TrAP of this virus is able to inhibit the HR; explaining why in these hosts ToLCNDV infection does not induce a HR. The appearance of necrotic lesions along the veins of melon plants inoculated with ToLCNDV suggests that this plant species has resistance to the virus; thus encodes a resistance gene which recognizes an avirulence gene encoded by the virus. It is thus possible that the mutation in DNA B of MYLCV is a response to the resistance of melon. This idea is supported by the findings of Hussain *et al.* (2005) who showed that deletion of 60 amino acids at the N-terminus abolished the ability of NSP to elicit necrosis. Thus truncation of the N-terminal sequences of NSP in MYLCV DNA B suggests a novel strategy of the virus to escape a host defense mechanism.

The truncation of NSP renders the MYLCV DNA B functionally impaired, as discussed earlier. The question thus arises as to what this molecule contributes to the synergistic infection of melon with ZYMV. MYLCV DNA A is unable to support systemic infection of *N. benthamiana* (or melon) but is maintained in infections in which an intact DNA B (that of ToLCNDV) is supplied. Likely, in this case, the MYLCV DNA B is maintained as a satellite (although the question remains as to whether the MP gene is expressed). Possibly then, in melon, the MYLCV DNA B is
Discussion

maintained as a poorly-functional satellite which is not required for the infection. Further investigation will be needed to address this question. The presence of this defective DNA B in many plants and in geographically distant fields of melon, as well as in various cucurbit species, however, suggests that this molecule may play an important part. A satellite that provides no selective advantage to a virus would be expected to be lost quite rapidly.

Synergistic interactions where one of the viruses is a potyvirus have been shown to be mediated by HC-Pro (Pruss et al., 1997). The helper component proteinase (HC-Pro) is a key protein encoded by plant viruses of the genus Potyvirus that is involved in aphid transmission, replication, virus cell-to-cell and systemic movement as well as countering host defence mechanism by suppression of post-transcriptional gene silencing (Syller, 2004). It thus seemed reasonable to assume that, in the case of synergism between ZYMV and MYLCV, the basis of the synergism would reside with HC-Pro. The experiments involving the expression of ZYMV HC-Pro from a PVX vector suggest that this is the case. These experiments show HC-Pro to complement the missing DNA B functions when MYLCV DNA A is co-inoculated to N. benthamiana. However, it is unclear whether it is the cell-to-cell movement function or the suppression of PTGS functions of HC-Pro that is mediating this movement of a begomovirus DNA A component. A similar complementation of ToLCNDV DNA B functions has recently been shown by Saeed et al. (2007). They showed that, in the absence of DNA B, ToLCNDV DNA A could move systemically in the presence of a DNA β satellite. In common with HC-Pro, the single gene product encode by DNA β satellites, known as βC1, is a suppressor of PTGS (Cui et al, 1995; Gopal et al., 2007; Kon et al., 2007). This may suggest that, for both the complementation of movement of ToLCNDV DNA A by HC-Pro and DNA β, it is suppression of a host encoded resistance preventing movement rather than the proteins actually mediating cell-to-cell movement. However, further investigation will be needed to confirm this suggestion.

