GENETIC ENGINEERING OF POTATO FOR BROAD SPECTRUM RESISTANCE AGAINST RNA VIRUSES

By

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

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IN
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Islamabad

“We, the supervisory committee, certify that the contents and form of thesis submitted by Mr. Muhammad Arif 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 parents.
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Muhammad Arif
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Full Name</th>
<th>Acronyms</th>
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<tbody>
<tr>
<td><em>African cassava mosaic virus</em></td>
<td>ACMV</td>
</tr>
<tr>
<td><em>Ageratum yellow vein virus</em></td>
<td>AYVV</td>
</tr>
<tr>
<td><em>Alfalfa mosaic virus</em></td>
<td>AMV</td>
</tr>
<tr>
<td><em>Andean potato mottle virus</em></td>
<td>APMoV</td>
</tr>
<tr>
<td>Aphid Transmission</td>
<td>AT</td>
</tr>
<tr>
<td><em>ARGONAUTE</em> protein</td>
<td>AGO</td>
</tr>
<tr>
<td><em>Barley stripe mosaic virus</em></td>
<td>BSMV</td>
</tr>
<tr>
<td><em>Bean dwarf mosaic virus</em></td>
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</tr>
<tr>
<td><em>Bean golden mosaic geminivirus</em></td>
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</tr>
<tr>
<td><em>Bean yellow mosaic virus</em></td>
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<tr>
<td><em>Bemisia tabaci</em></td>
<td><em>B. tabaci</em></td>
</tr>
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<td><em>Bhendi yellow vein mosaic virus</em></td>
<td>BYVMD</td>
</tr>
<tr>
<td><em>Cauliflower mosaic virus 35S</em></td>
<td>CMV 35S</td>
</tr>
<tr>
<td><em>Cactus virus X</em></td>
<td>CVX</td>
</tr>
<tr>
<td><em>Chrysanthemum stem necrosis virus</em></td>
<td>CSNV</td>
</tr>
<tr>
<td>Coat protein</td>
<td>CP</td>
</tr>
<tr>
<td>Coat Protein-Mediated Resistance</td>
<td>CPMR</td>
</tr>
<tr>
<td>Complementary DNA</td>
<td>cDNA</td>
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<tr>
<td><em>Cotton leaf curl disease</em></td>
<td>CLCuD</td>
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<tr>
<td><em>Cotton leaf curl virus</em></td>
<td>CLCuV</td>
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<tr>
<td><em>Cucumber mosaic virus</em></td>
<td>CMV</td>
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<tr>
<td>Cylindrical Inclusion Protein</td>
<td>CI</td>
</tr>
<tr>
<td><em>Cymbidium mosaic virus</em></td>
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</tr>
<tr>
<td>Defective interfering</td>
<td>DI</td>
</tr>
<tr>
<td>DNA methyl transferase</td>
<td>DMTase</td>
</tr>
<tr>
<td>Double anti-body sandwich ELISA</td>
<td>DAS ELISA</td>
</tr>
<tr>
<td>Double stranded DNA</td>
<td>dsDNA</td>
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<tr>
<td>Double stranded RNA</td>
<td>dsRNA</td>
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<tr>
<td>Endoplasmic Reticulum</td>
<td>ER</td>
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<td><em>Figwort mosaic virus</em></td>
<td>FMV</td>
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<tr>
<td><em>Groundnut rings spot virus</em></td>
<td>GRSV</td>
</tr>
<tr>
<td>Hairpin RNA</td>
<td>hpRNA</td>
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<tr>
<td>Helper component protease</td>
<td>HC-Pro</td>
</tr>
<tr>
<td>Histone deacetylase</td>
<td>HAD</td>
</tr>
<tr>
<td>Hypersensitive Response</td>
<td>HR</td>
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<tr>
<td>Intergenic region</td>
<td>IR</td>
</tr>
<tr>
<td>Messenger RNA</td>
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<tr>
<td>Movement protein</td>
<td>MP</td>
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<tr>
<td>Neomycin phosphotransferase II</td>
<td><em>npt II</em></td>
</tr>
<tr>
<td>Non-translated Regions</td>
<td>NTR</td>
</tr>
<tr>
<td>Nucleoside triphosphate</td>
<td>NTP</td>
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Nuclear Inclusion Protein a  
Nuclear Inclusion Protein b  
Octopine synthase  
*Okra yellow vein mosaic virus*  
Open Reading Frames  
*Plum pox virus*  
Pathogen derived resistance  
Pokeweed antiviral protein  
Post Transcriptional Gene Silencing  
*Potato leaf roll virus*  
*Potato spindle tuber viroid*  
*Potato virus X*  
*Potato virus Y*  
*Pepper mild mottle virus*  
Recombination dependent replication  
Replication associated protein  
Replication enhancing protein  
Ribonuclease III  
Ribosomal RNA  
Rice yellow mottle virus  
RNA dependent RNA polymerases  
RNA dependent DNA methylation  
RNA induced silencing complex  
RNA induced transcriptional gene silencing  
Satellite conserved region  
Satellite RNA  
Single stranded DNA  
Size exclusion limit  
Small interfering RNAs  
*Tobacco rattle virus*  
*Tobacco etch virus*  
*Tobacco mosaic virus*  
*Tobacco bushy stunt virus*  
*Tobacco chlorotic spot virus*  
*Tobacco golden mosaic virus*  
*Tobacco leaf curl virus*  
*Tomberry mosaic virus*  
*Tomato spotted wilt virus*  
*Tomato yellow leaf curl Sardinia virus*  
*Tomato yellow leaf curl virus*  
Transcriptional activating protein  
Transcriptional gene silencing  
Transfer DNA  
Transfer RNA  
Triple gene block
<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Abbreviation</th>
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<tr>
<td>Turnip mosaic virus</td>
<td>TuMV</td>
</tr>
<tr>
<td>Turnip crinkle virus</td>
<td>TCV</td>
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ABSTRACT

Pathogens and pests always have been and still is a potential threat to agricultural production worldwide. Potato (Solanum tuberosum L.) is nutritionally a balanced staple food; therefore, it has potential to minimize the pressure on cereal crops in Pakistan. Viral diseases are major problem in stable crop production, especially in vegetative propagated plants such as potato where diseases are easily transmitted from one clonal generation to the next. Conventional methods of virus control are limited to use of virus-free seed tubers and chemical control of insect vectors. However, development of resistant cultivar is the only effective, economical and environmentally safe method of disease control. The use of RNA silencing has become the tool of choice for gene silencing in many crop species. The key element of this technology is the discovery of double-stranded RNA (dsRNA), diced into small interfering RNAs (siRNA), which is a potent trigger for RNA silencing. By arranging transgenes as inverted repeats encoding self-complementary hairpin RNA (hpRNA), which is diced into siRNA after transcription, it is possible to obtain strong silencing of expression of homologous RNA. Using this technology efficient simultaneous knock-down of multiple genes of three different viruses have been achieved by using a single RNAi construct in potato. In this study, the transgenic resistance in potato was obtained based on the construction of hairpin RNA plant expression cassettes I and II containing the sequences of different genes of three important potato viruses. The cassette I containing the short sequences of Nucleotide Triphosphate binding helicase (NTP) gene of Potato Virus X (PVX), Helper Component Protease (HC-Pro) gene of Potato Virus Y (PVY) and Coat Protein (CP) gene of Potato Leaf Roll Virus (PLRV), while the expression cassette II contains the sequences of CP genes of PVX, PVY and PLRV. The sense and anti-sense fragments of these genes were fused separately to form a chimeric N gene and arranged in an RNAi vector as inverted repeats, under the CaMV 35S promoter, separated by intron. These expression cassettes were transformed separately in potato cv Kuroda and Desiree through Agrobacterium mediated transformation by using Agrobacterium tumefaciens strain LBA 4404. Fourteen independent transgenic lines of each cassette were developed and transferred to containment after analysis of T₀ transgenic plants by PCR and Southern hybridization for the presence of transgenes. The transgenic expression of these
cassettes showed that up to 20% of the transformed plant lines were resistant and 46% were tolerant to all three viruses. The analysis of the resistant plants showed accumulation of siRNA as compared to susceptible transgenic and non-transformed control plants. This indicates that the resistance is due to simultaneous RNA silencing of the three target genes in each construct. Overall, the work presented here demonstrates a simple procedure to obtain broad spectrum virus resistance in two commercial potato cultivars Kuroda and Desiree by RNA silencing technology. At present, another independent study is being conducted to multiply and evaluate the field performance of putative transgenic potato lines after obtaining approval of National Biosafety Committee (NBC) of Environment Protection Agency (EPA), Government of Pakistan. In future, studies to improve frequency of developing multiple virus resistant plants could be attempted by extending the transgenes construct with a large number of smaller fragments of target genes. Moreover, it is possible that present strategy can be extended to other plant species to obtain broad spectrum resistance against many other devastating viruses.
CHAPTER 1

Introduction and Review of Literature

1.1 Potato crop

Potato (Solanum tuberosum L.) and other tuber bearing Solanum species first time originate in the highland regions of the Andes in Peru and Bolivia (Burton, 1966). Potato is the fourth most important crop in production and fifth in area among crop plants grown for human consumption world wide. Potato is being appreciated for its nutritional value as well as its uses in the starch and food processing industry (Ross, 1986). Most of the world's potato crop is grown in the industrialized countries of the northern temperate zone. During the last three decades, potato in developing countries has experienced the world’s highest annual growth rate in production. In the early 1990s, the contribution of developing countries to the global potato production was 30% as compared to 11% in the early 1960s (Schmiediche, 1997). It is now a valuable cash crop in almost all countries of this region and ranked as the 2nd to 4th most important crop in relation to other crops (Niederhauser, 1993).

Potato has emerged as high yielding cash crop in Pakistan during the last decade and the area under its cultivation has increased rapidly since independence. The total estimated production is 2.63 million tons from an area of 138 thousand hectares (MINFAL, 2007). However, per hectare average yield is very low (16 to 19 tons/ha) as compared to developed potato grown countries (45 to 50 tons/ha). Pakistan has very unique climate in the world for the cultivation of potato crop. Irrigation system combined with climatic condition allowing the cultivation of three crops round the year in various agro-ecological zones from sea level to 3000m altitude. Autumn crop in plains and in southern Punjab, plains of Balochistan and Sindh, spring crop is cultivated in the plains and lower hills of Balochistan, North-West-Frontier Province; and one summer crop is cultivated in the high hilly northern areas (Gilgit and Skardu), North Western Frontier Province and Azad Jummu Kashmir (Khalid et al., 2000). Bulk of the production comes from Okara, Sahiwal, Sialkot, Lahore, Kasur and Jhang districts of Punjab which
contributes about 50% of the total production. Other important areas for production are Pishin and Kalat districts of Balochistan and Kaghan, Swat, Dir and Peshawar valleys of the North Western Frontier Province. Potato is nutritionally a balanced staple food as compared to rice, corn, wheat and carrot. Therefore it has a great potential to supplement the food resources of the country and minimize the pressure on cereal crops. The rapid growth rate of potato production has declined in many developing countries because of three major constraints: disease and pest control, seed production and scarce economic resources (Ross, 1986; Niederhauser, 1993).

1.1.1 Potato Diseases
Potato is susceptible to many diseases and pests, and the amount of chemical pesticides applied annually to this crop is greater than that of any other food crop. The cost or availability of pest control techniques could be a limiting factor in further development of potato in developing countries. Many pathogens like bacteria, fungi and viruses can attack on potatoes. The important bacterial diseases of potatoes are Bacterial wilt (*Pseudomonas solanacearum*), Black Leg, Soft rot (*Erwinia caratovora*), Ring rot (*Corynebacterium sepedonicum*) and important diseases due to fungi includes Common scab (*Streptomyces scabies*), Late blight (*Phytophthora infestans*), Early blight (*Alternaria solani*), Silver scurf (*Helminthosporium solani*), Pink rot (*Phytophthora erythroseptica*), Black scurf (*Rhizoctonia solani*), Verticillium wilt, *Fusarium* wilt, Powdery scab (*Spongospora subterranea*), Powdery mildew and *Fusarium* dry rot (Khalid et al., 2000).

Potato is an asexually reproducing crop, which makes it highly susceptible to systemic and viral disease. Potato-infecting viruses are among the most important group of disease agents with potato late blight pathogen (*Phytophthora infestans*) and the bacterial wilt agent (*Ralstonia solanacearum*). Next to tulip, the potato is the oldest sufferer from virus diseases. Potatoes are susceptible to about 40 viruses and two viroids (Jeffries et al., 2005). Of these most important viruses in Pakistan are potato virus M (PVM), potato virus S (PVS), potato virus X (PVX), potato virus A (PVA), potato virus Y (PVY) and potato leaf roll virus (PLRV) (Khalid et al., 2000). Among all the viruses that have been reported to infect potato, PVX,
PVY and PLRV are considered to be the most destructive. Yield reduction in potato by these viruses may reach up to 80% in susceptible cultivars, but an even greater loss might be incurred when PLRV occurs in mixed infection with PVX or PVY (Thomas et al., 1997; Mughal, 1990; Khalid et al., 2000).

1.2 Viruses

Being the Latin origin “virus” had the meaning of slimy liquid, poison. According to the Oxford English Dictionary, in the 18th century, “virus” got the meaning of a morbid principle or poisonous substance produced as a result of some disease in the body especially in the case of infectious disease in which these poisonous substances are capable of being introduced into other body of human or animal. Viruses are obligate parasites and can not be cultivated on artificial media in the same fashion as other organisms like bacteria. A virus can be define as “a simplest microscopic parasite consists of core RNA or DNA surround by a protein coat, causes a number of diseases in plants, animals and bacteria”.

Viruses are found everywhere in nature or it can be said that wherever cellular life occurs, viruses also occur. Viruses infect all of the kingdom Animalia and kingdom Planta. Virus particles are not complete cells so they cannot carry out functions of their own. The size of most of the viruses falls in the range of 30 and 100 nm. However, there are many exceptions, like the poxviruses has oval shape virions and have size of 200 to 400 nm. The genetic material of viruses can be either DNA or RNA, occurring as either single stranded or double stranded molecules in nature. The genetic material of viruses is overlaped by a protein coat. Some viruses may possess a wrap over a protein coat; other may have internal proteins (Hull, 2002).

1.2.1 Plant viruses

Plant viruses are obligate intracellular parasites, like all other viruses, that do not have the molecular machinery to replicate. The history of plant virus diseases starts from the early sixteenth century. The first plant virus disease reported in 1576 was tulip flower breaking.
disease (tulip mosaic) (Smith, 1935). Plant viral diseases are serious threats to crops and cause economic losses in variety of crops by reducing yield and compromising quality (Kang et al., 2005). There are about 1000 plant-infecting viruses known today and most plant viruses have a broad host-range (Hull, 2002). The first plant virus discovered was Tobacco mosaic virus (TMV). Plant viruses have been classified in 73 different genera, of which 49 have been divided into families. On the basis of genome, the plant viruses can also be classified into two groups i.e. DNA and RNA virus (Khan and Dijkstran, 2006).

1.3 Transmission of plant viruses

After the discovery of viruses, it was clear soon that these contagious agents differ in their transmissibility. No transmission of virus occurred when the virus titer in the inoculum was too low. Also the presence of some substances (phenolic compounds) in the inoculum, which inhibited the infection process, hampered the transmission of viruses. Besides these phenolic compounds, some plants have been reported to contain certain proteins which inhibit the infection by making the plants less susceptible to viruses (Allard, 1918). However, the viruses have adopted a number of means for its transmission.

1.3.1 Mechanical or sap transmission
Grafting or budding is an old established method to propagate the plants vegetatively. This is the easy way of transmission of virus from the scion or bud to rootstock through sap. The effectiveness of inoculation of sap-transmissible viruses can be increased by dusting the leaves by fine carborendum powder prior to inoculation (Rawlins and Tompkins, 1936). The reported sap-transmitted virus includes CMV, TMV, PVX and some geminiviruses. It implies direct transfer of sap accidentally from wounded plants to healthy plants on tools, hands, clothes or machinery. Potato virus X can easily be spread by farm implements. The ability of these viruses to be spread by sap in the field is due to their extreme stability.
1.3.2 Virus transmission by insects

In nature, most of the viruses are transmitted by vectors, i.e., organisms able to carry-over the virus from one plant to another over a short or long distance. Insects are most common and economically important means among vectors. First report of involvement of insect in transmission of virus came from a publication on rice stunt disease from Japan (Bennett, 1940). Insects transfer the virus in persistent and non-persistent manner (Watson and Roberts, 1939). Persistently transmitted viruses are acquired from a diseased plant and the vector cannot transfer it to healthy plant immediately. The virus has to circulate within the mid gut of the insect and finally reach to the salivary system. The period between the acquisition of virus by vector and transmission to healthy plant is called latent period. In non-persistent manner the vector acquires the virus and transmits it in a few seconds. The potato virus Y (PVY) is transmitted in non-persistent manner, while potato leaf roll virus (PLRV) is persistent in its vector, *Myzus persicae*.

1.3.3 Virus transmission by nematodes

Many widespread and important viruses have been shown to be transmitted via soil-borne nematodes. The three genera of nematode i.e., *Xiphinema, Logidorus* and *Trichodorus* of the order *Dorylaimida* are known to transmit plant viruses. Nematodes vectors feeds on cells of root tips with their stylet, acquiring virus. The virus is retained within the gut or esophagus and transmitted to plants on feeding of nematodes. There are 38 nepoviruses and three tobraviruses already have been reported to be transmitted by soil-borne nematodes (Williamson and Gleason 2003).

1.3.4 Pollen and seed transmission of viruses

The virus transmitted by pollen may infect the seed and the seedlings which grow from it or it can also infect the plant through the fertilized flower. The pollen transmission is known to occur mainly in fruit trees like sour cherry. The *Ilarviruses* are known to transmit through pollen. Many important virus diseases are known to be transmitted by seeds. *Bean common mosaic virus* (BCMV) and *Cucumber mosaic virus* (CMV) were among the first reports on transmission of viruses through seeds (Doolittle and Gilbert, 1999; Reddick and Stewart,
1919). The earliest possible infection of seedlings is the result of viruses transmitted through seed. *Pea seed born mosaic virus* has been dispersed throughout the world in infected seeds.

### 1.4 POTATO VIRUS X (PVX)

#### 1.4.1 Hosts, Symptoms, Distribution

*Potato virus X* (PVX) causes significant damage to plants of the families of *Amaranthaceae, Cruciferae, Solanaceae* and some member of *Leguminosae*. Most important damaged crop plants are potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), brassica (*Brassica campestris* ssp. *rapa*), tomato (*Lycopersicon esculentum*). The damage becomes more significant when PVX attack with association of other viruses like PVY and PLRV. PVX is often a latent virus i.e. the symptoms are not clearly visible to the naked eye. It may show symptoms ranging from a mild mottling of the leaf to a severe mottling of the plant with roughening and reduced leaflet size. Mottling may be more visible in the cloudy weather, and may be not existent after a few days of sunny weather. The overall growth of plant may be stunted with small leaves. In some cases, the tips of the plant may die (Rich 1983).

The PVX is distributed worldwide in potato grown areas. It is transmitted mechanically by plant to plant contact (leaves, shoots and roots), machinery, cutting tools and animals. Chewing insects such as grasshoppers have also been suspected as a means of spreading the disease. There must be wounding and an exchange of plant sap for infection to occur.

#### 1.4.2 Virus structure and composition

Potato virus X belongs to genus *Potexvirus*. The genus *potexvirus* is one of eight genera belonging to the family *Flexiviridae* containing about 40 species (Adam *et al.*, 2004). The members of this family are characterized by flexuous, filamentous virions between 470 and 580 nm in length, built of subunits of a single coat protein (CP). *Potexviruses* have monopartite, positive-strand RNA genomes encoding five open reading frames (ORFs). Methylguanosine cap is present on the 5’ end and the 3’ end has a poly (A) tail (Huang *et al.*, 2004). The first ORF encodes the viral replicase. The central region of the genome is divided
into three overlapping ORFs, known as the triple-gene block (TGB). The TGB encodes three movement proteins (MP) named TGBp1, TGBp2 and TGBp3. These are required for virus cell-to-cell movement (Verchot et al., 1998; Verchot, 2005). The final ORF is the viral capsid protein CP, which is required for genome encapsidation and virus cell-to-cell movement (Fedorkin et al., 2001).

**Figure: 1.1. Genome map and protein produced from single-stranded RNA of Potato virus X.**

<table>
<thead>
<tr>
<th>ORF</th>
<th>Molecular Weight</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>ORF1</td>
<td>166 kDa</td>
<td>RNA-Replicating protein</td>
</tr>
<tr>
<td>ORF2</td>
<td>25 kDa</td>
<td>Triple gene block involve</td>
</tr>
<tr>
<td>ORF3</td>
<td>12 kDa</td>
<td>in viral transport &amp; replication</td>
</tr>
<tr>
<td>ORF4</td>
<td>8 kDa</td>
<td></td>
</tr>
<tr>
<td>ORF5</td>
<td>25 kDa (CP)</td>
<td>Virus particles formation, protection</td>
</tr>
</tbody>
</table>

### 1.4.3 Properties and functions of gene product of potexviruses

The open reading frame 1 (ORF1) of potexvirus produces RNA-Replication protein. The replicase protein of potexvirus is a single protein that involves in the RNA helicase, methyltransferase and RNA polymerase activities. The template-dependent PVX replicase isolated from the infected tobacco plants disclosed the association of replicase with cellular membranes (Plante et al., 2000). Two RNA stem-loop structures (5′SL1 and 5′ SL2) are present with in the first 182 nucleotide of ORF1 which are very essential for the replication of PVX (Kim and Hemenway, 1996; Miller et al., 1999). The 5′ SL1 element is multifunctional and involved in virus replication, virion disassembly and virus cell-to-cell movement. Kwon and Kim (2006) proved by using SELEX (systemic evolution of ligands by exponential enrichment) that the GAAA sequences in the terminal loop and 5′SL1 are essential for the synthesis of plus-strand RNA.
Triple Gene Block (TGB) is a collective term used for the ORF 1, 2 and 3. The region of triple gene block is conserved in the viruses belong to genera *Potexvirus, Pecluvirus, Carlavirus, Allexivirus, Hordeavirus, Pomovirus* and *Foveavirus*. The mechanisms of cell-to-cell movement are same for these viruses (Verchot, 2005). TGB encodes three movement proteins TGBp1, TGBp2 and TGBp3 which provide separate activities for the virus transport and replication. TGBp1 is a multifunctional protein which in connection with CP forms a complex with the viral RNA which traffics to the plasmodesmata. Cellular proteins interact with the viral ribonucleoprotein (vRNA) complex at the opening of plasmodesmata which initiates the expansion of the pore to allow trafficking between cells (Verchot, 2005; Lucas, 2006). The evidence also suggested that TGP1 also interacts with RDR6, a factor in production of short interfering RNA (siRNA) and meristem exclusion of viruses (Schwach *et al*., 2005; Xie and Gua, 2006).

The other two proteins TGBp2 and TGBp3 are ER-binding proteins. Sequence analysis of TGBp1 showed that it has two transmembrane domains while a single, N-terminal transmembrane domain is present in TGBp2. Mutations analyses also showed that the disruption of membrane association of these proteins suppress viral movement, indicating that association of ER is very important (Krishnamurthy *et al*., 2003; Mitra *et al*., 2003). It was reported in early infection of *Potato mop top virus* (PMTV) that TGBp2 and TGBp3 proteins were associated with motile granules, which moved towards plasmodesmata (Haupt *et al*., 2005). Similar granules have also been observed in the confocal microscopy images. It is considered that these granular bodies which are described in the PMTV model may be the infectious agents carrying towards the plasmodesmata (Haupt *et al*., 2005; Ju *et al*., 2005).

The final ORF encode viral capsid protein (CP), which is essential for genome encapsidation and viral cell-to-cell movement. The expression of CP subunits of *papaya mosaic virus* (PapMV) and PVX CP in *Escherichia coli* form virus like empty particles and discs that have been proved instrumental for studying the requirements for particle assembly (Tremblay *et al*., 2006). The accumulation of sufficient amount of discs and viral RNA in the cells initiates
the virion assembly. Particle packaging occurs when these discs bind RNA and then assemble along the central RNA into full length virion (Tremblay et al., 2006). Earlier studies of PVX, CVX and FMV demonstrated that the CP of potexvirus resides in the plasmodesmata and enhances infection (Rouleau et al., 1995; Oparka et al., 1999). However, in later experiment it was shown that TGBp1 can initiate the plasmodesmata gating, suggesting that CP and TGBp1 proteins work jointly and promote plasmodesmal gating and virus cell-to-cell transport (Lough et al., 2000). CP of PVX is also considered as the elicitor for Rx-mediated resistance in potato. Bendahmane et al., (1999, 2000) demonstrated that Rx is a protein that normally causes extreme resistance in inoculated leaves, but the expression of CP of PVX causes hypersensitive resistance (HR).

1.5 Potato Virus Y (PVY)

1.5.1 Host, Symptoms and Distribution

Potato Virus Y (PVY) causes significant yield loss in variety of crops of solanaceous family including potato (Solanum tuberosum L.), tobacco (Nicotiana tabacum L.), tomato (Lycopersicon esculentum) and pepper (Capsicum spp. L.), wherever they are cultivated (De Bokx and Huttinga 1981). The yearly transfer is mainly via potato tubers. Different strains show different symptoms. Primary infection with PVYO and PVYC strain group isolates induces necrosis, mottling or yellowing, necrotic leaf spots or rings, leaf drop and premature death of stems. Sometimes necrosis in leaf, dwarfing and crinkling symptoms causes in potato by secondary infection of PVYO and PVYC (Hooker 1981). PVYN produces milder form of leaf mottling. Plants infected with PVYO, PVYC and PVYN produces the tubers with no symptoms. However with PVYNTN produces tubers with irregular brownish colored rings on skin, which forming necrotic arc in the flesh and cracking the skin at the surface.

Isolates of PVY are placed in different strain groups. This division is based on the mosaic symptoms (PVYO, PVYC and PVYZ) or necrotic symptoms (PVYN) induced in tobacco and potato (Jones 1990, Valkonen et al., 1996). PVYN has a subgroup of isolates, designated as PVYNTN that includes those isolates causing necrotic ringspot in the tubers (Beczner et al.,
1984). PVY\(^O\) (ordinary type) occurs in most potato growing countries worldwide, PVY\(^C\) in Eastern Australia, South America, New Zealand, Europe, South Africa and North America, and PVY\(^N\) in North America, New Zealand, Europe, South America and Africa. PVY\(^{NTN}\) is a new type of strain reported in Europe, New Zealand, Middle East, North America and Japan.

### 1.5.2 Virus structure and composition

PVY is the type member of the genus *potyvirus*. The genus *potyvirus*, a member of *potyviridae* family is the largest and most economically important genus of the viruses (Shukla *et al.*, 1994). The members belonging to the potyvirus genus are rod-shape flexuous filaments 680-900 nm long and 11-13 nm wide. The virion contains a monopartite, positive sense single-stranded RNA about 9.7 kb in size (Shukla *et al.*, 1994). A typical potyvirus genome is started with short non-translated regions (NTR), usually less than 200 bp long. A genome-linked viral protein, VPg, is covalently bound at the 5’ terminus (Murphy *et al.*, 1991) and a poly (A) tail is located at the 3’ end (Hari *et al.*, 1979). The ORF encodes large poly-protein which is subsequently processed by viral proteinases into 10 functional proteins (Adams *et al.*, 2005). These protein are (5’ to 3’) P1, HC-Pro (helper component proteinase), P3, 6K1, CI (cylindrical inclusion protein), 6K2, VPg (viral protein genome linked), NLa (nuclear inclusion protein a), Nlb (nuclear inclusion protein b) and CP (coat protein).

![Genome Map and Protein Produced from Single-Stranded RNA of Potato virus Y](image)

**Figure:** 1.2. Genome map and protein produced from single-stranded RNA of *Potato virus Y*.

### 1.5.3 Properties and functions of gene product of *potyvirus*

The genome of *potyvirus* encodes the poly-protein which comprises of the following gene product. The first N-terminal protein P1 is a proteinase (similar to chymotrypsin-like serine
proteinase) responsible for its cleavage at its C-terminus. This region is extremely variable in size (26-60 kDa) and sequences among potyviruses (Adams et al., 2005). In TEV, it acts as an adjunct factor for genome amplification and binds non-specifically to RNA suggested that P1 may be involved in viral movement (Brantley and Hunt, 1993).

HC-Pro is multifunctional helper component-proteinase (about 50 kDa) that cleaves itself from the poly-protein and is involved in aphid transmission, cell-to-cell movement of virus within the host plant and suppression of host RNA silencing mechanism. Virus transmissibility was tested with four aphid species and was showed that the food canal of aphid species differs in their ability to interact with the HC-pro which affects the ability of aphids to hold the virus in their mouth parts (Wang et al., 1998). The C-terminal region of HC-Pro is generally assumed to be important for cell-to-cell movement. A deletion of 87 AA partially or totally prohibited cell-to-cell movement of heterogeneously expressed protein in microinjection studies (Rojas et al., 1997). Kassachau et al. (1997) showed in the mutational analysis that the central region of the TEV HC-Pro (AA 100-300) is assumed to be important in genome amplification, synergism and systemic movement within host plants. HC-Pro of potyvirus has also been shown to be highly effective suppressor of RNA silencing in transient silencing-suppression assays (Hamilton et al., 2002; Silhavy and Burgyan, 2004) and in transgenic plants (Llave et al., 2000). The expression of P1-HC-Pro protein in Arabidopsis thaliana reduces the accumulation of miRNA in transgenic plants (Mallory et al., 2001). Similar results have also been obtained when the PVY derived P1-HC-Pro was transgenically expressed, causes significant reduction in the accumulation of 21 nt small interfering RNA (siRNA), but has less effect on 24 nt siRNA accumulation (Dunoyer et al., 2004).

P3 is a third gene product in the ORF and has a size of about 40 kDa. The role of P3 protein has been reported in pathogenicity by interaction with some other viral proteins. It was shown by Suehiro et al (2000) that the C-terminal region of the P3 in the Turnip mosaic virus (TuMV) genome carries a pathogenicity determinant, which is necessary for the infection of a particular host. Similarly, the role of P3-6K1 complex in the C-terminal region of Plum pox virus (PPV) has been determined as a pathogenicity determinant (Saenz et al., 2000). 6K1 is a
small protein located next to P3. However, in potyvirus such as TEV the cleavage between 6K1 and P3 has not been observed. Immunological data showed that P3+6K1 carry a determinant for the pathogenicity as both P3 and P3+6K1 product were present in infected cells (Schaad et al., 1997).

Cylindrical inclusion protein (CI) is the next protein in the ORF. It is the largest potyvirus gene product (around 70 kDa) and a major component of the replication complex. CI is an RNA helicase and contains seven conserved sequence motifs typical of the RNA helicase of the superfamily SF2 (Fernandez et al., 1997). Carrington et al. (1998) showed in a mutational experiment that CI protein facilitate potyvirus cell-to-cell movement by interaction directly with plasmodesmata and capsid protein-containing ribonucleoprotein complexes. In addition, the data also suggests that the function of cell-to-cell movement is genetically dissociable from its RNA replication functions of CI protein. 6K2 is a second small peptide present next to cylindrical inclusion protein. 6K2 is involved in the replication of virus genome because it anchors the replication apparatus to the endoplasmic reticulum (Schaad et al., 1997).

The N-terminal part of the NIa consists of genome linked viral protein (VPg) which has a size of about 49 kDa. This is the only viral protein, apart from the CP, found covalently attached to the 5’ end of the genomic RNA via a tyrosine residue (Murphy et al., 1991). The VPg is involved in the replication of virus and it was already shown by the mutation analysis of Tobacco vein mottling virus (TVMV). The mutant virus of the tyrosine residue, that links the VPg to the viral genome, did not accumulate to the detectable level (Murphy et al., 1996). It was observed that VPg also interacts with the plant translational initiation factors, like elf4E (Wittmann et al., 1997; Leonard et al., 2004). Recently the role of VPg in long distance movement has also been identified (Kang et al., 2005).

The first component of nuclear inclusion protein is the nuclear inclusion protein a (NIa). Although NIa is structurally related to serine proteases, it has a cysteine at the active site. Furthermore, the protease domain of NIa matches P1 and HC in having nonspecific RNA-binding activity (Adams et al., 2005a). NIb is the second component of nuclear inclusions
located next to the NIa. It is the RNA-dependent RNA polymerase (RdRp) having a size of 58 kDa. The polymerase activity of NIb was determined in Tobacco vein mottling virus (TVMV) in which NIb was able to utilize full-length TVMV RNA as a template for RNA synthesis. Further the mutation of the highly conserved GDD motif significantly reduced the polymerase activity of the TVMV NIb (Hong and Humt, 1996).

The last protein in the ORF is the coat protein (CP) having the size about 30-36 kDa. Many roles have been assigned to this protein like, making the virus particle, protect the genomic RNA and play role in movement. CP is a well characterized potyvirus protein which is divided into N-terminal, C-terminal and core domains. The N-terminal region is found to be important for efficient transmission of aphids (Lopez-Moya et al., 1998). Mutation analysis showed that N-and C-terminal regions of the CP of TEV were essential for the systemic movement, while the core region was necessary for the cell-to-cell movement (Dolja et al., 1995). The CP and HC-Pro are the two movement proteins that have ability to increase the size exclusion limit (SEL) of plasmodesmata and therefore, help in cell-to-cell movement of virus (Rojas et al., 1997). One of the major roles of the CP is particle assembly. Recent studies showed that both the C-terminal and N-terminal are dispensable for particle assembly (Anindya and Savithri, 2003; Kang et al., 2006).

1.6 Potato Leaf Roll Virus (PLRV)

1.6.1 Host, symptoms, distribution
PLRV is also economically important plant virus because it causes significant losses in major crops. PLRV belongs to family Luteoviridae, which is one of the most economically significant plant virus families because it contains barley yellow dwarf virus (BYDV), cereal yellow dwarf virus (CYDV), potato leaf roll virus (PLRV) and beet western yellow virus (BWYV) which cause significant losses in major crops (Mayo and Arcy, 1999). PLRV has comparatively narrow host range. Potato is it’s only known natural hosts. The majorities of experimentally infected hosts are in the family Solanaceae, and include Atropa belladonna, Capsicum annum, Datura stramonium, Lycopersicum esculentum and Nicotiana spp.
Susceptible plants in other families include *Amaranthus caudatus*, *Celosia argentea* and *Gomphrena globosa* (Dragoljub et al., 1999). Plants emerging from infected tubers show symptoms first on the lower leaves and the symptoms gradually progress upward. The resulting symptoms include, rolling of the upper leaves at the top of the plants that assume a brittle, leathery texture, marginal yellowing and thickness of apical leaves (Khalid et al., 2000).

PLRV has worldwide distribution and probably occurs wherever potato is grown. Incidence of PLRV, however, remains low when seed stocks are frequently replaced and systemic insecticides are used. PVX and PVY are transmitted easily to a narrow range of hosts by sap and by several species of aphids in a non persistent manner; whereas aphids transmit PLRV in a persistent manner of which *Myzus persicae* is the most important and efficient vector. The virus replicates in the tissues, specifically in the phloem of the infected plants (Radcliff et al., 1993; Ragsdale et al., 1994).

### 1.6.2 Virus structure and composition

PLRV belongs to the genus *Luteovirus*, family *Luteoviridae*. PLRV particles are spherical, containing a positive sense genomic RNA (5880-5990 bp). Particles are icosahedral, with a diameter of 24-30 nm. Virions contain 30% RNA and 70% protein (Miller, 1999). PLRV is transmitted by aphid vectors that introduce particles into the vascular tissue of plants where PLRV multiplies and remains largely restricted within phloem tissue (Barker, 1987; Heuvel et al., 1995; Barker et al., 2001). The nucleotide sequence of the genome of PLRV consists of six open reading frames (ORF). These open reading frames (ORFs) are ORF0, ORF1 (PRO putative protease VP genome linked protein), ORF2 (POL RNA dependent RNA polymerase), ORF3, CP (coat protein), ORF4 MP (movement protein) and ORF5 AT (aphid transmission) (Miller, 1999).
6.0 kb

**Figure: 1.3. Genome map of Potato leaf roll virus (PLRV).**

- **ORF 0:** 28 kDa protein of unknown function
- **ORF 1/2:** 70/69 kDa Replicase Protein
- **ORF3:** 23 kDa Coat Protein
- **ORF4:** 17 kDa Movement Protein
- **ORF5:** 56 kDa Aphid Transmission

### 1.6.3 Properties and functions of gene product of *Luteovirus*

The potato leaf roll virus encodes six open reading frames (ORFs) in which ORF 0 encodes a 28 kDa protein. The translation product p28 of this ORF has been suggested to play a role in host recognition (Wilk *et al.*, 1997). The ORF 1 and 2 encode the replicase protein necessary for RNA replication. The ORF 2 does not encode a separate gene product and is only expressed as a C-terminal fusion with ORF1. The RNA dependent RNA polymerase (RdRp) component of the replicase is encoded by ORF 2 (Mohan *et al.*, 1995). ORF 3 encodes a major Coat Protein have subsequently been reported to be located near the centre of the genome (Reutenauer *et al.*, 1993). The 17 kDa protein sequence of ORF 4 are completely laid within the coat-protein ORF but in different frame. It has been proposed that this protein also encodes for the genome-linked viral protein (VPg), based on its size similarity with the VPg isolated from BYDV (Murphy *et al.*, 1989). The product of ORF 4 is required for systemic infection and cell-to-cell movement (Chay *et al.*, 1996). The ORF 5 produces the largest 56 kDa protein. This protein is required for aphid transmission. The N-terminal portion of ORF 5 is necessary and sufficient for aphid transmission (Delmer *et al.*, 1997).

As the viruses are intracellular parasites so the chemical treatment cannot be applied against them. Consequently, viral problems must be solved by using virus-resistant cultivars, virus-free planting materials and appropriate crop rotation in the field. Virus-resistant cultivars can be developed by using traditional way of breeding or through modern biotechnology by
developing transgenic plants. For developing virus resistant cultivars, it is important to understand the molecular biology of the virus and its interaction with the particular host plant.

### 1.7 Viral infection cycle

The genome of plant viruses may consist of DNA (single or double-stranded) or RNA (positive-, negative-, or double-stranded). The large group of plant viruses has a positive-strand RNA genome that is encapsidated into viral particles by the viral coat protein (Hull, 2002). Viruses are obligate parasites they are unable to replicate on their own. Instead they must enter a host cell and utilize the host machinery for nucleic acid and protein synthesis to be able to replicate in the host cell. The host machinery must be compatible to invading virus in order to cause successful infection.

Plant viruses first lands on plant surface through aphids or by mechanical means and enter into plant cells through wounds made mechanically or by aphids. By entering into cell, virus starts to disassemble and releasing its RNA to the cytoplasm. Subsequently, the viral RNA is translated on ribosomes, resulting in viral proteins required for viral replication. First a negative strand is synthesized using the positive strand with the help of virus-encoded replicase (RNA-dependent RNA polymerase [RdRp]). The negative strand then serves as a template for the generation of positive stranded RNA molecule (Hull, 2002).