There is mounting evidence to suggest that ToLCNDV (and its close relatives such as MYLCV) is not a typical bipartite begomovirus. ToLCNDV is, with increasing frequency, being identified in the field in association with DNA β (Briddon et al., 2006). Although no one has yet shown that in a crop there is a productive interaction
between ToLCNDV and DNA β (an interaction where DNA β provides a selective advantage). Saeed et al. (2007) have shown experimentally, in tomato using clones of a ToLCNDV isolate not associated with DNA β and a DNA β isolated from cotton, that DNA β can complement movement of DNA A in the absence of DNA B. Similar results have been obtained by Saunders et al. (2002). They showed that Sri Lankan cassava mosaic virus (SLCMV) could dispense with its DNA B and systemically infect Ageratum conyzoides in the presence of the DNA β of Ageratum yellow vein virus. SLCMV DNA A is infectious to the experimentally host N. benthamiana in the absence of its DNA B and induces an upward leaf curl phenotype. This phenotype is usually associated with monopartite viruses. Although ToLCNDV DNA A is not infectious in the absence of its DNA B, it does induce the typical upward leaf curl phenotype in N. benthamiana in the presence of the DNA B, which is indistinguishable from that induced by monopartite begomoviruses and SLCMV DNA A. Here the movement of ToLCNDV DNA A has been shown to be complemented by HC-Pro, in the absence of DNA B, and to induce the upward leaf curl phenotype. This shows that there is a symptom determinant on ToLCNDV DNA A. For bipartite viruses, symptoms are usually determined by DNA B (Von Arnim and Stanley 1992; Hussain et al., 2005) These findings indicate that ToLCNDV is not typical of bipartite viruses. It will thus be interesting to investigate whether complementation by HC-Pro is also possible for other bipartite begomoviruses and whether there is the possibility of further such synergistic interactions occurring or whether this synergism happens only with unusual viruses such as ToLCNDV; a virus with monopartite-like characteristics.

Synergism can be unidirectional or mutualistic but, in either case, synergism implies that proteins from one virus (helper virus) can enhance infection by the other (dependent), resulting in diverse viral traits including expansion of host range, acquisition of mechanical transmission, enhanced specific infectivity, enhanced cell-to-cell and long distance movement, novel vector transmission and elevated viral titer (Jonathan et al., 2008). Geminivirus infections of cucurbits are not abundant as compared to solanaceous crops. Similarly DNA β are not commonly reported in cucurbits. DNA β associated with monopartite begomoviruses (Briddon and Stanley, 2006) are required, although not strictly essential, to infect hosts efficiently and induce symptoms. Possibly the satellite is required to overcome host plant defenses by
suppressing post-transcriptional gene silencing, a double-stranded RNA-mediated process which is believed to be part of a plant’s defense against foreign nucleic acids, including viruses (Dunoyer and Voinnet, 2005; Voinnet, 2001). Failure of attempts to detect DNA β in infected muskmelon samples suggests that the combination of viruses in muskmelon does not need a DNA satellite for successful infection in the presence of strong suppressors such as HC-Pro encoded by ZYMV and AC2 encoded by MYLCV. During the last few years DNA β satellites have been reported to be associated with bipartite begomoviruses such as MYMIV (Rouhibakhas and Malthi, 2005) and also with ToLCNDV. For both these viruses, the presence of both the DNA β satellite and the DNA B of the virus cause more severe symptoms than either the DNA A and DNA B or the DNA A with the satellite (Dr V. G. Malathi, personal communication). The absence of DNA β and a functional DNA B suggest that muskmelon may be resistant to begomoviruses. Dissociation of DNA β with cucurbit infecting viruses needs a broader study to understand this phenomenon. The infection of RNA viruses such as potyviruses may have provided opportunity to infect these hosts as secondary infection once the host defense is compromised due to infection of RNA viruses. The origin of potyviruses now prevalent in the Old World is not known. One possibility is that these viruses were introduced from other parts of the world with imported seeds in the colonial period