After entry to the cell, viruses must move intracellularly from site of replication to the plasmodesmata (PD). Macromolecules use the cytoskeletal elements such as microtubules and microfilaments with the association of endoplasmic reticulum (ER) (Langford, 1995; St Jonston, 1995). It was suggested that to reach to the PD the plant viruses use the normal intracellular system of the host (Krishnamurthy et al., 2002). The opening of the PD has a diameter of about 2.5 nm, limiting the transfer of macromolecules between cells. PD can limit small molecules and metabolites with a molecular mass of up to 1 kilodalton (kDa) (Lucas et al., 1993). The plasmodesmatal size exclusion limit can be increased to allow intercellular movement of endogenous protein or invading viruses (Lucas, 1995). Many types of viral MP have been identified, which were found to be promoting cell-to-cell spread of virus through
PD (Carrington et al., 1996; Lee et al., 2003). In some viruses like TMV, single MP is able to modify the PD and facilitate transport of itself, while in other viruses like PVX and PMTV, more than one MP protein facilitate the cell-to-cell movement. These viruses contain a set of MP genes called TGB, which encodes three proteins that are involved in virus cell-to-cell transport through PD (Petty & Jackson, 1990; Beck et al., 1991; Herzog et al., 1998, Krishnamurthy et al., 2002). Viruses move from cell-to-cell through PD and have to passes through several types of cells i.e. epidermal (EP), mesophyll (MS), bundle sheath (BS), phloem parenchyma (PP) and companion cells (CC) until they are loaded into the sieve element (SE) (Lucas & Lee, 2004). SE can transport the virus over long distances to other parts of plant where the viruses must exit the SE and re-establish replication and cell to cell movement in tissues distant from initial infection site (Cronin et al., 1995).
**Figure: 1.4. Virus infected field of potato crop.** (A) Mix virus infected field of potato showing widespread disease. The whole field shows devastation and thus crop loss. (B, C) Symptoms of PVY vary greatly in severity and type, depending on the sensitivity of the potato cultivar and the virulence of the virus strain: (B) veinal necrosis, (C) Rugose leaf symptoms (uneven leaf surface). (D) Mild leaf mottle symptoms of PVX. (E) Rolling upward of the top leaves, typical symptoms of Potato Leaf Roll Virus. (F) Green peach aphid (Myzus persicae), main aphid vector of potato viruses, especially of Potato leaf roll virus.

### 1.8 Viral disease resistance

Viruses cause devastating plant diseases of many crops around the world. Viral diseases are a limiting factor in agricultural production and have plagued world economic resources for centuries. Viral diseases are specifically problematic for tropical and subtropical regions. The losses due to viral diseases amount in billions of dollars annually. (Bawden, 1995; Pappu et al., 1994). Reducing such losses has long been a high priority objective for agriculture. Plant viruses seem nearly impossible to control, instead, practical attempts are made to keep them in check and manage their existence within a crop. Numerous traditional practices to minimize virus infection and spread including production of virus free propagating material, controlling of insect vectors with chemical or biological agents, cultural practices including sanitation, crop rotation, breeding resistant varieties and using integrated disease management (combining two or more of the above strategies) have been proven of less utility in a highly demanding scenario of combating viral diseases. The situation has urged the need to search for more efficient, durable and cost effective control methods. One of the most desirable approaches is to develop host-plant resistance to viral infection. Development in plant tissue culture, plant transformation and regeneration and improvement in techniques to isolate and manipulate viral genes have led to the exploitation of the concept of “Cross-Protection”: turning the virus onto itself and controlling it with its own gene. Approaches used for virus resistance including expression of viral coat protein, untranslatable sense or anti-sense RNA, satellite RNA, virus specific “neutralizing” antibody gene, viral replicase, protease or movement proteins and defective interfering RNA have been successful for developing resistance against many viruses (reviewed by (Lin et al., 2007)).
1.8.1 Natural Resistance

Plants are routinely challenged by different pathogens like viruses, bacteria, fungi and insects. In response, plants have evolved active and passive defence mechanism when recognized these invading pathogens. The most common mechanism associated with the active defence is hyper sensitive response (HR). During this process the surrounding cells of the primary infection site of the virus die due to a rapidly induced programmed cell death (Goldbach et al., 2003). The passive defence mechanism is based on the presence of existing barriers like the stiff cell wall.

Plant breeders have identified several natural resistance genes, and numerous varieties resistant to one or more viruses have been obtained by conventional breeding. The predicted products encoded by these resistance genes show a high degree of similarity and can be grouped into five classes based on the presence of conserved structural components (Bent, 1996). However, breeding programs often encounter difficulties to transfer resistance characters from one plant species or variety to another without loosing important traits of the recipient cultivars. Most natural resistance genes identified until now have not been localized precisely, which makes their cloning difficult. Considerable progress was made towards the isolation of various genes using restriction fragment length polymorphism (RFLP) mapping approaches (Ohmori et al., 1995; Simcox et al., 1995). The first natural resistance gene, the TMV resistance gene N, was isolated and cloned (Whitham et al., 1996). Specifically, N interacts with the helicase domain (p50) of the viral replicase protein (Ueda et al., 2006). This interaction has also been envisaged to play a role in the oligomerization of N prior to resistance activation (Mestre and Baulcombe, 2006). To date, many pathogen resistance (R) genes characterized from plants, mostly conferring resistance for fungal, bacterial and viral pathogens (Hammond-Kosack and Parker, 2003). Some well known signal molecules like salicylic acid, jasmonic acid, MAP kinases and ethylene amplified the local defence response throughout the plant (Zhang and Klessig, 2001, Glazebrook, 2001). In many plants salicylic acid (SA) is an essential signaling compound for activation of systemic and local defence against pathogens (Zhang et al., 1999). Diseases caused by plant viruses are difficult to manage and their control mainly involves the use of insecticide to kill insect vectors, the use
of virus free propagating materials, and the selection of plants with appropriate resistance genes. Virus free stocks are obtained by virus elimination through heat therapy and/or meristem tissue culture, but this approach is ineffective for viral diseases transmitted by vectors. While insecticides can control vectors, often the virus has already been transmitted to the plant before the insect vector is killed. The use of resistant cultivars has been the most effective means of control, however, plant virus resistance genes are frequently unavailable and their introgression into some crops is not straightforward (Simcox et al., 1995). RNA silencing known as Post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) is a natural defence system evolved by plants against transposons, viruses and viroids. This plant defense mechanism can be efficiently triggered by double-stranded transgene RNA which specifically targets cognate viral RNA providing a high level of virus resistance.

1.8.2 Cross Protection

The phenomenon of cross protection has been known for a long time and in theory it provides an efficient means of controlling virus diseases. McKinney (1929) was the first to show that plants already infected with a virus are normally protected against infection by a related strain of the virus. Thus, plants infected with a mild strain could be protected against infection by severe isolates or strains of that virus. One assumption of cross-protection was that there were limited virus-specific multiplication sites in the cell. If one virus occupied all these sites, an incoming related strain would not be able to multiply (Bawden & Kassanis, 1945). Another proposed mechanism was that the first strain used all essential metabolites required by the second strain to multiply (Matthew, 1991). Recently it is known that cross-protection is based on RNA silencing (Ratcliff et al., 1999). In a series of cross-protection experiments, it was shown that PVX carrying the reporter gene β-glucuronidase (GUS) plus part of the green fluorescent protein (GFP, PVX-GUS.GF) were not able to infect TMV-GFP infected plants.

However, cross protection referred here as classical protection and has been used to control virus diseases of a few crops (Fulton, 1986). There are a number of reasons for the limited use and application of classical protection: First, the fact that a "live" virus is used to deliberately infect plants makes researchers and growers reluctant to use this approach on a practical scale.
Second, it is generally difficult to obtain mild strains that are of practical value. Third, there is some possibility that the mild strain might mutate and cause even greater problems than the control strain itself. In some cases the two viruses act synergistically to produce more severe infection than either virus would cause on its own. Furthermore, the possibility of infection of neighboring field with the protecting strain of virus must be considered. Because of this farmers are often reluctant to use classical cross protection to control virus disease.

1.8.3 Engineered Resistance

Engineered resistance approaches to plant virus have widely explored since the earliest experiments whereby transgenic tobacco plants expressing TMV coat protein were challenged with TMV and shown to be resistant (Goldbach et al., 2003). It is now possible to engineer resistance and tolerance to plant viruses using transgenes derived from a wide range of organisms including plant-derived natural R genes, pathogen-derived transgenes, and even nonplant and nonpathogen-derived transgenes. As compared to conventional breeding for virus resistance, genetic engineering provides a quicker and more precise technology to obtain plants that are resistant to viruses. The concept of pathogen-derived resistance (PDR) has stimulated research on obtaining virus resistance through genetic engineering (Sanford and Johnston, 1985).

Plant viruses have small genomes containing limited number of genes. Nowadays the functions of all these genes during the viral replication cycle have been well elucidated, so these pathogens are suitable targets for engineered resistance concepts based on the principle of PDR. The mechanism of PDR is considered more efficient against viruses of all the engineered resistance mechanisms (Goldbach et al., 2003). PDR is a phenomenon whereby transgenic plants containing genes or part of gene sequences of a pathogen are protected against harmful effects of the related pathogens (Sanford and Johnston, 1985). The successful development of virus resistance plants through PDR was first achieved by Beachy’s group in 1986. They showed that tobacco and tomato plants expressing the coat protein gene of TMV exhibited resistance or delayed infection when challenged inoculated with TMV (Powell-Abel et al., 1986; Nelson et al., 1988).
Initially, it was thought that pathogen-derived resistance was operating through the expressed viral protein (Powell-Abel et al., 1986). It was proposed that the presence of transgene-derived CP blocked disassembly of the virus particle and thereby prevented release and translation of the viral genome. However, it was later shown that transgenic plants expressing an untranslatable form of Tobacco etch virus (TEV, genus Potyvirus, family Potyviridae) CP were able to confer resistance to TEV (Lindbo & Dougherty, 1992a). This type of resistance was based on RNA and became known as RNA-mediated resistance. Consequently, two mechanisms work in pathogen-derived transgenic plants: 1) Protein-mediated resistance 2) RNA-mediated resistance.

1.8.2.1 Protein-mediated resistance

There are some examples which explain that accumulation of Coat Protein rather than the CP mRNA is involved in the resistance. Several theories have been suggested to explain the mechanism of Coat Protein-mediated resistance (CP-MR). The model which is the most accepted suggest that the transgenic CP is responsible to prevent TMV virions from disassembly, which is an important event of infection (Wu et al., 1990). Another model explains that the presence of transgene-derived CP in the cell may favor assembly instead of disassembly and thereby suppress uncoating of the invading virus particle (Register et al., 1989). The disassembly of TMV depends on repulsive interactions between carboxyl-carboxylate groups of amino acids residues present on the interface of CP subunits (Bancroft, 1970). The interaction of these negatively charged groups can be facilitated by changes in pH and Ca$^{2+}$ ion concentration and thus destabilized the virions and initiating virus disassembly (Culver, 2002). Therefore it was suggested that the dominating cell environment would prefer virion assembly instead of disassembly, in TMV CP transgenic plants (Schuster et al., 1980).

1.8.2.2 RNA-mediated resistance

It was initially thought that for virus resistance the expression of transgenic protein is required. However, the constructs containing untranslatable transgene that were actively transcribed in the nucleus could provide a high level of resistance or immunity to the plant (Lindbo and Dougherty, 2005). RNA-mediated resistance operates through diverse pathway
and does not require the translation of the transgene. The virus may have initially infected the transformed plants but 3-5 weeks after inoculation, the plants recovered and symptomless leaves develop from the infected plants (Swaney et al., 1995; Guo and Garcia, 1997). Theses asymptomatic leaves are resistance to new infection of the same virus; this phenomenon is known as “recovery”. The phenomenon of recovery has been analyzed by Lindbo and Dougherty, (1992) in detail by transforming the tobacco with the CP-encoding region of tobacco etch virus (TEV). No viral protein was detected in the recovered tissues and a drastic decrease (12-22 folds) of transgene-derived mRNA was observed (Lindbo and Dougherty, 1992). However, run-off assay of transgenic nuclei reveals that there is no change in the transcription rate (Lindbo et al., 1993). These studies indicated that the decrease in the mRNA expression level must be due to the mechanism of RNA degradation prevails in the cytoplasm. Thus, it was assumed that the presence of high level transgene RNA transcript in the cytoplasm would initiate a sequence-specific post transcriptional RNA degradation mechanism leading to the silencing of gene.

1.9 Strategies used for transgenic resistance

The T-DNA insertion vectors, modified from the naturally occurring tumor inducing plasmid of Agrobacterium tumefaciens, have been extensively used in the study of gene expression. The genetic transformation of number of crop plants through this natural method has been increasing day by day. These, along with the tremendous advances in our understanding of plant-virus interaction in the process of transmission, infection and resistance, have unfolded the hypothesis of genetic engineering approaches for the development of virus resistance in plants (Dasgupta et al., 2003).

Based on source of gene used, two main approaches were used for the development of genetically-engineered virus resistance plants. The genes may be from the pathogenic virus itself or from the source other than virus. Based on pathogen-derived resistance (PDR) concepts first proposed by Sanford and Johnston (1985), numbers of transgenic approaches
based on viral gene and sequences were applied to several plant species. In PDR the gene or part of gene transformed into the plant subsequently interfere with the essential steps in the life cycle of infecting virus. This mechanism was first demonstrated by the group of Roger Beachy (Beachy et al., 1990) who introduced the CP gene of TMV into tobacco and noted TMV resistance in transgenic tobacco. The type of resistance obtained by introducing CP gene is conventionally called coat protein-mediated resistance (CPMR). In variety of crops effective transgenic resistance has been developed by introducing viral genes, or part of gene, in the target crop by genetic transformation. The genes which are usually used in pathogen-derived approaches for generating resistance against viruses are; Coat protein (CP), Replicase protein (RP) and Movement protein (MP). Other PDR approaches reported in the literature include the expression of untranslatable sense or anti-sense, satellite RNA and defective interfering RNA or DNA.

1.9.1 Coat protein mediated virus resistance

The Coat Protein mediated virus resistance was first published by Powell-Abel et al. (1986), describing tobacco plants transformed with the coat protein (CP) gene of *Tobacco mosaic virus* (TMV), showing improved resistance against TMV. The CP expressing seedlings of transgenic plants confirmed significant delay in symptom development when inoculated with TMV. The process of symptom development was prolonged and was correlated with the expression level of the CP.

Since this first demonstration that transforming a plant with the coat protein gene of a virus can confer resistance to the corresponding virus, this approach has been widely used for resistance against numerous viruses belonging to different groups in many crop plants as well as ornamentals and fruits plants (e.g. Pang et al., 2000; Lehman et al., 2003; Liao et al., 2004; Yang et al., 2004; Pongrit et al., 2007; Bazzini et al., 2006; Voloudakis et al., 2005; Srivastava and Raj, 2008; Zanek et al., 2008). CP-mediated resistance (CPMR) approach can also be applicable commercially. One of the examples is *Papaya ring spot virus* (PRSv), discovered in Puna, Hawaii, which soon damaged severely the Hawaiian papaya industry. The
experimental work of CPMR against this virus was initiated soon, which was successful. In 1998 two varieties were developed and available for commercial use and the papaya industry started to recover (Gonsalves, 2002). The CPMR mechanism seems to act differently in different viruses. For some viruses the resistance is protein-mediated, requiring the expression of the coat protein (Bendahmane et al., 1997; Lehman et al., 2003), and for other viruses resistance is mediated also by untranslatable or truncated forms of the CP-gene implicating an RNA-based mechanism (Masmoudi et al., 2002; Liao et al., 2004). The mechanism of CPMR was also proved to be very effective in potato. The CP gene of a necrotic strain of *Potato virus Y* (PVY) has been engineered in potato cultivars and tested under field conditions, out of which three lines proved to be highly resistant against two different strains of PVY in provocative experiment (Józsa et al., 2002). Introduction of a gene expression cassette with two copies of a PLRV CP gene, in which the nucleotide sequence was modified to improve expression of the gene, has been shown to be effective for conferring resistance to PLRV infection in potato (Thomas et al., 1997). RNA silencing using CP genes was successfully applied to engineer potato plants which are resistant to potato virus Y (PVY). In an experiment, double stranded (ds) RNA derived from the 3’ terminal part of the coat protein gene of PVY, which is highly conserved in sequence amongst different PVY isolates, expressed in transgenic potato. Twelve out of fifteen transgenic lines produced siRNAs and were highly resistant to three strains (PVY$^N$, PVY$^O$, and PVY$^{NTN}$) of PVY (Missiou et al., 2004).

### 1.9.2 Replicase or polymerase protein-mediated resistance

A replicase protein is another type of genes used for conferring Pathogen Derived Resistance mechanism. Different experiments were performed by introducing either full-length, truncated, mutated versions or read-through portions of replicase genes and resistance has been accomplish against at least 14 different viruses representing 10 different taxonomic groups (Palukaitis & Zaitlin, 1997). The selection of appropriate sequence for replicase-mediated PDR has greater influence on resistance level, because the replicase genes are structured differently in different virus genera. Introduction of full-length replicase genes has been shown to be effective for conferring resistance to Potex-, Poty-, Luteo- Sobemo- and Poleroviruses (Braun & Hemenway, 1992; Audy et al., 1994; Koev et al., 1998; Pinto et al., 2004).
1999; Thomas et al., 2000). This strategy has also been proved effective in field trials and a commercial product has been developed in which the resistance to the Potato leaf roll virus (PLRV) was combined with resistance to Colorado potato beetle in transgenic Russet Burbank potatoes (Lawson et al., 2001).

The 57-kDa read-through domain of the replicase gene of TRV has been cloned in tobacco. From the total of six lines containing the viral transgene, four displayed various levels of resistance against rub-inoculated and nematode transmitted virus (Vassilakos et al., 2008). The read-through portion of replicase gene of other Tobamovirus and Tobraviruses were also used for resistance against viruses (Golemboski et al., 1990; MacFarlane & Davies 1992; Tenllado et al., 1995). Other mutated or truncated forms of replicase genes have also been shown to be able to confer virus resistance (Anderson et al., 1992; Brederode et al., 1995; Longstaff et al., 1993; Tsukasa et al., 2002). PLRV resistance potato lines have been developed by transforming the potato with the replicase gene of PLRV (Rovere et al., 2000). Three version of ORF2b (non-translatable sense, translatable sense with an engineered ATG and anti-sense) were constructed. The resistant lines obtained after infection experiment has shown resistance in transgenic plants which could be obtained with any one of the construct suggesting that the resistance mechanism is independent of protein expression and is RNA mediated. Also for DNA plant geminiviruses, PDR by transformation of plants with replication-related genes has been obtained (Hong & Stanley, 1996; Noris et al., 1996). Replicase mediated resistance often confers a high level of protection but it is very specific and generally is effective against the viruses only, which are closely related to the source of the transgene. This observation combined with other observations such as association between high levels of resistance with low levels of transgene mRNA indicated that this kind of resistance is mediated by RNA rather than protein. Some studies have also shown that the post-transcriptional gene silencing or RNA silencing is involved in the resistance mechanism (Marano & Baulcombe 1998; van den Boogaart et al., 2001). However, in many other studies e.g. the use of modified replicase genes, protein-mediated mechanisms is also involved, possibly combined with RNA-mediated mechanisms (Wintermantel & Zaitlin, 2000; Goregaoker et al., 2000).
1.9.3 Movement protein-mediated resistance

After getting entry to plants, the viruses have to move from cell-to-cell in order to spread infection. For this purpose, the plant viruses encode movement proteins (MPs) which are the agent for viral cell-to-cell movement. The most common mechanism by which plant viruses move between plant cells via plasmodesmata. The MPs contain nucleic acid binding domains and localize to plasmodesmata where they modify the function of the plasmodesmata which facilitate the viral transfer to adjacent cells. The resistance to plant viruses can be achieved by expressing the dysfunctional MPs. This mechanism has been shown experimentally by expression of mutated versions of MP genes from the *Luteovirus* PLRV and the *Tobamovirus* TMV, while no effect was seen when the wild type MPs were expressed. The type of resistance shown by mutated gene of MP was effective not only to homologous virus and related viruses but also to viruses belonging to other virus groups (Lapidot et al., 1993; Cooper et al., 1995; Tacke et al., 1996). In some viruses like the genre Potex-, Carla-, Pomo-, and *Pecluvirus* the MP is expressed by a set of three genes block called triple-gene-block (TGB). When this TGB was mutated and expressed, it shows a broad spectrum resistance as has been shown for white clover mosaic virus (WClMV) (Beck et al., 1994) and *Potato virus X* (PVX) (Seppänen et al., 1997). These two viruses belong to the genus *Potexvirus*. In both cases the introduced resistance was very broad, affecting other *Potexviruses* as well as other TGB-containing viruses such as Potato virus S and M that belong to the genus *Carlavirus*.

Expression of modified viral protein-mediated silencing involving MP, results in a relatively broad resistance. This kind of resistance is generally protein-mediated requiring the expression of the dysfunctional protein. It was thought to be the result of preformed, dysfunctional transgene of MPs or TGB encoded proteins that disrupt the viral transfer system through plasmodesmata (Lapidot et al., 1993; Tacke et al., 1996; Seppänen et al., 1997). Transgenic tobacco plants expressing the Potato leaf roll virus movement protein gene gave the evidence for expression level dependent modulation of carbohydrate status and virus resistance (Hofius et al., 2001). Soluble sugars and starch contents in leaves decreased when the expression of MP17 and MP17: GFP was low, while increased MP17 protein levels led to carbohydrate accumulation and stunted growth. It was suggest that decreased level of sugar
and starch contents was possibly due to changes in plasmodesmata permeability. The expression level of MP17 also affects the resistance against unrelated virus species like *Potato virus Y* (PVY) N strain. The wild type and mutated BV1 or BC1 movement proteins gene of *Bean dwarf mosaic virus* (BDMV) was transformed in tomato plants (Hou et al., 2000). The transgenic R₀ plants, expressing either wild type or mutated BV1 or BC1 proteins, presented a significant delay in TMV infection, compared with control plants. R₁ progeny also showed a delay in infection but this delay was not as significant as in R₀. Ares et al., (1998) also observed delay in symptoms development when transgenic tobacco plants, expressing the MP gene of TMV, were inoculated with PVX.

### 1.9.4 Satellite RNA mediated resistance

Baulcombe et al., (1986) expressed the *Cauliflower mosaic virus* (CMV) satellite RNA in transgenic tobacco plants. When these transgenic lines challenged with CMV, showed resistance and attenuated symptoms development. Moreover, the tobacco plants expressing the anti-sense satellite RNA also resisted the cognate virus and symptom development was delayed, ultimately. Kong et al, (1997) determined that satellite RNA attenuates symptoms of TCV containing an alteration in the initiating AUG of the CP reading frame. Satellite RNA reduced the accumulation of TCV by more than 25 % in protoplasts while reducing the level of TCV by 90 to 100 % in un-inoculated leaves.

### 1.9.5 Defective interfering (DI) RNA or DNA mediated resistance

Defective interfering RNAs are small sub-viral replicons which are non-coding deletion mutants of the virus that maintain cis-acting RNA elements necessary for replication of host virus (Ray and White, 2003). In situ analysis of infected plants containing DI RNAs revealed that the DI RNAs dramatically elevate the level of virus-specific siRNA in viral infections resulting in the silencing of viral RNA (Zoltan et al., 2004). The data also shows that activation of post transcriptional gene silencing (PTGS) plays a pivotal role in DI RNA-mediated interference. The plants engineered with the DI DNA also delayed diseased symptoms, coupled with increased resistance (Kunik et al., 1994).
1.9.6 Anti-sense RNA mediated resistance

Anti-sense RNA constructs basically are complementary (c) DNA sequences inserted in a vector fused with a promoter so as to be expressed as RNAs complementary to a particular mRNA. There are many reports in which anti-sense RNA-mediated transgenic resistance technique was used successfully. To check the intensity of resistance of anti-sense RNA, Hammond and Kamo (1995) transformed the *Nicotiana benthamiana* with three anti-sense construct (i: 660 nt long fragment of *Bean yellow mosaic virus* (BYMV) CP including the carboxy-terminal portion, ii: complete 3’ noncoding sequence and iii: a short poly (A) tail). Selfing of T₀ was done and homozygous T₁ plants were challenged with purified virus sap. 10 lines were examined in which only one transgenic line was highly resistant to BYMV infection. A P1 sequence of PVY was transformed to potato cultivar Pito in anti-sense orientation (Maki-Valkama *et al.*, 2000). Five transgenic lines showed a high level of resistance against mechanical inoculation of PVY. The low transgene levels in the resistance lines suggested that the resistance might be based on gene silencing. In another experiment Lim *et al.*, (1999) made a construct of CP gene of *Cymbidium mosaic virus* (CyMV) Korean isolated in anti-sense orientation in the plant expression vector pMBP1. The construct was transferred via *A. tumefaciens* mediated transformation in *Nicotiana occidentalis*. The T₁ transformed plants were found resistant to CyMV infection.

1.9.8 RNA-mediated resistance

RNA silencing is a natural defence mechanism which operates through diverse pathways. The process is triggered by sequence-specific processing of long double-stranded RNA (dsRNA) into small interfering RNA (siRNA) fragments of 21-24 nucleotides in length. The host-encoded ribonuclease III (RNase III) like enzyme termed Dicer is involved in the processing of these siRNA (Berstein *et al.*, 2001). Dicer requires ATP and other host encoded proteins for this activity. RNA-induced silencing complex (RISC) guided by these siRNA to degrade specifically homologous sequence for degradation or inhibit translation, or at the level of DNA, to promote epigenetic modifications (Tabara *et al.*, 2002; Dunoyer and voinnet, 2005). In plants the siRNAs are divided into two size classes; the short siRNA (21-22 nt long) and the long siRNA (24-26 nt long). These two classes are involved in different functions; the
short siRNA class is responsible for the degradation of mRNA, while the long siRNA class correlates with systemic silencing and methylation of DNA (Hamilton et al., 2002).

1.10 RNA silencing

1.10.1 History of RNA silencing
Post-transcriptional gene silencing (PTGS) or RNA silencing or RNA interference (RNAi) is used as a generally applicable antiviral strategy. This process was first described in plants when Napoli, van der Krol and their respective co-workers were attempting to make purple petunias an even darker purple by over-express chalcon synthase (CHS) in transgenic petunia plants (Napoli, 1990; van der Krol et al., 1990). These scientists were trying to increase the flower pigmentation by transforming CHS genes using different constructs. Rather than obtaining more intensive violet colour some flowers were completely colorless i.e., they loss the pigment synthesis. It was shown that the transgene RNA was suppressing its own expression and also suppress the endogenous gene. This phenomenon was called ‘co-suppression’. Not much later, another encounter was made with RNA silencing where the concept of pathogen-derived resistance (PDR) was being exploited for the development of virus resistance plants.

Plant virologists deserve credit for several major discoveries in the understanding of RNA silencing. There were many reports that demonstrated that the expression of viral protein was not required for virus resistance, but the untranslatable viral RNA is sufficient to trigger the silencing signals (Lindbo & Dougherty, 1992; de Haan et al., 1992; van der Vlugt et al., 1992). Later on this process have also been found in other organisms like Neurospora crassa, in which it is called quelling, and in other different animal systems. Initially, progress of research in this field was very slow. However, the real break-through came when in an experiment Fire and co-workers discovered that through the injection of very low amounts of dsRNA into Caenorhabditis elegans could induce what they called RNA interference (RNAi) (Fire et al., 1999; Vauchert et al., 1998; Kooter et al., 1999; Matzke et al., 2001). Over the last few years RNA silencing or RNAi is considered more valuable tool in the studies of gene
functions (Kennerdell and Carthew, 2000; Schmid et al., 2002). The study of RNAi has become increasingly more honoring and continually flourishing after the completion of several genomes sequencing project such as the *Arabidopsis* and Human genome project.

### 1.10.2 Different classes of RNAs

Before going to discuss the process of RNA silencing a brief description of different classes of RNA involve in RNA silencing seems appropriate. Ribosomal RNA (rRNA) is the most abundant type of RNAs inside the cell followed by transfer RNAs (tRNAs) and messenger RNAs (mRNAs). In addition, there are four more classes of RNAs, which take active roles in RNA silencing. These are hairpin RNAs (hpRNAs), double stranded RNAs (dsRNA), small interfering RNAs (siRNAs) and micro RNAs (miRNAs). These classes will be described here in some detail.

#### 1.10.2.1 Double stranded RNA (dsRNA)

Double stranded RNA is formed by the complementary base pairing of two single-stranded fragments of RNA and plays an active role in RNA silencing pathway (Agrawal et al., 2003). The dsRNA found naturally in the cell and is derived generally from the replacement of transposons (Schramke and Allshire, 2004) or virus induction. The process of RNA silencing is triggered when the dsRNA is formed.

#### 1.10.2.2 Small interfering RNA (siRNA)

Small interfering RNA also known as short interfering RNA or silencing RNA, is a type of 20 to 25 nucleotide-long double-stranded RNA molecules with a 3’ two nucleotide overhang, play a variety of roles in the cell. This is formed from the long dsRNA by the cutting activity of Dicer. Short interfering RNA is involved mainly in RNA interfering pathway, where it involves in disrupting the function of a gene by interfering with the RNA expressed by that gene. siRNA was first discovered as part of post transcriptional gene silencing (PTGS) in plant by the group of David Baulcombe in Norwich, England (Hamilton and Baulcombe, 1999). Not much later in 2001, synthetic siRNAs have been reported to be involved in RNAi initiation in human cell line (Elbashir et al., 2001). This discovery has been revolutionizing
the field of gene-function analysis. Short interfering RNAs believed to have an important role in viral resistance and in preventing transposons transposition (Lippman et al., 2003).

1.10.2.3 Hairpin RNA (hpRNA)
Hairpin RNA is another form of dsRNA. It is deduced from a long piece of single stranded RNA containing inverted repeat and connected by a hairpin (Wesley et al., 2001). This long piece of single stranded RNA is produced by a vector introduced in the cell. A constitutive promoter, U6 in animal cells and cauliflower mosaic virus 35S promoter (CaMV35S) in plants, is used to ensure the expression of hpRNA continuously in cell. Hairpin RNA is transcribed by RNA polymerase III in animal cell and by RNA polymerase II in plants.

1.10.2.4 Micro RNAs (miRNAs)
These are small non-coding single stranded molecules of about 21-23 nucleotides in length, that negatively regulate gene expression, which were first discovered in nematode C. elegans, while screening the genes that control developmental timing (Lee et al., 1993), and today hundreds of miRNAs have been identified in plants and animals, including the hundreds unique miRNA from Arabidopsis alone (Lee and Ambros, 2001; Llave et al., 2002; Reinhart et al., 2002; Millar and Waterhouse, 2005). Micro RNAs are formed from the precursor single-stranded RNA transcripts that have the ability to fold back onto themselves to produce imperfectly double-stranded stem loop precursor structures. The main function of miRNA appears to be gene regulation (Grosshans and Slack, 2002).

1.11 The Biochemistry of the RNA silencing machinery
The first clues of RNA silencing were found in plants and fungi, and these pathways are now known to operate in nearly all eukaryotic species. The investigation of RNA silencing machinery has caught up considerable speed after its discovery in the animal model systems. Parts of the RNA silencing machinery have been studied comprehensively in many organisms like plants, insects, mammals and protozoa. The main action of RNA silencing involves the
cytoplasmic degradation of sequence-specific RNA molecules. The dsRNA and siRNA alone can not degrade mRNA. Several silencing-associated factors are involved in the silencing process includes the Dicer-like (DCL) proteins, RDRs, protein of the Argonaute family (AGO) and RNA helicases. The intermediary element in the RNA silencing pathway is dsRNA, and the degradation of this element triggers the process of RNA silencing. Ribonuclease III-like enzyme DICER recognized and degrades the dsRNA into small (21-23 nucleotides long) siRNA. These siRNA subsequently serves as guide for cleavage of homologous RNA molecules, mediated by RISC. Some of the recent advances in RNAi biochemistry and structural biology are summarized in the next section.

1.11.1 Dicer
The expression of transgene, arranged as inverted repeats can produce dsRNA artificially. There are several other molecular processes which can generate small RNAs. Naturally occurring small RNAs can be: (1) endogenous siRNA (also known as repeat associated siRNA); (2) miRNAs involved in gene regulation; (3) transposon-derived small RNAs; (4) virus-derived siRNAs. All siRNAs are the products of degradation of long dsRNA by RNase III-like enzyme family, first discovered in Drosophila (Bernstein et al., 2001). This enzyme was named Dicer in animals or Dicer-like (DCL) in plants. Dicers are multifunctional protein and contain one or more dsRNA binding domain. The number of dicer may be varying in different organisms. Many animals encode only a single Dicer, Drosophila encodes two (Lee et al., 2004), and four DCL homologues (DCL1, DCL2, DCL3 and DCL4) have been identified in Arabidopsis thaliana that function differentially in siRNA and miRNA biogenesis (Schauer et al., 2002). However, other organisms (like C. elegans and humans) used only a single DCL protein to process both categories of silencing initiation.

The role of these Dicer proteins in plants has been investigated in many experiments. DCL1 is mainly responsible for the processing of miRNAs (Herr et al., 2005). It was shown that some other factors, such as HEN1 and HYL1 (a dsRNA binding protein) also helps the DCL1 in the generation of miRNAs (Vazquez et al., 2004; Xie et al., 2004). HEN1 is also involved in some other functions like, natural virus resistance and transgene silencing (Boutet et al.,
DCL2 was found to be involved in the production of viral-derived 22-nucleotide siRNA and antiviral defence (Gasciolli et al., 2005). This viral-derived siRNA production also required two RdRps (RDR1 and RDR6) depending on kind of virus which infect the plant (Muangsan et al., 2004; Xie et al., 2004). DCL3, in concert with RDR2, plays a role in the generation of longer class of endogenous siRNAs (24 nt) and RNA-dependent DNA methylation (Xie et al., 2004). These endogenous siRNA are involved in the initiation or maintenance of a heterochromatic state (Matzke et al., 2004). DCL4 is involved in RNA silencing in plants and appear to produce 21-nucleotide siRNA component of the cell-to-cell silencing signal (Dunoyer et al., 2005b). Recent proofs of complementary distribution of functions between different DCLs suggests that these proteins could have an interchangeable and overlapping role in both siRNA and miRNA pathways (Deleris et al., 2006).

1.1.1.2 RISC

The biochemical recognition of the RNA-induced silencing complex (RISC), which sets up on the dsRNA that activates silencing, was a crucial advancement. RISC is a multi-protein complex and most of the components have been identified to the date. RISC is involved in different catalytic functions in the process of RNA silencing as mRNA cleavage and translational inhibition. The sequence specificity is provided by the small RNA molecules to RISC. Another protein called ARGONAUTE (AGO) has been found to be part with RISC studied in all organisms. The AGO protein is essential for the slicing activity of mRNA. Argonaute proteins have been extensively studied in plants and 10 members of the Argonaute family have been identified. Two AGO protein, AGO1 and AGO4, have been studied extensively, in which the AGO1 expression is regulated by a miRNA (miR168) indicating that the AGO1 protein regulates its own expression in a negative feedback loop (Vaucheret et al., 2004). AGO1 involved in the slicing activity and process miRNA and certain classes of endogenous siRNAs but not the viral siRNAs (Baumberger and Baulcombe 2005). The role of AGO4 has been reported in the production of the long siRNA of about 24 bp. Its involvement in long siRNA mediated chromatin alteration (histone methylation) also has been reported (Zilberman et al., 2003). AGO2 is part of RISC and essential for siRNA-directed RNA silencing, as reported in Drosophila. AGO2 has no role in the processing of miRNA, but
the role of AGO1 was indicated (Okamura et al., 2004). Despite the extensive work done to characterize the proteins that form the RISCs, the function of all the AGO proteins is still not fully understood. It can be concluded that most of the AGO protein, if not all, are involved in different parts of RNA silencing and possibly defines the mode of action of RISC in which they are integrated (Baulcombe, 2004).

1.12 RNA silencing-based transgenic virus control strategies

Transgene-mediated virus resistance is a classical example of RNA silencing. The research findings of Lindbo and Dougherty, (1992, 2005) on transgenic plants expressing the non-translatable CP gene of *Tobacco etch virus* (TEV) gives a breakthrough for RNA-mediated resistance in plants. Molecular analysis of these transgenic plants gave evidence that the sequence specific RNA degradation was initiated by the transgene-encoded RNA sequences. Since then there have been a number of examples of targeting RNA, DNA viruses and viroids through transgene-mediated resistance using viral sequences. Different strategies have been used to identify the efficient silencing mechanism induced by the transgene.

1.12.1 Construct design feature

The transgene-induced constructs were mostly transformed and checked in model plant species such as *N. benthamiana* and *N. tabacum*. Anti-sense RNA was used to engineered resistance against *Tomato yellow leaf curl virus* (TYLCV) in *N. benthamiana*. Waterhouse *et al.*, (1998) also obtained resistance against PVY by expressing the transgene RNA simultaneously in sense and anti-sense form in tobacco. Experiment shows that the constructs having both sense and anti-sense transcript and capable of producing dsRNA duplexes are proved to be efficient in producing resistance than the constructs producing only sense or anti-sense RNA alone (Waterhouse *et al.*, 1998). Although mechanisms of producing sense, anti-sense and dsRNA transcript are different, the steps following the processing of dsRNA, in the RNA silencing pathway, are common to all three transgene types (Beclin *et al.*, 2002).
The transgene constructs encoding a spliceable intron within a hairpin (hp) RNA structure was transformed in tobacco (Smith et al., 2000), which can induce PTGS with almost 100% efficiency. The ectopic expression of self complementary hairpin RNAs covering the P1 and HC-Pro genes of PPV were able to confer efficient resistance to PPV in N. benthamiana (Nicola-Negri et al., 2005). The percentage of PVY-resistant tobacco plants were 4% for anti-sense gene construct, 7% for sense gene construct, 58% for the hpRNA with sense and anti-sense arms separated by a non-spliceable intron, and 96% for same hpRNA with a spliceable intron separating sense and anti-sense transgene fragments. Hairpin RNA induced resistance was also proved efficient when Mitter et al., (2001, 2003) transformed with a construct, having 735 bp of the PVY Nla sequence as inverted repeats separated by an unrelated intron sequence, into tobacco plants. The resistance against PVY, varied from 40-50% in independent transformations.

Recently, it was shown that transgenically derived RNA silencing has also provided resistance to ssDNA viruses of the genus *Begomovirus*. While during replication ssDNA viruses do not produce dsRNA intermediate, their mRNAs may be targeted by RNA silencing machinery. For example, the essential replication associated protein encoding sequences of AC1 gene (Rep) was expressed in tobacco, which provided heritable resistance to cotton leaf curl disease in tobacco (Asad et al., 2003) and TYLCV in tomato (Yang et al., 2004; Bian et al., 2006).

### 1.12.2 Resistance to multiple viruses

Although the vector-based transgene siRNA approaches are used widely, presently most vector contains only a single siRNA expression cassette. A chimeric transgene construct composed of sequences from several viruses can confirm multiple virus resistance. Vectors containing multiple tandem siRNA expression cassettes (up to six) have been developed to maximize the efficiency and versatility of the vector (Wang et al., 2006). These vectors can be used not only for simultaneous suppression of the expression of multiple genes by producing different siRNA but also to maximize the silencing of a single gene by expressing multiple copies of siRNA targeting a single region of the gene. The N gene sequence...
fragment of the four major tomato-infecting tospoviruses, *Tomato spotted wilt virus* (TSWV), *Groundnut ring spot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and *Watermelon silver mottle virus* (WSMoV) was used in a single small chimeric hairpin (hp) RNA construct (Bucher *et al.*, 2006). Four gene fragments were combined and cloned into inverted repeat arrays around the intron, resulting in the sense-antisense IR-IN and the antisense-sense IR-OUT constructs. Sixteen independent IR-OUT and 16 IR-IN transgenic lines of *N. banthamiana* were generated. The self fertilized seed of S1 progeny were germinated and the plants were challenged with the tospoviruses TSWV, GRSV, TCSV and WSMoV either separately or in combination, in isolated green house. 81% of IR-OUT and 63% of IR-IN lines were found resistant to the infection of all four viruses.