The synergism described here raises some interesting epidemiological questions. ZYMV is a member of the genus Potyvirus (family Potyviridae); being aphid transmitted in a non-persistent manner (Gal-On, 2007). In contrast, the begomoviruses are transmitted by the whitefly Bemisia tabaci in a circulative manner (Duffus, 1987; Duffus, 1995; Zeidan and Czosnek, 1991). Whereas for synergistic interaction between ZYMV and CMV the viruses may be transmitted by the same aphid vector (Lecoq et al., 1981), transmission of MYLCV and ZYMV by two distinct vector insects and by two distinct mechanisms suggests that it is unlikely that the two viruses are co-transmitted by a single insect species. This aspect of the epidemiology of MYLCD will require further work to exclude this possibility. Were the viruses to turn out to be co-transmitted by a single insect species, it would not be the first case that a synergistic interaction between an RNA virus and a DNA virus was due to a change in vector specificity of one of the pair. The synergistic interaction between Rice tungro spherical virus (RTSV, a virus with an ssRNA genome; genus Waikavirus,
family Sequiviridae) and Rice tungro bacilliform virus (RTBV, a dsDNA virus; genus Badnavirus, family Caulimoviridae) depends upon a change of vector specificity of RTBV from being mealybug transmitted to being transmitted by the green leafhopper (Nephotettix virescens, the vector of RTSV) in the presence of RTSV (Caba et al., 1985). However, it is likely that the two viruses affecting muskmelon are independently transmitted. If this is the case, it means that the two viruses must be transmitted, independently by their respective vectors, to the plant at just the right time to establish a synergistic interaction. The timing and requirements for this to occur will be an interesting future avenue of research.

RNA interference has emerged as a breakthrough and robust technology for engineering resistance in plants to viruses. RNAi is a natural defense mechanism against viruses that is triggered by dsRNA and leads to down regulation of gene expression (Waterhouse et al., 2001; Qi et al., 2005). The concept of pathogen-derived resistance based on expression of viral genes in transgenic plants was well known even before RNA silencing was discovered (Goldbach et al., 2003). Efforts using the concept of PDR against RNA viruses were thoroughly investigated and it was evident that in the majority of such cases resistance resulted from RNA rather than protein. The involvement of double-stranded RNA in the process was evident from the fact that crossing of sense and anti-sense RNA transgenic plants resulted in better resistance (Waterhouse et al., 1998). Since HC-Pro of potyviruses is involved in proteolytic activity of polyprotein, long distance movement of viral proteins and synergistic interaction (Revers et al., 1999; German-Retana et al., 2000; Shi et al., 1997), targeting of these sequences along with coat protein sequences would interfere with both encapsidation and movement of ZYMV. Since MYLCV is dependent on ZYMV for movement in plants and appears defective, there is little concern that this virus will cause problems once ZYMV infection is prevented. Thus, these ZYMV sequences provide a better target for providing enhanced protection against the disease. However, these sequences are not conserved among potyviruses.

For silencing of both HC-Pro and CP through RNAi a simultaneous silencing approach has been adopted. One possible approach to silence the two components could be development of independent constructs targeting HC-Pro and CP sequences separately. Since the double stranded RNA produced by the RNAi construct is
digested into short RNA molecules by Dicer, the resulting short RNA would silence the two genes. Results obtained in this research show that the two components could be silenced simultaneously. There have been many examples of resistance development against potyviruses using CP gene (Lindbo and Dougherty, 1992; Silva-Rosales et al., 1994; Voloudakis et al., 2005) and Di Nicola et al (2005) indicated that RNA silencing of PPV P1/HCPro sequences through hair-pin construct result in an efficient and predictable PPV resistance. Amelioration of PVX-HC-Pro symptoms in transgenic plants inoculated with PVX-HC-Pro construct indicates successful blocking of HC-Pro gene of ZYMV and the blocking of movement of DNA A when inoculated with PVX-HC-Pro proves that the synergistic effect of the two unrelated viruses can also be overcome by a single construct. Since ZYMV is very common among cucurbits, our strategy to control this virus through blocking of multiple genes would help us greatly to alleviate losses in these crops. The involvement of ZYMV in both the Punjab and NWFP is prompted targeting of this virus for resistance. However sometimes, virus encoded suppressor of PTGS lead to concerns about the stability of such resistance. Mitter et al (2003) showed, that CMV was able to suppress dsRNA induced PTGS and associated PVY immunity in tobacco. Using RNAi as a tool to inhibit viral infection and synergism also avoids the hazards of transcomplementation and reduces the possibility of recombination with the transgene in field grown crops because of the fact that only a small portion of gene sequence is used. The ultimate aim of this work is to develop virus resistant muskmelon through genetic engineering. Transformation of these genes constructs in susceptible muskmelon varieties and evaluation of transgenic plants will ultimately result in deployment of genetically engineered strategy for the control of this important pathogen in Pakistan.