1.13 Durability of RNA-mediated transgenic virus resistance

The RNA-based sequence-specific defense mechanism against virus infection was proved very efficient, still there are many viruses that successfully infect plants and overcome the plant defense. This long-lasting constancy and efficiency of RNA-mediated transgenic virus resistance can be impaired by plant, viral and environmental factors. The potential effect of such factors has been investigated and some of these factors are discuss below:

1.13.1 Viral-encoded suppressors of RNA silencing (viral antisilencing strategy)

RNA-silencing or posttranscriptional gene silencing (PTGS) is a natural mechanism which acts as an antiviral defence system in plants, viruses have to suppress the antiviral defence pathway in order to create a successful infection. For this purpose viruses have evolved a variety (more than 30) of RNA-silencing suppressors that counteract the antiviral defence pathway in plants during the critical steps of the infection cycle like initiation, maintenance and signaling of PTGS (Carrington *et al.*, 2001; Voinnet 2005a). This behavior of viruses has led to the concerns about the utility of RNA-mediated transgenic virus resistance and solidity of this mechanism under open environment where plants might be exposed to the variety of viral pathogens. This mechanism has been investigated by several scientists and now there are several reports mentioning that the PTGS-based virus-resistance plants can revert to a
susceptible one, following by the infection of heterologous virus encoding RNA silencing suppressor proteins (Savenkov and Valkonen 2001; Guo & Ding, 2002; Simon-Mateo et al., 2003). More than 35 individual viral suppressor of RNA silencing (VSR) families have been identified from almost all plant virus types, sorting out a necessary and ubiquitous counterstrategy (Li and Ding, 2006). VSRs are remarkably distinct within and across kingdoms because they are often encoded by the genes which have quite different sequences from each other.

The first and best described viral suppressor protein of RNA-silencing is the HC-Pro protein of genus *potyvirus*. It was first reported as a suppressor of RNA silencing when plants, in which a reported gene was silenced, were infected with PVX carrying HC-Pro. Upon systemic infection with this chimeric virus the silenced reporter gene was expressed again (Anandalakshmi et al., 1998). Further experimental analysis revealed that the HC-Pro is also involved to reduce the degradation of reporter mRNA into siRNAs and could interfere with the silencing signal (Hamilton et al., 2002). This means that the HC-Pro interacts with the RNase-III like enzyme, involved in the processing of dsRNA into the siRNAs, of the RNA-silencing machinery and blocks its function.

Another reported suppressor protein of RNA-silencing is 2b protein of *Cucumoviruses*. Cucumber mosaic virus (CMV) 2b affects differently the RNA silencing pathway as compared to HC-Pro. 2b is unable to suppress the RNA silencing directly in the tissues where the process of RNA silencing is already started. However, it was shown that 2b acts efficiently in the newly emerging tissues and prevent the initiation of the RNA silencing in these tissues (Brigneti et al., 1998; Beclin et al., 2002). The first hint that the 2b functions in nucleus is given by Guo and Ding (2002), where they postulated that the 2b interferes with the restoration of transgene methylation. They also showed that signal-independent RNA silencing initiation of transgene and virus silencing can not prevent by 2b (Guo and Ding, 2002).
The P19 of *Tomusbvirus*es is another most instant suppressor of RNA silencing. The P19 suppress the RNA silencing by keeping the siRNA binded in their double stranded form. This activity of P19 hinders the availability of siRNA for RNA silencing effectors such as RISC (Lakatos *et al.*, 2004). The siRNA is also involved in the systemic spreading of silencing signals, P19 either suppresses the systemic signal by binding the siRNAs and preventing their cell-to-cell movement, or it inhibits the activity of an siRNA-primed RdRp which were thought to be involved in the formation of the systemic signal (Voinnet, 2001).
### Table: 1.1. Examples of RNA silencing suppressors found in plant viruses.

<table>
<thead>
<tr>
<th>Virus Group</th>
<th>Virus name</th>
<th>Protein</th>
<th>Possible interference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Begomovirus</td>
<td>TYLCV, ACMV</td>
<td>C2, AC2</td>
<td>DNA binding</td>
<td>Voinnet et al., 1999</td>
</tr>
<tr>
<td>Crinivirus</td>
<td>SPCSV</td>
<td>P22</td>
<td>?</td>
<td>Kreuze et al., 2005</td>
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<tr>
<td>Carmovirus</td>
<td>TCV</td>
<td>P38</td>
<td>?</td>
<td>Thomas et al., 2003; Qu et al., 2003</td>
</tr>
<tr>
<td>Cucumovirus</td>
<td>CMV</td>
<td>2b</td>
<td>Prevent systemic spread of silencing, nuclear localization</td>
<td>Voinnet et al., 1999</td>
</tr>
<tr>
<td>Nodavirus</td>
<td>FHV</td>
<td>B2</td>
<td>?</td>
<td>Li et al., 2002</td>
</tr>
<tr>
<td>Potexvirus</td>
<td>PVX</td>
<td>P25</td>
<td>?</td>
<td>Voinnet et al., 2000</td>
</tr>
<tr>
<td>Potyvirus</td>
<td>PVY, TEV, TuMV</td>
<td>HC-Pro</td>
<td>Prevents accumulation of siRNA</td>
<td>Brigneti et al., 1998; Kasschau &amp; Carrington, 1998; Dunoyer et al., 2002</td>
</tr>
<tr>
<td>Tospovirus</td>
<td>TSWV</td>
<td>NSS</td>
<td>?</td>
<td>Bucher et al., 2003</td>
</tr>
<tr>
<td>Tombusvirus</td>
<td>TBSV</td>
<td>P19</td>
<td>Binds siRNA, prevents incorporation in RISC</td>
<td>Shilhavy et al., 2002; Voinnet et al., 1999</td>
</tr>
<tr>
<td>Closterovirus</td>
<td>BYV</td>
<td>P21</td>
<td>?</td>
<td>Reed et al., 2003</td>
</tr>
<tr>
<td>Pecluvirus</td>
<td>PCV</td>
<td>P15</td>
<td>?</td>
<td>Dunoyer et al., 2002</td>
</tr>
</tbody>
</table>

ACMV: African cassava mosaic virus; BYV: Beet yellows virus; CMV: Cucumber mosaic virus; FHV: Flock house virus; PCV: Peanut clump virus; PVY: Potato virus Y; PVX: Potato virus X; SPCSV: Sweet potato chlorotic stunt virus; TBSV: Tomato bushy stunt virus; TCV: Turnip crinkle virus; TEV: Tobacco etch virus; TSWV: Tomato spotted wilt virus; TuMV: Turnip mosaic virus; TYLCV: Tomato yellow leaf curl virus.
1.13.2 Sequence specificity of RNA silencing
To obtain maximum protection against a target organism, RNA silencing mechanism need to present a high degree of sequence identity to the target RNA. This is also a potential drawback of the RNA silencing mechanism in biotechnological applications, as RNA-mediated resistance is only efficient against those virus strains which have more than 90% nucleotide sequence homology to the transgene (Missiou et al., 2004). This was practically demonstrated when barley plants, carrying a single copy of Barley yellow dwarf virus-PVA (BYDV-PVA; genus Luteovirus) polymerase hpRNA transgene, were co-inoculated with BYDV-PVA and Cereal yellow dwarf virus-RPV (CYDV-RPV; genus Polerovirus) show resistance against the homologous but susceptible to the heterologous virus (Wang et al., 2000). Similarly in example sugarcane transformed with Sorghum mosaic virus CP sense sequence was evaluated for resistance. The transgenic phenotype was resistance to the virus strain with 95% sequence identity to the transgene but not to the strain of Sugarcane mosaic virus with only 75% sequence homology to the CP (Ingelbrecht et al., 1999).

1.13.3 Gene dosage and plant developmental stage
The PTGS or RNA silencing strategies was compared in homozygous and heterozygous progeny and was proved that, increasing gene dosage by self-pollination or by crossing of plants carrying homologous transgenes resulted in virus resistance (Jan et al., 2000). The effect of increased gene dosage was also seen on resistance phenotype in transgenic papaya in Hawaii. Homozygosity of a single copy CP gene of Papaya ringspot virus (PRSV) changed over the phenotype from susceptible to highly resistant to heterologous PRSV isolates. Similarly the increased degree of resistance was also noted with increased age of the plant (Tennant et al., 2001). RNA silencing activity may vary between plant tissues. It may be very effective in one tissue while low in other tissue of the same plant. It was shown recently that transgenic resistance was less effective in roots as compared to leaves in N. benthamiana against Beet necrotic yellow vein virus (BNYVV) (Andika et al., 2005).
1.13.4 Environmental factors
Temperature is the limiting factor of RNA silencing amongst all the environmental factors studied so far. RNA silencing seems to be settled at low temperatures (≤15°C). Virus-resistant phenotypes of *A. thaliana, N. benthamiana* and potato made by sense, anti-sense and hpRNA transgene have been shown to be lost under cold conditions (Szittya *et al.*, 2003). The accumulation of transgene-derived siRNAs appears to be temperature-dependent due to the reduced segmentation of dsRNA to siRNAs at low temperature (Kalantidis *et al.*, 2002). All these findings show that the temperature may pose constraint for the commercial use of transgenic RNA silencing technologies in crop plants. However, recently transgenic anti-sense RNA insertion lines of potato were developed. These lines pose RNA silencing resistance even at low temperature (S´os-Heged˝us *et al.*, 2005).

1.14 RNA silencing-based transgenic resistance in crop plants
Since its discovery in plants, RNA silencing-based transgenic resistance strategies against RNA and DNA viruses have been applied in variety of crop plant species (Table 2). Only three virus-resistance species, barley, potato and tomato, have been reported to carry an hpRNA transgene and all other carry translateable or untranslateable sense or anti-sense fragments. The maize ubiquitin promoter was used in majority of monocots to drive a transgene whereas the CaMV 35S promoter was generally used for dicots. In most reported examples some plants showed high resistance at least in some plants, but some recovery phenotypes have also been obtained. Wang and Metzlaff (2005) reported that during recovery in transgenic plants, an initial virus infection triggers post transcriptional gene silencing, which leads to amplification of silencing and high level of resistance. High level transgenic resistance was reported in plum lines C5 against the *Plum pox virus* (PPV) (sense CP transgene). All the molecular characteristics showed by the transgenic resistance lines were typical of PTGS i.e., low level of transgene mRNA, high level of transgene transcription in nucleus and methylation of the silenced CP transgene (Scorza *et al.*, 2001). Some changes were made in the construct design for improvement of efficiency and have been assessed in *N. benthamiana*. To confer systemic PPV resistance without affecting local infection, a tissue-
specific promoter was employed (Pandolfini et al., 2003). The hp RNA construct having PPV P1 sequence under the control of the phloem-specific RolC promoter was resistant to systemic PPV infection.
### Table: 1.2. Examples of virus-resistance transgenic crop plants based on RNA silencing

<table>
<thead>
<tr>
<th>Crop plant</th>
<th>Target virus</th>
<th>Transgene</th>
<th>Resistance characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>Rice yellow mottle virus</td>
<td>Sense RNA polymerase gene; CaMV 35S promoter</td>
<td>Immunity; high nuclear transcription but low steady state transgene mRNA</td>
<td>Pinto et al., 1999</td>
</tr>
<tr>
<td>Barley</td>
<td>Barley yellow dwarf virus</td>
<td>1.6 kb hpRNA ORF1+(polymerases); 860 bp ORF2 fragment spacer; maize ubiquitin promoter</td>
<td>Mostly single transgene; immunity</td>
<td>Wang et al., 2000</td>
</tr>
<tr>
<td>Wheat</td>
<td>Wheat streak mosaic virus</td>
<td>Nib gene; ubiquitin promoter, CP gene; ubiquitin promoter</td>
<td>Recovery phenotype; no detectable transgene mRNA, Recovery phenotype; no CP expression mRNA degraded</td>
<td>Sivamani et al., 2000, 2002</td>
</tr>
<tr>
<td>Cassava</td>
<td>African cassava mosaic virus</td>
<td>Sense AC1 gene (replication associated protein); Cassava vein mosaic virus promoter</td>
<td>Broad spectrum resistance to geminiviruses; siRNAs detected; AC1 mRNA levels suppressed</td>
<td>Chellappa et al., 2004</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>Sorghum mosaic Virus</td>
<td>Untranslatable CP gene; up to 15 copies; ubiquitin promoter</td>
<td>Recovery and immune phenotypes; more resistant plants show high nuclear transcription, but low steady-state transgene mRNA</td>
<td>Ingelbrecht et al., 1999</td>
</tr>
<tr>
<td>Papaya</td>
<td>Papaya ringspot Virus</td>
<td>Untranslatable CP ORF sense; 35S promoter; up to 5 copies</td>
<td>Co-suppression of GUS-ORF2b in transient leaf assay; no correlation between copy number and resistance</td>
<td>Lines et al., 2002</td>
</tr>
<tr>
<td>Potato</td>
<td>Potato leafroll Virus</td>
<td>Replicase ORF2b; non-translatable sense, translatable sense, anti-sense; 35S promoter</td>
<td></td>
<td>Vazquez Rovere et al., 2001</td>
</tr>
<tr>
<td>Potato</td>
<td>Potato virus Y (PVY)</td>
<td>P1 sense transgene; resistance independent of copy number 605 bp hpRNA from conserved CP 3' terminus; 1255 bp phage λ spacer; 35S promoter</td>
<td>Low steady-state level of transgenic mRNA siRNAs detected in mature plants; broad, strong resistance against PVY strains from N, O, NTN subtypes</td>
<td>Maki-Valkama et al., 2000</td>
</tr>
<tr>
<td>Tomato</td>
<td>Tomato spotted wilt virus</td>
<td>Nucleocapsid gene sense; single insertion locus, 3 rearranged copies</td>
<td>Immunity</td>
<td>Accotto et al., 2005</td>
</tr>
<tr>
<td>Tomato</td>
<td>Tomato yellow leaf curl virus</td>
<td>Rep gene sense or anti-sense; 35S promoter</td>
<td>Immune phenotype with viruliferous whitefly challenge glasshouse and field</td>
<td>Yang et al., 2004</td>
</tr>
</tbody>
</table>

NI, nuclear inclusion; ORF, open reading frame; satRNA, satellite RNA; siRNA, small interfering RNA; CP, coat protein; hp, hairpin
In Africa, cassava crop was badly disturbed by DNA virus, *African cassava mosaic virus* (ACMV), in the beginning of this century. Multiple copies of the AC1 gene of ACMV were expressed in cassava. The accumulation of transgene-specific siRNAs were observed, which contributed high level resistance to ACMV and two heterologous cassava-infecting geminiviruses (Chellappan *et al.*, 2004). Recently a tomato plants were transformed with intron hpRNA construct of TYLCV inverted repeat *Rep* gene sequences separated by a castor bean catalase intron. The transgene-specific siRNA accumulation was observed in transgenic lines and was immune to TYLCV challenged by viruliferous whiteflies (Fuentes *et al.*, 2006).

### 1.15 Other functions of RNA silencing

As mentioned above RNA silencing is a natural mechanism reported in plants and nearly about all eukaryotes. The antiviral activity is not its only function in plants. Many other processes are accomplished by using the component of RNA silencing machinery. Perhaps the roles of these processes are very important in plants and even more in other multicellular organisms, which has many functions other than resistance against viruses. These processes are gene regulation by miRNA, transposons silencing, transcriptional gene silencing by sequence specific DNA methylation and chromatin condensation.

#### 1.15.1 Transcriptional gene silencing

The effect of RNA silencing machinery is not limited to cytoplasm where it degrades the cognate RNA and silences its function. The function of smallRNA was also reported in the nucleus. The first clue that the RNA is involved in the transcriptional gene silencing (TGS) in the nucleus was given by Wassenegger and co-workers (Wassanegger *et al.*, 1994). Small RNAs that are derived from transgene or inverted repeat sequences has the ability to direct either DNA methylation or histone methylation in plants (Chen *et al.*, 2004). This phenomenon was termed RNA dependent DNA methylation and several components of the RdDM pathway have been identified. The DNA methyltransferases (DMTase) DRM1 and DRM2 were described to be involved in the de novo RNA-directed methylation and the putative DMTase, histone deacetylase HDA6, preserve or enhance the methylation. The
HDA6 recruitment results in the CG methylation and finally heterochromatin is formed at the specific targeted loci (Matzke et al., 2004). AGO4 was recently shown to be involved in the long siRNA-directed DNA methylation and its sustainment (Zilberman et al., 2003).

1.15.2 Transposons and endogenous repeat associated gene silencing
Movement of transposeable elements from one place to another new insertion sites can cause major damage to the plant genome. In order to defend themselves against these genomic parasites plants have to develop a defence system, a mechanism that can distinguish self from non-self at the nucleic acid level. Recently elaborative studies of DNA methylation, RNA silencing and post-translational histone alterations in the plants have begun to disclose what seems to be just such an integrated genome defence system. It has been shown that longer type of siRNAs produced in plants has been derived from transposon. Cloning and sequencing of naturally occurring siRNA of A. thaliana revealed that these originate not only from transposon or retroviruses but also from highly repeated ribosomal DNAs (Hamilton et al., 2002; Xie et al., 2004). These siRNA can lead to the sequence specific RdDM and consequently transcriptional silencing of transposon. The RDR2 and other RNA processing factors are involved in transposon silencing, revealed by studies of mutant Arabidopsis (Bender, 2004). Similarly the extensive work done on C. elegans revealed that several factors involved in RNAi (mut-7 and RNaseD homolog, mut-16 and mut-14 an RNA helicase) are also required for transposon silencing (Sijen and Plasterk, 2003).

1.15.3 Chromatin modeling
RNA silencing mechanism is also involved in chromatin structure, centromeric cohesion and cell division, which perhaps is of greater magnitude than transcriptional gene silencing. The three main genes which encoded the central enzymes of the RNA silencing machinery, Argonaut, Dicer and an RdRp were shown to be crucial for this purpose. Similarly the mutational analysis also indicated that RNA silencing compounds were required for the pericentromeric organization in Schizosaccharomyces pombe (Volpe et al., 2003). A complex repeated dsRNA originating from pericentromeric heterochromatin required RdRp for its production. These RNA duplexes incorporated into the RNAi-induced transcriptional gene
silencing (RITS) complex after the rapid processing of Dicer. The Ago1 protein of *S. pombe* is a major element of RITS complexes which binds the siRNA. The RITS complex has high biochemical similarity to RISC (Verdel *et al.*, 2004). RITS function has also been reported in sequence specific methylation of centromeric regions and chromosome condensation in the dividing cells.
RNA silencing is triggered by dsRNA originating from replicating RNA viruses, DNA viruses or transgene mRNA. The dsRNA is cleaved by RNAse III-like enzyme (Dicer) into small interfering RNAs (siRNA) that are incorporated into the RNA-induced silencing complex (RISC) and guide it to RNA molecules with homologous sequence, which is degraded, generating more siRNA. siRNA can move systemically in the plants and cause systemic silencing. In addition siRNA are thought to be important for methylation of transgene DNA. Viral-encoded RNA silencing suppressors can interfere with different steps in the RNA silencing pathway.
Objectives of the study

Potato is asexually propagated crop which makes it more susceptible to systemic and viral diseases. About twenty different viruses infect potato in which most prevalent viruses in Pakistan are Potato virus X (PVX), Potato virus Y (PVY) and Potato leaf roll virus (PLRV). The yield losses are about 30% due to these viruses and even more when the viruses occur in combination. There is no natural virus resistance gene in the presently grown cultivar in Pakistan. Conventional approaches of virus control are limited to chemical control of virus vectors and to use of virus free seed tubers. Chemical pest control is very costly and of environmental concerns. Also the virus free seed tubers are not affordable by small farmers due to its high cost of in vitro propagation, inspection and certification. In crop production, development of resistant potato cultivars remains the most effective, economical and environmentally safe method of disease control. The conventional methods of breeding disease resistance genes are problematic in potato due to the complexity of inheritance in autotetraploid plants and loss of important agronomic characters during wide hybridization. Genetic engineering provides some novel approaches for selectively adding important traits, like disease resistance to the elite cultivars. Pathogen-derived resistance is one of the approaches which have been used to develop virus resistance through genetic transformation of virus-derived transgene that interfering with the essential steps in the life cycle of infecting virus. To address this issue, a study was initiated in 2005 with the major aim being to develop a broad spectrum resistance in potato against these devastating viruses. The overall objectives of the study include:

1) Optimization of conditions for the development of transgenic plants.
2) Engineerign resistance against multiple viruses in the local elit cutivars of potato to reduce the annual yield losses caused by viruses.
3) Producing indigenous virus free seed potato stock for local formers.
CHAPTER 2

Materials and Methods

2.1. Collection of viral infected plants samples

Viral infected plant samples were collected from the hilly potato growing areas of Kaghan, NWFP, during the summer season of 2005. The symptoms of each virus i.e. *Potato Virus X* (PVX), *Potato Virus Y* (PVY) and *Potato Leaf Roll Virus* (PLRV) were present as endemic in the area. On the basis of specific symptoms of each virus, infected plants were selected. The plants were uprooted with soil and transferred to the pots. These infected samples were brought to the laboratory and maintained *in-vitro* in large test tubes through micro-propagation.

2.1.1 Serology

Potato infecting viruses were suspected on the basis of symptoms. The samples were screened for the presence of PVX, PVY and PLRV by Double Antibody Sandwich ELISA using virus specific polyclonal antisera according to manufacturer’s instructions (Agdia, USA). Positive samples were maintained *in-vitro* as a regular source of viruses for carrying out further studies.

2.1.2 Isolation of total RNA

Total RNA was extracted from the each virus i.e. PVX, PVY and PLRV infected tissues by using Concert Plant Trizol Reagent (Invitrogen USA) according to the manufacture’s instructions. Before starting the procedure the centrifuge machine was set at 4 °C. Infected leaves weighing 200 mg to 400 mg were cut from each plant and ground into a very fine powder in pestle and mortar in the presence of liquid nitrogen and transferred to 1.5 ml RNase free tubes. Each sample was added with 0.5 ml cold Concert Plant RNA Reagent and mixed by flicking. The tubes were laid down horizontally and incubated for 5 minutes at room temperature. Solutions were clarified by centrifugation at room temperature for 2 minutes at
13000 rpm in micro centrifuge (Eppendorf Minispin 22331, Germany). Supernatant was collected in fresh tubes, 100 µl of 5 M NaCl and 300 µl Chloroform was added to each tube, mixed by inversion and centrifuged at 4 °C for 10 minutes at 13000 rpm. The top aqueous phase of each solution was transferred to fresh tube, equal volume of isopropanol was added, mixed thoroughly and allowed to stands at room temperature for 10 minutes. The tubes were again centrifuged at 4 °C for 10 minutes at 13000 rpm. Supernatant was discarded and the pellet was washed by resuspended in 80 % ethanol and spun at room temperature for two minutes at 13000 rpm. After a brief interval the air dried pellet was dissolved in 30 µl RNase free water. The integrity of RNA was checked on 1 % (w/v) agarose gel and stored at -80 °C.

2.1.3 RNA quantification
The concentration of each RNA sample was checked by using a SmartSpec Plus (Bio-Rad, USA) flourimeter. Dilutions were made of each RNA sample to uniform concentration by adding deionized H₂O and loaded in flourimeter. The final concentrations were found by multiplying the samples reading with dilution factor.

2.2 Isolation of genes through cDNA synthesis

2.2.1 Primer designing
Six pairs of specific primers were designed (Table. 2.1) on the basis of known gene sequence data available in database (NCBI, Gene Bank) for the amplification of Nucleoside Triphosphate (NTP-binding helicase 300 bp) and Coat Protein (CP 375 bp) genes of PVX, Helper Component Protease (HC-Pro 365 bp) and Coat Protein (CP 340 bp) genes of PVY and Coat Protein (CP 300 bp) gene of PLRV. For each primer the particular restriction sites were determined on vector as well as in the primer. It was confirmed that the same restriction sites were not present within the coding regions of the target gene fragments, primers were not complementary to each other in order to avoid primer dimmer formation and the GC contents of each primer were not less than 50 %.
Table 2.1. Primers used for amplification of sense and anti-sense fragments used in the fusion-PCR in order to clone the chimeric N-gene cassette. Restriction sites are underlined.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer description</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sense cloning*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S PVX F</td>
<td>GAC GTC GAC CTA GAG GCA TAC AGA AGC CAG</td>
</tr>
<tr>
<td>2</td>
<td>S PVX R</td>
<td>GAC CTG CAGCAA TGG CTA TCA CCT TCC CAA</td>
</tr>
<tr>
<td>3</td>
<td>S PVY F</td>
<td>GAC GGT ACC GCA TAA ACA TGC AAG AGA TGG</td>
</tr>
<tr>
<td>4</td>
<td>S PVY R</td>
<td>GAC GTC GAC ATT CCA GTT TGC CTT GGC AGA</td>
</tr>
<tr>
<td>5</td>
<td>S PLRV F</td>
<td>GAC CTG CAG GCC GCT CAA GAA GAA CTG GAG</td>
</tr>
<tr>
<td>6</td>
<td>S PLRV R</td>
<td>GAC CTC GAG TCG TAA TTT GGA ACT TGT TGA</td>
</tr>
<tr>
<td>7</td>
<td>SPXF</td>
<td>GAC GTC GAC TCT GGA AGG ACA T GA AGG TGC</td>
</tr>
<tr>
<td>8</td>
<td>SPXR</td>
<td>GAC CTG CAG TCA TTT CAG TTT CAG ACG GTG</td>
</tr>
<tr>
<td>9</td>
<td>SPYF</td>
<td>GAC GAA TTC GTC GCC GCT CAA GAA GAA CTG GAG</td>
</tr>
<tr>
<td>10</td>
<td>SPYR</td>
<td>GAC GTC GAC CCA CAT CCC GCA GAT TTT GAA</td>
</tr>
<tr>
<td>11</td>
<td>SPLF</td>
<td>GAC CTG CAG GCC GCT CAA GAA GAA CTG GAG</td>
</tr>
<tr>
<td>12</td>
<td>SPLR</td>
<td>GAC GGT ACC TCG TAA TTT GGA ACT TGT TGA</td>
</tr>
<tr>
<td></td>
<td>Anti-sense cloning**</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>A PVX F</td>
<td>GAC CTG CAGCAA TGG CTA CCT TCC CAA</td>
</tr>
<tr>
<td>14</td>
<td>A PVX R</td>
<td>GAC GAA TTC CTA GAG GCA TAC AGA AGG CAG</td>
</tr>
<tr>
<td>15</td>
<td>A PVY F</td>
<td>GAC ATC GATATT CCA GTT TGC TTT GGC AGA</td>
</tr>
<tr>
<td>16</td>
<td>A PVY R</td>
<td>GAC CTG CAG GCA TAA ACA TGC AAG AGA TGG</td>
</tr>
<tr>
<td>17</td>
<td>A PLRV F</td>
<td>GAC GAA TTC TCG TAA TTT GGA ACT TGT TGA</td>
</tr>
<tr>
<td>18</td>
<td>A PLRV R</td>
<td>GAC GGA TCC GCC GCT CAA GAA GAA CTG GAG</td>
</tr>
<tr>
<td>19</td>
<td>APXF</td>
<td>GAC CTG CAG TCA TTT CAG TTT CAG ACG GTG</td>
</tr>
<tr>
<td>20</td>
<td>APXR</td>
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<tr>
<td>21</td>
<td>APYF</td>
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<tr>
<td>22</td>
<td>APYR</td>
<td>GAC CTG CAG CTC GGG CAA CTC AAT CAC AGT</td>
</tr>
<tr>
<td>23</td>
<td>APLF</td>
<td>GAC GAA TTC TCG TAA TTT GGA ACT TGT TGA</td>
</tr>
<tr>
<td>24</td>
<td>APLR</td>
<td>GAC ATC GAT GCC GCT CAA GAA GAA CTG GAG</td>
</tr>
</tbody>
</table>

* The primers designed for the amplification of fragment in sense orientation
** The primers designed for the amplification of fragment in anti-sense orientation

2.2.2 First strand cDNA synthesis:

The first strand cDNA was synthesized using AMV (Avian Myeloblastosis Virus) First-Strand cDNA Synthesis Kit (Invitrogen, USA) following manufacturer’s instruction with slight modifications. The partial cDNA of NTP and CP genes of PVX, HC-Pro and CP genes of PVY and CP gene of PLRV were synthesized by using the genes specific reverse primers. The primers used were, S PVX R, SPXR for NTP and CP, S PVY R, SPYR for HC-Pro and
Materials and Methods

CP and S PLRV R for Coat protein respectively (Table 2.1). Five cDNA reaction mixtures were prepared in RNase free tubes on ice. Total volume of each reaction was kept 20 µl and contained 4 µl of total RNA (2 µg), 4 µl cDNA synthesis Buffer (5X), 2 µl dNTPs (10 mM), 1 µl RNase Out (40 U µl\(^{-1}\)), 2 µl reverse primer (10 µM), 1 µl reverse transcriptase enzyme (15U µl\(^{-1}\)), 1 µl DTT (0.1 M) and 5 µl DEPC-treated water. The reactions were incubated for 20 minutes at room temperature and 1.5 hour at 42 °C. The reactions were stopped by heating at 94 °C for 3 minutes and chilled on ice.

2.2.3 Polymerase Chain Reaction amplification:
The desired partial genes fragments of NTP (300 bp), CP (375 bp) (PVX), HC-Pro (365 bp), CP (340 bp) (PVY) and CP (300 bp) (PLRV) were amplified through PCR from their corresponding cDNAs by using specific forward and reverse primers for sense cloning given in Table 2.1. The PCR reactions were carried out using Taq DNA polymerase (Fermentas, Germany). Five separate reactions were carried out in separate tubes. The total volume of each reaction were 50 µl which contained 5 µl of 200 ng cDNA, 5 µl of 10X PCR Buffer, 3 µl of 25 mM MgCl\(_2\), 1 µl of 10 mM dNTPs, 1 µl each of 5 µM reverse and forward Primer, 0.5 µl of 5U µl\(^{-1}\) Taq polymerase, 33.5 µl of deionized H\(_2\)O. A negative control without cDNA was also included. The fragments were amplified for 34 cycles of 94 °C for 60 sec, 55 °C for 60 sec and 72 °C for 60 sec after an initial denaturation of 94 °C for 5 min in a thermal cycler machine (Eppendorf Mastercycler, Germany).

2.2.4 Agarose gel electrophoresis of PCR products
PCR amplified products were analyzed by gel electrophoresis using 1.0 % (w/v) agarose gels supplemented with ethidium bromide (100 µg ml\(^{-1}\)) for visualizing the DNA bands. The gel was submerged in 0.5X TAE (0.04 M Tris acetate, 0.001 M EDTA) (Appendices # 1) buffer in a gel tank, 10 µl of PCR product from each reaction was mixed with 3 µl of 6X loading dye (Fermentas, Germany) (Appendices # 2) and loaded to gel. The samples with 1kb DNA marker were electrophoresed at 80 volts for 40 minutes and visualized on an ultraviolet trans-illuminator. The photographs were taken using Stratagene Eagle Eye still video system to record the results.
2.3 Cloning of genes in a TA cloning vector pTZ57R

2.3.1 Purification and ligation of PCR product in TA vector
The five PCR amplified fragments using sense primer of genes (mentioned above) were purified individually using the CONCERT™ Rapid PCR Purification System (GIBCOBRL, UK) according to the manufacturer’s instructions with slight modifications. The approximate DNA concentration of each purified fragments were determined by comparing the intensity of band on agarose gel to a known amount of DNA size marker and ligated to the pTZ57R (Appendices # 11). The ligation procedure was carried out following the protocol described by Sambrook et al. (1989). Total volume of each ligation reaction mixture was 20 µl which contained 2 µl plasmid vector pTZ57R (0.165 µg µl⁻¹), 5 µl purified PCR fragment (0.09 µg µl⁻¹), 2 µl ligation buffer (10X), 2 µl PEG 4000 solution, 1 µl T₄ DNA Ligase (5U) and 8 µl nuclease-free water. The reactions were incubated overnight at 16 °C.

2.3.2 Preparation of competent cell of *Escherichia coli* strain DH5α
Competent cells of *Escherichia coli* strain DH5α were prepared according to the ECM 399 electroporation system (BTX Harvard Apparatus, USA) protocol. A single colony from a freshly grown *E.coli* plate was picked and transferred in 100 ml LB liquid medium (Tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹, and pH 7.0) in 500 ml flask and incubated at 37 °C overnight with vigorous shaking. Next day 2.5 ml of overnight culture was inoculated to 250 ml LB liquid 1000 ml flask and allowed to grow until O.D₆₀₀ reached between 0.5-1.0 (10¹⁰ cells ml⁻¹). The culture was transferred to 50 ml sterilized disposable polypropylene tube and cooled by keeping on ice for 10-20 minutes. Centrifugation was carried out at 5000 rpm at 4 °C for 10 minutes. Supernatant was discarded and the pellet was dissolve in 50 ml of cold deionized water. The cells were pelleted at 5000 rpm for 10 minutes at 4 °C. The cells were dissolved again in 25 ml cold deionized water and centrifuged at 5000 rpm at 4 °C for 10 minutes. This step was repeated and finally cells were washed with 10% glycerol, centrifuged at 5000 rpm for 5 minutes at 4 °C and dissolved in 500 µl of 10 % glycerol. Aliquots of 50µl each were freezed in liquid nitrogen and stored at -80 °C.
2.3.3 Transformation of ligation products into E. coli strain DH5α

The electroporator ECM 399 (BTX Harvard Apparatus, USA) was used to transform the ligation product into *E.coli* strain DH5α (Appendices # 6). The electroporation cuvettes of 2 mm gap (BTX, USA) were chilled on ice, 4 µl of each ligated product was mixed with 50 µl of competent cells separately and transferred to each cuvette. The pulse was given to each cuvette by using the following parameters: volts 2.5 KV, resistance 159 ohm, capacitance 25 µf and capacitance extender 125 µf. After given the pulse the cells in each cuvette was added with 1 ml of liquid LB medium and transferred to 15 ml tube after mixed gently. To recover from electric shock, the cells were incubated at 37 °C with vigorous shaking for 45 minute. Each transformation mixture was then spread on LB agar plate containing ampicillin (100 mg ml⁻¹) and incubated overnight at 37 °C.

2.3.4 Screening of the trans-conjugant clones

Ten colonies of trans-conjugant of each clone were picked with the help of toothpick and individually grown in glass culture tubes, containing 5 ml LB (Appendices # 3) broth with ampicillin (100 µg ml⁻¹), for 6 to 8 hours at 37 °C shaker. After the optimum growth of culture, miniplasmid isolation was carried out following the procedure of Sambrook *et al.* (1989). Each individually grown overnight culture was transformed to 1.5 ml eppendorf tube and spun at 14000 rpm for 2 minutes. The pellet of cells was resuspended by adding 200 µl of buffer 1 (25 mM Tris, PH 8.0, 10 mM EDTA, pH 8.0, RNase 100 µg ml⁻¹) (Appendices # 4). The cells were lysed by adding 200 µl lysis buffer 2 (0.2N NaOH, 1% SDS) (Appendices # 4) and mix by inverting the tubes many times. In each tube 200 µl of buffer 3 (3M potassium acetate) (Appendices # 4) was added to neutralize the reaction. Centrifugation was done at 1400 rpm for 10 minutes and supernatant was transferred to fresh eppendorf tube. Plasmids were precipitated by adding 300 µl ethanol then incubated at -20 °C for 30 minutes and centrifuged at 14000 rpm for 10 minutes. The plasmid pellets were washed with 70 % ethanol, air dried and dissolved in 20 µl nuclease free water.
2.3.5 Verification of clones

The verification of clones was carried out by digesting miniplasmid of each trans-conjugant colony separately. The restriction enzymes EcoR I and Pst I sites, which were present in the vector, used for the restriction confirmation of all the clones. The total volume of each restriction reaction was kept 20µl which contained plasmid DNA 2 µl (4 µg 20 ml⁻¹), 2 µl of 10X restriction buffer, 1 µl of RNase enzyme (0.01µg µl⁻¹), 0.5 µl of EcoR I and Pst I each (10 U µl⁻¹) and 14 µl nuclease free water. The clones were further confirmed by restricting with specific enzyme sites of each fragment which were inserted during primer synthesis. Specific enzymes used for each fragment in restriction reaction were Sal I, Pst I for NTP, CP (PVX), Kpn I, Sal I for HC-Pro, Xho I, Pst I for CP (PLRV), Sal I, EcoR I for CP (PVY) and Kpn I, Pst I for CP (PLRV). The reactions were incubated at 37 °C for 1 hour.

2.3.6 Agarose gel electrophoresis

The restricted plasmids were size fractionated on 1 % agarose gel as mentioned under section 2.2.4. The correct size of fragment was marked by comparing with 1 kb standard DNA marker (GeneRuler 1 kb DNA ladder, Fermentas, Germany), which was run in the gel beside the samples. The clones having the required band size were selected and further confirmed by PCR amplification using the gene specific forward and reverse primers.

2.3.7 DNA sequencing

The fragments cloned in pTZ57R plasmid were sequenced by using ABI PRISM 310 Genetic Analyzer (ABI, USA) machine. The PCR based version of Sanger’s dideoxy chain termination method (Sanger et al., 1997) was followed for DNA sequencing using fluorescence labeled dye terminator. The cloned gene fragments were multiplied by PCR reaction in a 0.2 ml PCR tubes which contained 2 µl of 1 µg µl⁻¹ highly pure plasmid DNA, big dye terminator mix 4 µl (ABI, USA), 1.5 µl of 10 ng µl⁻¹ primer, 2.5 µl of deionized H₂O. The PCR profile were set as for 24 cycles of 96 °C for 10 sec, 50 °C for 05 sec and 60 °C for 04 sec after an initial denaturation of 96 °C for 01 min in a thermal cycler machine (Eppendorf Mastercycler, Germany). The salt precipitation of each reaction was done by adding 1 µl of 20 mg ml⁻¹of glycogen, 2 µl of 3M Na acetate, 90 µl sterile deioninzed H₂O
and 250 µl absolute ethanol to the tube. All the reagents were mixed thoroughly and centrifuged for 30 minutes at 14000 rpm after incubation at room temperature for 15 minutes. The supernatant was aspirated without disturbing the tiny reddish pellet. The pellet was washed by resuspending with 200 µl of 80 % ethanol and centrifuged for 15 minutes at 14000 rpm. The supernatant was discarded completely and the tubes were left opened for some time at room temperature for complete dryness of DNA pellet. The 20 µl of Hi-diluted formamide was added to each tube and were mixed thoroughly by vortex mixer. The DNA fragments were denatured by giving heat shock of 95 °C for 2 minutes and chilled on ice immediately. The samples were mixed again by vortex mixer and centrifuged for 30 seconds at 13000 rpm to collect volume at the bottom of tubes. The sample were transferred to sample tubes and caped with rubber gasket. The properly sealed sample tubes were loaded in the machine for sequence analysis.

2.3.8 Amplification and cloning of genes using primers for anti-sense cloning

The five genes fragments were also amplified through PCR reaction and cloned individually in TA vector using specific primers designed for anti-sense cloning (Table 2.1). The reagents used for each PCR reaction was 2 µl of plasmid DNA having specific cloned gene, 5 µl PCR buffer (10X), 3 µl MgCl₂ (25 mM), 1 µl dNTPs (10 mM), 1 µl each anti-sense forward and reverse primer (5 µM), 0.5 µl Taq polymerase (5 U µl⁻¹) and 36.5 µl deionized H₂O. Each PCR amplified fragment was size fractionated on 1 % agarose gel, purified and ligated to vector pTZ57R (section 2.3.1). The ligated product was transformed through electroporation into DH5α and spread on LB agar plates (section 2.2.3). The trans-conjugants were screened and size fractionated on 1 % agarose gel after restricted with their specific enzymes as mentioned under sections 2.3.4, 2.3.5 and 2.3.6).