The work presented here has for the first time shown, in the field, a synergestic interaction between a DNA and an RNA virus that enhances symptoms. In the future it will be necessary to determine the geographical extent of this synergistic infection and the number of hosts it affects. It is likely that further such synergistic interactions are present or will develop in the future. For this reason it is necessary to determine the nature of the synergistic interaction and possible strategies to prevent losses from this. The study detailed here is a first step in this direction.
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References


References


References


References


References


References


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APPENDICIES

Appendix 1

CTAB method for isolating total genomic DNA modified by Doyle and Doyle, (1990)

1. Take 20 ml 2X cetyl triethylmethyl ammonium bromide (CTAB) and 100 µl mecraptoethanol in a 50 ml falcon tube. Keep the tube in a water bath at 65°C for 30 minutes. Collect fresh leaves in liquid nitrogen. Grind the sample (1 g) to a fine powder.
2. Pour the hot 2X CTAB in ground powder, suspend and incubate at 65°C for 30 minutes with occasional mixing.
3. Add an equal volume of chloroform: isoamyl alcohol (24:1) and mix gently.
4. Centrifuge at 9000 rpm for 10 min at room temp. Take the upper phase in a 50 ml tube and add 2.5 volumes of absolute ethanol. Leave the tubes overnight for viral DNA precipitation at room temp.
5. Next day centrifuge at 9000 rpm for 5 minutes. Decant the supernatant and wash the pellet with 1 ml absolute ethanol.
6. Centrifuge and dry. Dissolve the pellet in 1 ml double distilled autoclaved water, transfer to an eppendorf tube and spin for 2-3 minutes.
7. Take the supernatant in another tube and run 10 µl on 1% agarose gel to check the concentration of genomic DNA.

Reagents:

2X CTAB
2% CTAB (w/v)
100 mM Tris (pH8.0)
20 mM EDTA (pH8.0)
1.4 M NaCl
1% PVP (polyvinylprrolidone)
Appendix 2

Agarose gel electrophoresis
DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels in 0.5X TAE buffer containing ethidium bromide (10 mg/ml). Fragment sizes were estimated by comparison with Fermentas 1 kb ladder. Fermentas 6X DNA loading dye was used.

50X Tris-acetate EDTA buffer (TAE):
Tris base 242 gm
Glacial acetic acid 57.1 ml
0.5 M EDTA (pH 8.0) 100 ml
Make up the final volume with distilled water to 1000 ml.
Appendices

Appendix 3

Southern hybridization
Genomic DNA was run on 1% agarose gel. After that it was transferred to nitrocellulose membrane.

I- Transfer of DNA to nitrocellulose membrane

1. Agarose gel was gently agitated in 0.25 M hydrochloric acid for 15-20 minutes to depurinate the DNA, which improves transfer of high molecular weight and super coiled DNA.
2. DNA was denatured by soaking the gel for 15-20 minutes in several volumes of denaturation solution (0.5 M NaOH, 1M NaCl) with constant, gentle agitation (e.g., on a rotary platform).
3. The gel was briefly rinsed in de-ionized water, and then neutralized by soaking for 30 minutes in Neutralization solution [1 M Tris-HCl (pH 7.5), 1.5 M NaCl] at room temperature with constant gentle agitation.
4. The gel was transferred to a steel tray having 2X SSC solution and was put on a tray equal to the gel size. The nitrocellulose membrane was put on the gel and then a filter paper equal in size was put on it. For any unused area of the gel was trimmed away with a razor blade. Bottom left corner of the gel was cut off, which served to orient the gel during the second operation.
5. DNA was transferred overnight onto Hybond N-N filters (Amershame, Life Science) by capillary action in 20X SSC (0.3 M trisodium citrate, 3 M NaCl), pre-soaked in same buffer.
6. After blotting, the filters were washed in 2X, SSC, UV-crosslinked in UV Stratalinker 2400 (Stratagen) and air-dried. The filters were ready for hybridization.