2.4 Combining of the genes in different combination by fusion PCR

In a second round of PCR the individually cloned genes fragments were fused in different combination by ligation and PCR amplification. In first combination the NTP-binding helicase of PVX, HC-Pro of PVY and CP of PLRV were fused which resulted in a chimeric
965 bp \textbf{SN1} gene fragment (S stands for sense cloning) as shown in Fig. 2.1. In second combination the fusion of CP of PVX, CP of PVY and CP of PLRV resulted in a chimeric single 1015 bp long \textbf{SN2} gene fragment as shown in Fig. 2.2.

\subsection*{2.4.1 Digestion of the plasmid DNA}
Each fragment cloned in a TA vector was taken out by individually restricted the plasmid using specific restriction site of each clone. The restriction enzymes used and other conditions for restriction reaction were same as mention under section 2.3.5. The restricted fragments were size fractionated on 1 % agarose gel (section 2.3.6) and the exact size fragments were eluted.

\subsection*{2.4.2 Gel elution and}
Gel elution of each fragment was carried out by using Marligen Bioscience Gel Extraction System (GIBCOBRL, UK). The digestion reactions were run on 1 % agarose gel and visualized under the UV trans-illuminator. The fragments showing the correct sizes were excised from the gel and transferred to 1.5 ml tubes containing 30 µl gel solubilization buffer. The tubes were incubated at 50-55 °C for 15 minutes and inverted after every 3 minutes to dissolve the gel properly. Each mixture was transferred to a cartridge placed in a recovery tube and centrifuge at 13000 rpm for 1 minute. The flow through in the recovery tube was discarded and 700 µl of wash buffer was loaded to each tube. The tubes were incubated for 5 minutes at room temperature and centrifuge at 13000 rpm for 1 minute. The flow-through was discarded and this step was repeated to remove the traces of wash buffer completely. Finally the DNA of each sample was eluted by adding 50 µl of warm TE buffer (10 mM Tris and 1 mM EDTA) to cartridge followed by 1 minute incubation and 2 minute centrifugation at 1000 rpm. The concentrations of final eluted three fragments were checked on agarose gel and were combined by ligation.

\subsection*{2.4.3 Fusion of three fragments}
The reagents and their ratio used in the ligation reaction were optimized for two separate reactions. In the first reaction NTP (300), HC-Pro (365) and CP (300) (PLRV) were combined
through ligation reaction and subsequent PCR amplification. The reagents in the first reaction were 5 µl each of NTP, HC-Pro and CP (PLRV) eluted fragment (1 µg of each fragment), 2 µl of 10X ligase buffer, 1 µl of T₄ DNA ligase (5 U µl⁻¹) and 2 µl nuclease free H₂O. In the second ligation reaction CP genes of PVX (375 bp), PVY (340 bp) and PLRV (300 bp) were combined and the ligation reaction were set as 5 µl (1 µg) each of three CP eluted fragment, 2 µl of 10X ligase buffer, 1 µl of T₄ DNA ligase (5 U µl⁻¹) and 2 µl nuclease free H₂O. Reaction mixtures were incubated overnight at 16°C. The first ligation reaction were resulted in 965 bp long Xho I, Kpn I chimeric SN1 gene fragment (Fig. 2.1). The second ligation also resulted in a 1015 bp long EcoR I, Kpn I SN2 chimeric gene fragment (Fig. 2.2). Each ligation product was purified and used as template for PCR amplification. Other reagents and their ratio used in PCR reaction were same as described under section 2.2.3. The PCR amplified fragment was analyzed and detected on 1 % agarose gel. The fragment having the exact size was purified using the CONCERT™ Rapid PCR Purification System (GIBCOBRL, UK) according to the manufacturer’s instructions with slight modifications (section 2.3.1). The purified fragment was ligated in pTZ57R and transformed into DH5α through electroporation (section 2.3.3). The cultures of 30 tans-conjugants were grown individually in culture tubes and mini plasmids were isolated (section 2.3.4). The clones were verified by restricting plasmids individually and resolved on 1 % agarose gel (section 2.3.5, 2.3.6).
Figure: 2.1. Schematic representation of inverted repeat expression cassette I derived from 3 potato virus encoded genes with their cloning sites. (A) cDNA fragments of 3 genes were amplified using primers pairs as described in Table 2.1 and fused by cloning into a single SN1 gene. (B) Amplification and fusion of 3 anti-sense fragments into a single AN1 gene by ligation. (C) Inverted repeat cassette I of SN1 and AN1 genes in RNAi based vector pN6. CP: coat protein; aCP: anti-sense cp; NTP: nucleoside triphosphate; aNTP; anti-sense NTP; HC-Pro: helper component protease; aHC-Pro: anti-sense HC-Pro; 35S: CaMV 35S promoter; OCS: octopine synthase terminator.
2.4.4 Combining the anti-sense fragments by fusion PCR

The fragments which were amplified by using anti-sense primers and cloned individually in pTZ57R vector were also fused by ligation. Each fragment was excised from the plasmid by using their respective restriction sites inserted during primer designing. The NTP, CP (PVX) fragments were excised by EcoRI and PstI, HC-Pro by PstI, ClaI, CP (PVY) by PstI, HindIII, CP (PLRV) by EcoRI, ClaI and EcoRI, BamHI separately. Two separate ligation reaction were set same as mentioned under section 2.4.3. In the first reaction NTP, HC-Pro and CP (PLRV) were fused to a long AN1 (A stands for anti-sense) chimeric gene fragment of 965 bp (Fig. 2.1). In a second reaction the three CP genes were fused which resulted in 1015 bp long AN2 chimeric gene fragment (Fig. 2.2). The ligated fragments were amplified through PCR, purified and ligated in TA vector using the same procedure as mentioned under the sections 2.2.1, 2.4.2, 2.4.3 for sense fragment fusion and cloning.
Figure: 2.2. Schematic representation of inverted repeat expression cassette II derived from three CP genes of PVX, PVY and PLRV with their cloning sites. (A) cDNA fragments of 3 genes were amplified using primers pairs as described in Table 2.1 and fused by cloning into a single SN2 gene. (B) Amplification and fusion of 3 anti-sense fragments into a single AN gene by ligation. (C) The inverted repeat cassette II of SN2 and AN2 genes in RNAi based vector pN6. CP: coat protein; aCP: anti-sense cp; 35S: CaMV 35S promoter; OCS: octopine synthase terminator.
2.5 Construction of two plant expression cassettes in RNAi based vector pN6

Two expression cassette I and II were constructed in the RNAi based vector pN6 (Appendices # 11) provide with the courtesy of Dr. Ali Rezaian Adelaide Australia). This plasmid has multiple cloning sites between the CaMV 35S promoter and OCS terminator. Multiple cloning sites are separated by an intron sequence, which is a non-translatable sequence and helps in making hairpin loop structure after transcription (Fig. 2.3).

2.5.1 Cloning of SN1 and SN2 genes fragment

2.5.1.1 Digestion of plasmid DNA

The pTZ57R vector carrying SN1 and SN2 fragments was digested individually to take out the 965 bp long \textit{Xho} I, \textit{Kpn} I SN1 and 1015 bp long \textit{Kpn} I, \textit{EcoR} I SN2 gene fragment. The reagent and their ratio used for each digestion reaction was as 16 µl of plasmid DNA, 2 µl of 10 X buffer and 1 µl (10U) of each \textit{Xho} I, \textit{Kpn} I enzyme for SN1 gene and \textit{Kpn} I, \textit{EcoR} I enzyme for SN2 gene. All other conditions for digestion reaction were same as described in section 2.3.5. The restriction reaction was size fractionated on 1 % agarose gel and the exact size fragment was eluted from the gel (section 2.2.3, 2.4.2).

2.5.1.2 Digestion of vector pN6

The RNAi vector pN6 was also restricted in separate reactions using the same \textit{Xho} I, \textit{Kpn} I enzymes for SN1 and \textit{Kpn} I, \textit{EcoR} I for SN2 fragment. Two separate restriction reactions were carried out in 1.5 ml tube and each reaction contained containing 16 µl of vector DNA, 2 µl of 10X buffer and 1 µl (10U) of each \textit{Xho} I, \textit{Kpn} I in first reaction and \textit{Kpn} I, \textit{EcoR} I in second reaction. The reactions were incubated at 37 °C for 1 hour. The digested product were purified by adding 1/10 volume of 3M sodium acetate and 0.6 volume of isopropanol and centrifuged for 10 minutes at 13000 rpm after an incubation at -20 °C for 20 minutes. The supernatant was discarded and the pellet was washed with 70 % ethanol and dissolved in nuclease free water.
2.5.1.3 Ligation reaction

The SN1 and SN2 fragment were ligated to pN6 vector individually in separate ligation reactions. The 3:1 ratio of the fragment and the vector DNA were used. The total volume of each reaction was 10 µl and contained 2 µl (200 ng) of purified digested plasmid, 6 µl (600 ng) of purified 1 kb fragment, 1 µl of ligase buffer (10 X) and 1 µl of T₄ DNA ligase (5 U). The reaction was incubated at 16 °C overnight in circulating water bath (Poly Science, USA). The ligation product was transformed in *E.coli* strain DH5α by electroporation using the same procedure as described under section 2.3.3.

The trans-conjugants clones were screened using the restriction enzymes *Xho* I, *Kpn* I for SN1 and *Kpn* I, *EcoR* I for SN2 and other restriction conditions were same as mentioned in the section 2.3.5. The clone was further verified by restricted the internal specific genes and size fractionated on agarose gel (section 2.3.6). The clone having the exact size fragments was purified and used for the anti-sense cloning.

2.5.1.3 Cloning of the AN1 and AN2 genes fragment

The pN6 vector having SN1 fragment cloned was restricted with *Cla* I, *BamH* I restriction enzymes and purified as mentioned in section 2.5.1.2. The plasmid having cloned the AN1 fragment was also restricted with same *Cla* I, *BamH* I restriction enzymes to take out the AN1 fragments. The fragments were size fractionated on agarose gel and exact size fragment were eluted from gel (sections 2.3.6, 2.4.2). To complete the construction of plant expression cassette I, the AN1 fragment was placed as an inverted repeat of SN1 fragment in pN6 as described by Chen *et al.* (2004) (Fig. 2.1). Similarly to complete the expression cassette II, AN2 fragment was excised from the pTZ57R by using the restriction enzyme *Cla* I, *Hind* III and placed at the same site in pN6 as an inverted repeat of SN2 (Fig. 2.2). Ligation reactions of cassette I, cassette II were carried out by using the same procedure described under section 2.5.1.3. The ligation reaction were transformed into *E. coli* strain DH5α and the trans-conjugants were screened by restricting with the enzyme *Cla* I, *BamH* I for cassette I and *Cla* I, *Hind* III for cassette II (section 2.3.3, 2.3.4). The restriction reactions were resolved on 1 %
agarose gel (section 2.3.6). The clone having released the exact sized fragment was identified and used for large plasmid preparation.

2.5.1.4 Plasmid preparation

Large plasmid preparation was carried out separately from positive clones of cassette I, II using Concert™ rapid plasmid purification system (GIBCO BRL, USA) according to the supplier’s instructions. 100 ml of overnight grown culture was centrifuged in 50 ml centrifuge tube at 5000 rpm for 10 minutes. The supernatant was discarded and the cells were resuspended in cell suspension buffer. The cells were lysed by adding 6 ml of cell lyses solution and incubate for 5 minutes at room temperature followed by adding 8 ml of neutralization solution. The contents were mixed by inverting the tubes 5-6 times. The column was placed in a 50 ml centrifuge tube; mixture was loaded into the column and centrifuged at 5000 rpm for 2 minutes. The flow through was discarded and the column was washed by adding 15 ml of wash buffer and centrifuged with same speed for 5 minutes. Finally the plasmid was eluted in a fresh tube by adding 3 ml of TE (10 mM Tris and 1 mM EDTA, pH 8.0) in the column and centrifuged at same speed for 5 minutes. The eluted plasmid was re-verified as described under the section 2.3.6 and stored at -20 °C in 1.5 ml tubes.

2.6 Cloning of expression cassettes in plant transformation binary vector pGreen.

The binary vector pGreen (Appendics # 11) was used for the plant transformation. This plasmid contains the npt II selectable marker gene and multiple cloning cites between the T-DNA borders. The pGreen was restricted with a single enzyme Not I and purified. The restriction reaction contained 16 µl of vector DNA (pGreen), 2 µl of 10X buffer and 1 µl (10U) of Not I and other conditions were kept same as described under section 2.5.1.2. Dephosphorylation of plasmid was done by adding 1 ul (10U) of alkaline phosphatase (Fermentas, Germany) in the same restriction reaction and the incubation time was further extended for 15 minutes. The restricted vector was resolved on 1 % agarose gel along with the DNA ladder, purified by adding 1/10 volume sodium acetate and 0.6 volume isopropanol and centrifuged for 10 minutes at 13000 rpm after an incubation at -20 °C for 20 minutes as described under section 2.5.1.2.
Two plant expression cassettes I and II were cloned in pGreen vector in two separate ligation reactions. In first ligation reaction a Not I fragment about 4.96 kb of cassettes I (Fig. 2.1) restricted from pN6 vector, was sub-cloned in pGreen at the Not I site between the left and right border (Fig. 2.4). In a second ligation reaction a Not I fragment of about 5.0 kb, excised from pN6, was ligated to pGreen at the Not I site (Fig. 2.5). Each ligation reaction was performed in 0.5 ml PCR tubes which contained 2 µl (200 ng) of purified dephosphorylated plasmid, 6 µl (600 ng) of purified fragment, 1 µl of 10X ligation buffer, 1 µl of T4 DNA ligase (Fermentas, Germany) and incubated at 16 °C overnight as described under section 2.5.1.3.

Each ligation reaction was transformed into *E.coli* strain DH5α and was spread on LB solid plate containing antibiotic (kanamycine 100 mg l⁻¹). About 20 colonies of trans-conjugants of each clone were screened and the genes fragments were confirmed by restriction. The restriction enzymes used for the confirmation of cassettes I insertion were *Xho* I, *Kpn* I and *Cla* I, *BamH* I and for the confirmation of cassettes II were *EcoR* I, *Kpn* I and *Cla* I, Hind III. The restriction reactions were size fractionated on agarose gel (2.3.6). The positive clones were marked and were used for the preparation of large plasmid as described under section 2.5.1.3.
Figure: 2.3. Schematic representation of T-DNA map of binary vector pGreen (cassette I); R: Right Borger; L: Left Border; NptII: Neomycin phosphotransferase II; NOSP: Nopaline synthase promoter; NOST: Nopaline synthase terminator; CaMV35S: Cauliflower mosaic virus promoter; OCST: Octopine synthase terminator.

2.7 Preparation of competent cell of Agrobacterium tumefaciens strain LBA4404

Electrocompetent cells of Agrobacterium tumefaciens strain LBA4404 (GIBCO BRL, USA) were prepared as described (Mersereau et al., 1990). Single colony from a freshly grown plate of LBA4404 was picked and inoculated into 100 ml LB liquid medium containing antibiotic streptomycin (100 µg ml⁻¹), in 250 ml autoclaved flask using sterile toothpick and inoculated at 28 °C for 48 hours with vigorous shaking. 5 ml of the 48 hours grown culture was re-inoculated into 1 liter flask containing 250 ml of the same LB medium and antibiotic and incubated at 28 °C until the OD₆₀₀ of cells were become 0.5-1.0 (10¹⁰ cells ml⁻¹). The cells were transferred aseptically to ice cold 50 ml polypropylene tube and kept cool on ice for 30 minutes. The cells were then centrifuged, resuspended and freezeed as described under the section 2.3.2.
2.8 Transformation of recombinant plasmids in *Agrobacterium tumefaciens* strain LBA4404

The purified pGreen plasmids carrying three partial genes RNAi cassettes I and II were transformed into *Agrobacterium tumefaciens* separately with the help of helper plasmid pSoup through electroporation. About 1 µg (2 µl) of plasmid from each cassette and 1 µg of helper plasmid pSoup were mixed separately with competent cells of LBA4404 in two 1 mm gap cuvettes (BTX, USA) and fixed in an electroporator for electric shock (section 2.3.3). After electroporation the cells were diluted with 1 ml liquid LB and incubated for 4 hours at 28 °C. The cells of each construct were transferred separately to two 1.5 ml tubes and pelleted by centrifugation. After discarding 900 µl supernatant the pellet was resuspended in 100 µl of remaining supernatant and spread on LB solid medium plates containing antibiotics (100 mg l⁻¹...
streptomycine, 50 mg l\(^{-1}\) kanamycine 3 mg l\(^{-1}\) tetracycline). Plates were incubated at 28 °C for 48 hours for the growth of *Agrobacterium* colonies.

### 2.8.1 Verification of cloned plant transformation binary vector pGreen in LBA4404 by PCR

Six colonies of the trans-conjugant from each cassette I and II clone were grown individually into 2 ml of liquid LB medium containing antibiotics (100 mg l\(^{-1}\) streptomycine, 50 mg l\(^{-1}\) kanamycine 3 mg l\(^{-1}\) tetracycline). The cultures were grown overnight at 28 °C and PCR reaction were carried out using 2 µl of overnight grown culture of each colony as template DNA. The specific genes as well as npt II gene were amplified for verification of clones. All other reagents for the PCR reaction used were same as described under section (2.2.3). The PCR amplified fragment was resolved on 1 % agarose gel same as described under section 2.3.6.

### 2.9 Plant Transformation

*Solanum tuberosum* cv Kuroda and Desiree were transformed with two inverted repeat hairpin RNA (hpRNA) cassette I and II with the help of *A. tumefaciens* strain LBA4404, that harboring the hpRNA construct, in separate reactions. The sliced internodal explants method was used for potato transformation as described by Beaujean *et al*, (1998).

#### 2.9.1 Plant material

Potato plants, cultivars Kuroda and Desiree, were propagated in vitro in large test tubes (150 x 25). Each test tube has Murashige and Skoog (1962) medium (MS medium) (macro-elements, micro-elements (Appendices # 7) and MS vitamins (Appendices # 8)), containing sucrose 30 g l\(^{-1}\) and gelatin 3.6 g l\(^{-1}\) (Carl Roth, Germany), pH 5.7 and autoclaved at 120 °C for 20 minutes. Internodal stem cuttings from 4-weeks old plants were used as explants for transformation. Potato explants were prepared by cutting the upper stems sections from the *invitro* grown plants into internodal sections approximately 4 to 6 cm in length. Internodal explants were further cut lengthwise down the middle to exposed more vascular bundle zone to *Agrobacterium* infection, which allowed the production of large number of transgenic buds.
Table: 2.2: Media compositions used in potato tissue culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>CIM</td>
<td>MS + ZR (0.8 mg l(^{-1})) + 2, 4-D (2 mg l(^{-1})) + kanamycin (100 mg l(^{-1})) + cefotaxime (300 mg l(^{-1}))</td>
</tr>
<tr>
<td>SIM</td>
<td>MS + ZR (0.8 mg l(^{-1})) + GA(_3) (0.1 mg l(^{-1})) + kanamycin 100 mg l(^{-1}) + cefotaxime (300 mg l(^{-1}))</td>
</tr>
<tr>
<td>RIM</td>
<td>MS + IAA (0.1 mg l(^{-1})) + kanamycin (50 mg l(^{-1})) + cefotaxime (125 mg l(^{-1}))</td>
</tr>
</tbody>
</table>

CIM: callus inducing media; SIM: shoot inducing medium; RIM: root inducing medium; MS: Murashige and Skoog; ZR: zeatin riboside; 2,4-D: 2,4-dichlorophenoxyacetic acid; GA\(_3\): gibberelic acid 3; IAA: indole-3-acetic acid.