II- Preparation of radioactively labeled DNA probe
Probe was prepared by Random primer method, using rediprime™/kit. NSP gene of ToLCNDV.
The following protocol was used for probe preparation.

1. The DNA to be labeled was diluted to a concentration of 2.5-25 ng in 45 µl of 10 mM Tris HCl pH 8.0, 1 mM EDTA. (TE buffer).
2. The DNA sample was then denatured by heating to 95-100°C for 5 minutes in a boiling water bath.
3. The DNA was snap cooled by placing on ice for 5 minutes after denaturation.
4. After snap cooling the DNA was centrifuged briefly to bring the contents to the bottom of the tube.
5. Then the denatured DNA was added to the already prepared reaction tube.
6. 5 µl (0.01mci/µl) of radioactive $^{32}$P dCTP was added and mixed by pipetting up and down about 12 times, moving the pipette tip around in the solution and left for one hour at 37°C.
7. The reaction was then stopped by adding 5 µl of 0.2 M EDTA. For use in hybridization, the labeled DNA was then denatured by heating to 95-100°C for 5 minutes, and then snap cooled on ice for 5 minutes.
8. The tube was then centrifuged briefly and contents of the tube were mixed.
9. 14 µl of the labeled probe was then used per 5 ml of hybridization buffer.

III- DNA hybridization

1. The filters were placed in the hybridization tubes, to which was added 30 ml of pre-hybridization solution (6X SSPE, 0.08% (w/v) polyvinly pyrrolidine, 0.08% (w/v) ficoll, 0.08 % BSA, 1% SDS and 4µg/ml denatured calf thymus DNA) and pre-hybridized at 65°C for two hours in a hybridization oven (HYBAID, MIDI 14).
2. Hybridization was performed by adding the denatured radioactively labeled probe to the prehybridization solution and incubation at 65°C overnight.
3. After hybridization, blots were washed in 2X SSC and 0.1 % (w/v) SDS at 65°C until no more hybridized probe was left, then blots were air dried on blue towel and wrapped in cling film.

4. Autoradiography was carried out at –70°C using intensifying screens on Fuji RX film.

**IV- Production of Radioactive Probes**

The reaction mixture is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ngDNA</td>
<td>21 µl</td>
</tr>
<tr>
<td>dATP</td>
<td>1 µl</td>
</tr>
<tr>
<td>dGTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>dTTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>Buffer 32p)</td>
<td>20 µl</td>
</tr>
<tr>
<td>αP^{32} dCTP</td>
<td>5 µl</td>
</tr>
<tr>
<td>klenow</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Incubate at 25°C for 1 hr.

Add 5 µl stop buffer.

Denature the probe and add to the prehybridization solution.

**V- Reagents used in Southern hybridization**

**Depurination solution (Solution I)**

- HCL: 0.25N

**Denaturation solution (Solution II)**

- NaOH: 0.5M
- NaCl: 1.5M

Dissolve in water to make the final volume 1L.

**Neutralization solution (Solution III)**

- Tris base: 0.5M
- NaCl: 1.5M
**Appendicies**

EDTA  1.0mM
Dissolve in water to bring the final volume to 1L. Conc. 0.5M HCl is used to adjust the pH to 7.2.

**20X SSC**
- NaCl  3M
- Sodium Citrate  0.3M
- EDTA  1mM
Adjust pH to 7.6 with 10 N NaOH.
Adjust volume to 1L and autoclave.

**Denhardt’s Reagent (50X)**
- Ficoll  1% (w/v)
- PVP  1% (w/v)
- BSA  1% (w/v)
Total volume was made up to 1L and filter sterilize.

**Prehybridization solution**
- 20X SSC  15ml
- 50X Denhardt  5ml
- CT DNA boiled  5mg
- 10%SDS  1%
- dH₂O  25 ml
Total  50 ml
Appendix 4

I- Preparation of heat shock competent \textit{E. coli} 10b cell

1. A single colony from a freshly grown plate of \textit{E. coli} was picked and transferred into 50 ml LB (Appendix-8-I) medium in a 250ml flask and incubated at 37°C overnight with vigorous shaking.

2. 2 ml of the overnight culture was taken and diluted to 250 ml liquid LB in a 1L flask and shaken vigorously at 37°C until OD of 0.5-1.0 (10^{10} cells/ml).

3. Culture was cooled by placing on ice for 30 minutes. The cells are transferred aseptically to sterile disposable 50 ml propylene tubes

4. The cells were pelleted by centrifugation at 4000 rpm at 4°C for 5 minutes and resuspend in 5 ml of 0.1 M MgCl$_2$.

5. The cells were pelleted by centrifugation at 4000 rpm at 4°C for 15 minutes and resuspend in 5 ml of 0.1 M CaCl$_2$ and kept on ice for 30min.

6. The cells were again pelleted by centrifugation at 4000 rpm for 5 minutes and suspend finally in 10ml of 0.1M, CaCl$_2$ and sterile cold glycerol in thr ratio of 3:7 respectively.

7. The cells were stored in aliquots of 100 µl or 200 µl at –70°C.

II- Transformation of heat shock competent cells of \textit{E. coli} strain 10B

Transformation of competent cells was carried out using methods described by Sambrook \textit{et al.}, (1989). Frozen, competent cells (100 µl) were thawed on ice for 10 min. DNA (0.2-0.5 µg) was added, mixed gently and incubated on ice for 1 hr. The cells were heat-shocked by placing the tubes in a 42°C water-bath for 2-3 min. and replaced on ice for another 20-60 min. LB broth (1 ml) was added, shaken gently and incubated at 37°C in water bath for 1h. Cells were collected by centrifugation at 11,600 g for 30 sec, and resuspended in 100 µl LB. Dilutions of the cell suspension in 10 fold steps were made. Using an ethanol-dipped, flame-sterilized and cooled glass spreader, the cells were spread onto LB plates containing selective antibiotic. Plates were incubated overnight at 37°C.
Appendix 5

**Phenol-chloroform extraction**

The PCR product was diluted with d$_3$H$_2$O to 100 µl. An equal volume of phenol and chloroform (1:1) was added. The mixture was shaken gently and centrifuged for six minutes. The supernatant was taken into a fresh eppendorf tube. 1/10$^{th}$ volume of 3 M sodium acetate (10 µl), pH 5.4 and 2.5 volume of absolute ethanol (250 µl) were added. The mixture was placed at –20°C for an hour and then centrifuged for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The mixture was centrifuged for two minutes, the supernatant was removed and the pellet air dried. Finally the pellet was dissolved in 20 µl d$_3$H$_2$O.
Appendix 6

Plasmid isolation from *E. coli*

The following protocol was used for the isolation of plasmid DNA from *E. coli*.

1. A single *E. coli* colony was cultured in 25 ml liquid LB (Appendix-8-I) medium containing appropriate antibiotic and grown overnight at 37°C.
2. The *E. coli* culture was centrifuged in 1.5 ml eppendorf tube at 14000 rpm for 5 minutes.
3. 100 µl of solution I (Appendix-8-II) was added to eppendorf tube and the pellet was suspended in the solution with the help of a vortex mixer.
4. 150 µl solution II (Appendix-8-II) was added to eppendorf tube and mixed well by inverting gently.
5. 200 µl of solution III (Appendix-8-II) was added to eppendorf tube mixed well and centrifuged at 14000 rpm for 5 minutes.
6. The supernatant was taken into a fresh eppendorf tube and two volumes of 100% ethanol were added.
7. The eppendorf tube was kept at –20°C for 20 minutes and centrifuged at 14000 rpm for 5 minutes.
8. The supernatant was discarded and the pellet was washed with 70% ethanol.
9. After centrifugation the supernatant was discarded and pellet was vacuum dried.
10. 10-20 µl of distilled water was added to the pellet to dissolve DNA and stored at –20°C.
Appendix 7

I- Preparation of electro competent cells of *Agrobacterium tumefaciens* strain LBA 4404/ GV 3101

1. A single colony from a freshly grown plate of LBA 4404/GV3101 was picked and inoculated into 100 ml LB liquid (Appendix-8-I) medium in 250 ml autoclaved flask using sterile toothpick and incubated at 28°C for 48 hours with vigorous shaking.

2. 5 ml of the 48 hours grown culture was reinnoculated into 1L flask containing 250 ml of the same LB medium and incubated at 28°C until OD$_{600}$ of cells became 0.5-1.0 (10$^8$ cells/ml).

3. The cells were transferred aseptically to ice cold 50 ml propylene tube and kept cool on ice for 10 minutes.

4. The cells were then centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was decanted and the cells were pelleted, then resuspended in 50 ml of sterile cold d$_3$H$_2$O.

5. The cells were again centrifuged at 4000 rpm at 4°C for 10 minutes. The supernatant was decanted and the cells were pelleted, then resuspended in 25 ml of sterile cold ddH$_2$O.

6. After another wash cells were resuspended in 10ml sterile cold d$_3$H$_2$O containing filter sterilized cold 10% glycerol. This wash was repeated.

7. Finally the cells were resuspended in 1-1.5 ml filter sterilized cold 10% glycerol, aliquoted in 50 µl and stored at –70°C.

II- Transformation of clones in *Agrobacterium tumefaciens* strain LBA 4404/GV 3101 by electroporation

Only 2 µl of each clone was used for electro-transformation by electro cell manipulator 600 (BTX San Diego, California).

For electroporation the following protocol was used.

1. Electroporation cuvettes 1 mm gap were placed on ice.
2. Vials of frozen electro-competent cells of *Agrobacterium* were allowed to thaw on ice.

3. 1 μg DNA of the recombinant plasmid was mixed with 50 μl of electro-competent cells in the electroporation cuvettes on ice.

4. The electro-competent cells containing the DNA mixture were transferred to electroporation cuvette.

5. Pulse was given and 1 ml of liquid LB medium is added immediately, mixed gently and transferred to a 1.5 ml eppendorf tube and incubated at 28°C for 1 hour with vigorous shaking.

6. 200 μl and 400 μl of transformed culture were spread on petriplates containing solid LB medium supplemented with appropriate antibiotic so that only transformed cells should multiply.

7. When the liquid was absorbed completely the plates were sealed with para film and kept at 28°C for 2-3 days.

8. At the end of incubation colonies were picked with sterile toothpicks and cultured in 5 ml liquid LB medium in 50 ml tube containing appropriate antibiotic.

9. Culture tubes were kept at 28°C on shaker in *Agrobacterium* growth room for 48 hours with vigorous shaking.

10. Transformants were confirmed through PCR using primers specific to the gene or insert.
Appendicies

Appendix 8

I- LB (Luria-Bertini) broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1.0 %</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 %</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 %</td>
</tr>
</tbody>
</table>

Adjust pH 6.2-7.2 with NaOH and autoclave.

I- LB agar (Luria-Bertini) medium

Prepared as above with the addition of 1.5% agar prior to autoclaving.

II- Mini Prep Solutions

Solution I (Suspension buffer)

- 0.38 g Tris HCl pH 8 (25 mM)
- 0.37 g EDTA pH 8 (10 mM)
- 0.9 g Glucose (50 mM)

Make total vol. 100 ml with d$_2$H$_2$O.

Solution II (Denaturation soln.)

- 0.8 g NaOH (0.2 N)
- 1 g SDS (1 %)

Make total vol. to 100 ml with d$_2$H$_2$O.

Solution III (Neutralization soln.)

- 60 ml Potassium acetate (5 M)
- 11.5 ml Glacial acetic acid

Make total vol. to 100 ml with ddH$_2$O.
Appendices

Appendix 9

MS0 medium
1) MS Salts 4.4 g/L
2) Sucrose 30 g/L
Adjust pH to 5.8 with 0.1 N KOH before autoclaving.

Seed germination medium
1) MS Salt 4.4 g/L
2) Sucrose 30 g/L
3) Phytagel 1 g/L
Adjust pH to 5.8 with 0.1 N KOH before autoclaving.

Co-culture medium:
1) MS Salts 4.4 g/L
2) Sucrose 30 g/L
3) Phytagel 5.6 g/L
Adjust pH to 5.8 with 0.1 N KOH.
After autoclaving add filter sterilized:

4) B5 vitamin (100X) 10 ml/L
5) 1-naphthylacetic acid (NAA) (1mg/ml) 0.1 mg/L
6) 6-benzylaminopurine (BAP) (1mg/ml) 1 mg/L

Selection medium:
1) MS Salts 4.4 g/L
2) Sucrose 30 g/L
3) Phytagel 5.6 g/L
Adjust pH to 5.8 with 0.1 N KOH.
After autoclaving add filter sterilized:

4) B5 vitamin (100X) 10 ml/L
5) nephtylacetic acid (NAA) (1mg/ml) 0.1 mg/L
6) 6-benzylaminopurine (BAP) (1mg/ml) 1 mg/L
### Rooting medium

1. **MS Salts**  
   4.4 g/L  
2. **Sucrose**  
   20 g/L  
3. **Phytogel**  
   5.6 g/L  

Adjust to pH to 5.8 with 0.1 N KOH.  
After autoclaving add filter sterilized:

4. **1-naphthylacetic acid (NAA) (1mg/ml)**  
   0.1 mg/L  
5. **Cefotaxime (100mg/ml)**  
   250 mg/L  
6. **B5 vitamin (100X)**  
   10 ml/L
Appendix # 10

Stock solutions

Vitamin B5 (100X)

1) Thiamine HCl 1 g/100 ml
2) Pyridoxin HCl 100 mg/100 ml
3) Nicotinic Acid 100 mg/100 ml
4) Myoinsitol 10g/100 ml

Stock was filter sterilized and frozen.

Kanamycin (stock 100 mg/ml)

Kanamycin-sulphate 50 mg/L

Double distilled deionized water was used for stock and final stock was filter sterilized using Millipore filters of 0.22 µm and stored at –20 °C in aliquots.

Gulfosinate ammonium (stock 10 mg/L)

Gulfosinate ammonium 10 mg/l

Double distilled deionized water was used for stock and final stock was filter sterilized using Millipore filters of 0.22µm and stored at –20 °C in aliquots.

BAP (stock 1 mg/ml):

6-benzylaminopurine 1 mg/L

First dissolved in 1ml NaOH (1M), after making volume with double distilled deionized water, it was filter sterilized.

NAA (stock 1 mg/ml)

1-naphthylacetic acid 0.1 mg/L

First dissolved in 1 M NaOH, after making volume with double distilled deionized water, it was filter sterilized.

6X Gel loading buffer

1) Bromophenol blue 0.25% (w/v)
2) Xylene cyanol FF. 0.25% (w/v)
3) Glycerol 30.0% (v/v)
Dissolve in distilled water.

**IPTG (isopropyle-thio-β-D-galactoside) Stock solution (0.1M):**
Make a stock solution of 50 mg/ml in distilled water. Use 100 µl/100 ml.

**X-Gal stock solution:**
X-Gal (5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside) is available as 40 mg/ml stock in N, N dimethyle formamide. Use 0.5 ml/100 ml LB.