2.9.2 Transformation and regeneration

*Agrobacterium* strain LBA4404 containing cassette I and II in pGreen chimeric plasmid were incubated separately in two glass flasks for 16 hours with shaking at 28 °C in 50 ml liquid LB medium containing antibiotics (100 mg l\(^{-1}\) streptomycin, 50 mg l\(^{-1}\) kanamycin, 3 mg l\(^{-1}\) tetracycline) (Appendices # 9). The cultures were spun and pelleted by centrifugation in sterilized polypropylene tubes at 5000 rpm for 5 min. the pellets of each tube was resuspended in 2 volume of MS liquid medium (MS salts and vitamins MS, 3 % sucrose, pH 5.7) to an OD of 10\(^9\) cfu at 600 nm and mixed both the cells and medium. MS Media containing recombinant *Agrobacterium* cells of two construct were poured onto petri dish (9 cm dia) separately and stem cuttings explants of cultivars Desiree and Kuroda were immersed in each petri dish. After 30 minutes of incubation, explants were blotted on sterilized filter paper carefully not to let them dry out and cultured on Callus Inducing Medium (CIM) without antibiotic (Table 2.2). Internodal explants were placed with cut face in contact with medium. The petri dishes were sealed with sealing film, labeled properly and co-cultivated for 72 hrs (26 °C ± 2 °C) at low light intensity 16 hours photoperiod.

After co-cultivation the explants were washed with MS liquid medium (MS salts and vitamins MS, 3 % sucrose, pH 5.7) containing one g l\(^{-1}\) cefotaxime (Aventis pharmaceuticles, USA) for 30 minutes. After dry blotting, the explants were transferred to selection medium CIM (Table
2.2) and incubated as described above. The plates were checked regularly for any bacterial growth and were sub-cultured every 2-3 weeks to refresh the medium. After 3-4 weeks well developed calli were transferred to Shoot Inducing Medium (SIM) with antibiotics (Table 2.2). Shoot initiations were started after 3-4 weeks. Well-defined green shoots, after reaching a length of 1-2 cm, were excised and transferred to a large test tubes (150 x 25 mm) containing root inducing medium (RIM) (Table 2.1) with selective antibiotics (Kanamycin 50 \( \text{mg l}^{-1} \), Cefotaxime 250 mg l\(^{-1}\)). The putative transgenic shoots developed roots after one week of transfer on rooting medium and multiplication of shoots was carried out by sub-culturing the auxiliary buds on to the rooting medium to increase the survival rate. The plants were transferred to plastic pots containing mixed sand and soil. The humidity was retained by covering the plastic pots with closed vents plastic domes. Vents were opened progressively after 7 days until the plants acclimatized to the ambient humidity. Plants were transferred finally to earthen pots having soil and shifted to containment for developing T\(_0\) progeny.

2.10 Molecular characterization of transgenic plants

To confirm the presence of transgenes in T\(_0\) plants, total DNAs were isolated from all transformed and untransformed (negative control) plants. The DNAs were subjected to PCR and Southern hybridization for the confirmation of the presence of transgenes.

2.10.1 Isolation of total genomic DNA

The method of Iqbal et al. (1997) was followed for the isolation of total genomic DNA. The water bath temperature was set at 65 \( ^{\circ} \text{C} \) and 2 X CTAB DNA extraction buffer (2 % Cetyl-triethylammonium bromide, 100 mM Tris-HCL, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1 % PVP) (Appendices # 5) with 2 % 2-mercaptoethanol was preheated. About 2 gm of fresh leaves were ground to fine powder in pre-cooled pestle mortar in the presence of liquid N\(_2\). The powder was transferred to 50 ml sterile polypropylene tube and 15 ml (preheated at 65 \( ^{\circ} \text{C} \)) 2 X CTAB buffer was added to the tubes before the frozen powder started thawing. The tubes were inverted several time to mix the paste and incubated in water bath with gentle
shacking at 65 °C for 30 minutes. After incubation, tubes were allowed to cool at room
temperature for some time and 15 ml of chloroform: isoamylalcohol (24:1) was added to

tubes. The tubes were inverted gently to mix the solutions and centrifuged at 9000 rpm for 10

minutes at room temperature (Eppendorf centrifuge 5810, Germany). The top aqueous phase
was transferred to a new 50 ml tube and the remaining chloroform phase was discarded. The

DNA was precipitated by adding 0.6 volumes chilled isopropanol to each tube and mixed
gently. The tubes were incubated at -20 °C for 30 minutes and centrifuged at 9000 rpm for 10

minutes. The supernatant was discarded and the DNA pellet was washed with 70 % ethanol.

Air-dried the DNA pellet and dissolved in 1 ml nuclease free water. Isolated DNA was treated
with 5 µl of RNase (10 mg ml⁻¹) for 1 hour at 37 °C. The RNase was removed by adding an
equal volume of chloroform-isoamylalcohol (24:1) followed by centrifugation at 13000 rpm
at room temperature for 10 minutes. Supernatant was transferred to fresh tubes and DNA was
precipitated by adding 0.6 volume isopropanol and 1/10 volume of 3 M sodium acetate. After
spinning at 13000 rpm for 10 minutes, the pellet was washed with 70 % ethanol, air-dried and
resuspended in deionized water.

2.10.2 DNA quantification

DNA was quantified by determined its concentration by spectrophotometer (SmartSpec
BIORAD, USA). The absorbance readings were taken at 260 nm wavelength and the
conversion factor was O.D₂₆₀ 1=50 µg ml⁻¹. Each sample was diluted to a certain level before
loading the sample in the cuvette (trUView Cuvette-BIORAD) and dilution factor was set in
the machine. The reading of machine was blanked by loading the water, which was used for
DNA dilution to subtract the background reading.

2.10.3 Detection of transgenes in transgenic plants by polymerase chain reaction

The SN1 and SN2 transgenes were determined by PCR amplification using genomic DNA of
transgenic plants. The specific genes fragments of NTP, CP of PVX, HC-Pro, CP of PVY and
CP of PLRV were also determined by PCR amplification using specific primers. The reaction
was carried out in 0.5 ml PCR tube and contained 2 µl (200 ng) of plant DNA, 5 µl PCR
Buffer (10X), 3 µl MgCl₂ (25 mM), 1 µl dNTPs (10 mM), 1 µl each forward and reverse
Primer (5 µM), 1 µl forward Primer (5 µM), 0.5 µl Taq polymerase (5 U µ l⁻¹), 36.5 µl deionized H₂O. Two negative control were included in each reaction which corresponded to i) isolated DNA of an untransformed potato plant and ii) water control, the PCR mixture without DNA. The corresponding plasmid DNA was used as template in positive control reaction. All other conditions and reagents used for PCR were the same as described in section 2.2.3.

The presence of neomycin phosphotransferase (npt II) gene was also determined by PCR. The primers for amplification of npt II gene were custom synthesized (GIBCO, BRL, USA) using sequence of Kunik et al. (1994). Forward and reverse primers sequences are as follows:

npt II F 5’ CACGCAGGTTCTCCGCGCCG 3’
npt II R 5’ TGCGCTGCGAATCGGAGCG 3’

Conditions for preparing PCR reaction mixture were the same as described above. The profile used for PCR was as follows: 1 cycle 95 °C for 5 minutes, then 35 cycles of 95 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute. PCR products were analyzed on 1 % agarose gel electrophoresis as described under the section 2.3.6.

2.10.4 Southern blot analysis of transgenic plants

The transgene insertion in potato plant genome was also determined by Southern hybridization performed on total genomic DNA. For this purpose 10 µg of total genomic DNA of selected potato lines was digested with Hind III and incubated at 37 °C overnight. The digested DNA was size fractionated on 0.8 % agarose gel supplemented with 10 µg ml⁻¹ ethidium bromide at 40 V in TAE buffer for 5 to 6 hours. The DNA image was obtained under UV light in gel documentation apparatus (section 2.2.4). DNA was depurinated by submerging the gel in glass tray contained 250 ml depurination solution (0.25 M HCl) twice for 15 minutes on an orbital shaker and rinsed briefly with deionized water. Denaturation of DNA was carried out by floating the gel twice in denaturation solution (1.5 M NaCl and 0.5 N NaOH) for 15 minutes and washed thoroughly with deionized water. Finally neutralization was done by soaking the gel twice in neutralization solution (1 M Tris (pH 7.4), 1.5 M NaCl) for 15 minutes. DNA in the gel was transferred on nylon membrane (Hybond-Amersham) by capillary method using 10 X SSC (150 mM sodium citrate and 1.5 M NaCl) as transferred
buffer for 20 hour (Sambrook et al., 1989). The membrane was removed from gel and
crosslinked in UV crosslinker (CL-1000 Ultraviolet Crosslinker-UVP) at 120 mJ cm\(^{-2}\) energy.
The crosslinked membrane was further washed in a solution containing 0.1X SSC, 0.5 % SDS
at 65 °C for 45 minutes to remove residual agarose. To block the attachment of probe to non-
specific nucleic acid binding sites, the membrane was treated with 0.2 ml cm\(^{-2}\) pre-
hybridization solution [6X SSC, 5X Denhardt’s solution (0.1 % each of BSA, Ficol and PVP),
50 % deionized formimide, 0.5 % SDS and 50 µg ml\(^{-1}\) salmon sperm DNA] in a hybridizer
for 2-4 hours at 42 °C.

Biotin DecaLabel DNA Labeling kit (Fermentas, Germany) was used for labeling the purified
DNA products (section 2.4.3) of specific genes following the manufacturer’s instructions. The
reaction mixture for DNA labeling was prepared in 1.5 mL microcentrifuge tube containing
100 ng to 1 µg DNA template (usually PCR amplified purified product), 10 µl decanucleotide
in 5X reaction buffer and total of 44 µl of volume was made up by nuclease free water. The
reaction mixture was mixed briefly, spinned down and DNA was denatured in a boiling water
bath for 5-10 minutes. The tube was cooled immediately on ice, added 5 µl biotin labeling
mixture and 1 µl Klenow fragment, mixed briefly by vortexing and spinned down quickly.
The tube was incubated at 37 °C for 1 to 20 hour and the reaction was stopped by adding 1 µL
0.5 M EDTA (pH 8.0) to reaction. DNA labeled probe was again denatured at 100 °C for 5
minutes chilled on ice and added to the pre-hybridization solution (25-100 ng ml\(^{-1}\)). After the
treatment of pre-hybridization solution for 2-4 hours, the membrane was treated with
hybridization solution (60 µl cm\(^{-2}\)) in the hybridization tubes and incubated overnight in a
hybridizer at 42 °C. The membrane was first washed twice with 2X SSC and 0.1 % SDS for 10
minutes at room temperature then twice with 0.1X SSC and 0.1 % SDS at 65 °C for 20
minutes. To detect the biotin-labeled DNA the membrane was floated in 30 ml
Blocking/Washing Buffer (provided by the manufacturer) for 5 minutes at room temperature.
To block non-specific binding sites, membrane was treated with 30 ml Blocking Solution for
30 minutes. The membrane was incubated for 30 minutes in Streptavidin-AP conjugate
diluted in 20 ml blocking solution. The next washing was of 60 ml Blocking/Washing buffer
twice for 15 minutes and incubated for 10 minutes in 20 ml Detection Buffer. Finally 10 ml
Substrate Solution was prepared and the membrane was incubated in it at room temperature in the dark until blue-purple precipitate became visible. The reaction was terminated by washing the membrane with distilled water for few seconds after discarding the Substrate Solution.

2.11 *In vitro* assay of virus resistance

To assess the effectiveness of inverted repeat cassette I and II (2.3, 2.4) in the transformants, *in vitro* assay of virus resistance was performed in green house at the regenerated stage $T_0$. The PLRV infected plants were kept in cage and aphids were reared on it for 15 days. Approximately 14 *in vitro* grown transgenic lines each of cassette I and II were hardened for 15 days. Three replicates of each transgenic line were shifted to the temperature-controlled, insect-proof green house with a 16 h natural daylight photoperiod and 25/22 °C day/night temperature regime. To infect the transgenic plants, the pure sap of PVX and PVY (plants were maintained as infection source in growth room) was obtained by grinding leaves of each systemically infected plant in 0.5 % Na$_2$SO$_3$ solution. Extracts were filtered through double-layered gauzes, transferred to falcon tubes, and mixed with carborundum (1 mg/ml). The extracts were inoculated to the test transgenic plants separately and also mix. In mixed inoculum experiments equal amount of diluted sap from each grinded plant was mixed and was applied by softly rubbing the leaves using carborundum powder as an abrasive material (Lim *et al.*, 1997). For PLRV inoculation the plants were exposed to viruliferous aphids, which were reared on infected PLRV potato plants, in cages. After an inoculation feeding period of 72 h, aphids were eliminated with insecticide. For each inoculum used in all experiments, non-transformed control plants were inoculated after the inoculation of transgenic lines. The plants were then monitored for 60 days for symptoms development. The plants were analyzed by ELISA after 15 days of interval to exclude symptomless infections.
2.12 Double Antibody Sandwich (DAS) Enzyme Linked Immuno Sorbent Assay (ELISA)

The inoculated transgenic plants (upper leaves) were tested for the detection of PVX, PVY and PLRV 15, 30 and 50 days post-inoculation (dpi) by Double Antibody Sandwich Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) (Clark and Adams, 1977). Commercially available ELISA detection kit (Agdia, USA) was used for the detection of each virus. Each coating antibody (Purified polyclonal antibody of PVX, PVY and PLRV) was diluted 1000 times in coating buffer (1.59 g Na$_2$Co$_3$, 2.94 g NaHCO$_3$, 0.5 g NaN$_3$, pH 9.6, add deionized H$_2$O to 1000 ml total volume) and add 200 µl each to 96 wells microtiter plates. The covered plates with a lid are placed in a humid box (wet tissue on the bottom of the box) and incubated at 37 °C for 3 hour. Plates were washed three times with washing buffer PBST (0.1 % Tween-20 in 0.1 M PBS (8 g NaCl, 1 g KH$_2$PO$_4$, 14.5 g Na$_2$HPO$_4$, 0.5 g NaN$_3$, pH 7.4 with NaOH, add deionized H$_2$O to 1000 ml total volume)). Each infected leaf sample were ground in SEB (0.1 % Tween-20, 2 % polyvinylpyrrolidone (PVP-25), 0.2 % ovalbumine in 0.01 M PBS). The positive samples were diluted 10 times in SEB. Each coated microtiter well was loaded with 200 µl of grounded leaf samples, included 3 wells each with positive and negative control. The plates were washed 4 times with washing buffer (PBST) after an overnight incubation at 4 °C and 200 µl of AP conjugate (Alkaline Phosphate conjugated polyclonal virus-specific antibody (IgG)) was added to each dried well. The plates were covered with a lid and incubated at 37 °C for 3 hours. The plates were again rinsed 4 times with washing buffer and 200 µl of substrate was loaded to each dried well of the microtiter plates. The plates were covered with a lid and incubated at room temperature until the positive control and positive samples were colored yellow. The concentration of virus in each sample was determined by ELISA plate reader after 60 minutes incubation at room temperature.

2.13 siRNA analysis of the transgenic plants

Nucleic acids of transgenic plants were extracted and siRNAs enriched as described by Bucher et al. (2004) with slight modifications. Total nucleic acids of transgenic resistance,
susceptible and untransformed plants were extracted with the help of Concert Plant Trizol Reagent (Invitrogen USA) using the same procedure as described under section 2.1.2. The final pellet was dissolved in 150 µl of QRL1 solution in RNA/DNA Mini Kit (QIAGEN) and low molecular weight RNA were separated from DNA and higher molecular weight RNAs according to the manufacturer’s instructions. The precipitate of low molecular weight RNA of each sample was dissolved in 15 µl of formamide. The tubes were heated at 65 °C for 15 minutes and chilled on ice. Twenty micrograms of total small RNAs were separated by denaturing polyacrylamide gel electrophoresis (PAGE) at 200 V for 2.5 hour and transfer to Hybond-N⁺ membrane (Amersham Pharmacia) by capillary blotting (Sambrook and Russell, 2001). The membrane was rinse with 2 X SSC buffer and dried at room temperature. The RNA was fixed on the membrane by crosslinking in UV crosslinker (CL-1000 Ultraviolet Crosslinker-UVP) at 120 mJcm⁻² energy.

The probe was labeled with digoxigenin using PCR consisting of three steps: denaturation at 94 °C for 5 minutes; 30 cycles at 94 °C for 30 seconds; 53 °C for 30 seconds and 72 °C for 1 minute; and a final extension at 72 °C for 5 minutes. The membrane was soaked in pre-hybridization solution for 1 hour at 40 °C. The probe was denatured by heating and chilling on ice and added to the pre-hybridization solution. Hybridization of the membrane was carried out at 40 °C for 12 hour followed by washing twice with washing buffer for 15 minutes at 50 °C. The signal detection process including blocking the membrane, reacting hybridized probes with Anti-Digoxigenin-AP Fab fragments and washing the membrane was done according to manufacturer’s instructions (DIG Northern Starter Kit, Roche). The membrane was equibrated by equibration buffer and exposed to x-ray film (Fuji film) after soaking in CDP-Star solution (Applied Biosystems, USA).
CHAPTER 3

Results

3.1 Cloning of Potato Virus X, Y and Potato Leaf Roll Virus genes sequences

3.1.1 Isolation of genes sequences through cDNA synthesis

Five regions of genes, Nucleoside Triphosphate (NTP-binding helicase, 300 bp) and Coat Protein (CP, 375 bp) genes of PVX, Helper Component Protease (HC-Pro, 365 bp) and Coat Protein (CP, 340 bp) genes of PVY and Coat Protein (CP, 300 bp) gene of PLRV, were synthesized by making their complementary DNA (cDNA). Total RNA were isolated from viral infected potato tissues and used as template for synthesis of corresponding cDNA (section 2.2.2). Using cDNA as template these fragment were amplified by Polymerase Chain Reaction using five pairs of specific forward and reverse primers (Table 2.1) (section 2.2.3). PCR products were resolved on 1 % agarose gel electrophoreses along with 1 kb DNA ladder (section 2.2.4). The Fig. 3.1 shows the pattern of PCR amplified fragments of five partial gene regions.

![PCR amplified products of five genes fragments](image)

**Figure: 3.1. PCR amplified products of five genes fragments;** Lane 1: 1 kb DNA marker; Lane 2: HC-Pro of PVY; Lane 3: NTP-Binding helicase of PVX; Lane 4: CP of PLRV; Lane 5: CP of PVX; Lane 6: CP of PVY; Lane 7: Negative control (no DNA).
3.1.2 Purification and ligation in PCR cloning vector pTZ57R

The PCR amplified fragments of five genes corresponding to NTP, CP of PVX, HC-Pro, CP of PVY and CP of PLRV were purified and ligated individually in the pTZ57R (section 2.3.1). These ligated products were transformed into *E.coli* through electroporation. The plasmid DNA was isolated from 10 trans-conjugant colonies for each of the five clones. The positive clones were identified on the basis of exact size fragment released (section 2.3.6). The clones were further verified by PCR amplification using the specific forward and reverse primers.

3.1.2 Sequence analysis of amplified fragments

The authenticity of cloned gene fragments was verified by sequence analysis. Custom sequencing of five genes were performed with M13 forward and reverse primer (section 2.3.7). Sequences of these five regions are shown in Fig. 3.2. A sequence similarity search (blast) was performed by comparing the local isolates and those of other isolates of different regions, present in the NCBI Genbank Data Base, of the world. In the case of PVY, sequences of different strains from the different regions of the world were compared to the local isolates. The results showed that the sequences of viruses isolated from a local strain of Pakistan have a high level of identity to most of the sequences of viruses isolated in different regions of the world present in the data base. The highest sequence identities with different isolates are shown in Table. 3.1.
A.  
CTAGAGGCATACAGAAGCCAGGACCTATTCTGAGGGCACTTCTGAATTCTGCACACCGAGTGATCTTGTT
TGACAACACACACACACACACACCATACACGGCAGCATTGTGCTGAGGAGAGACCTGAGATCTGC
B.  
TCTGGAAGGACATGAAAGGTGGCCACACGACACTATGGCAAGGCTGCTTGGGACTTAGTCAGACACT
GTGCTGATGTAGACACTGCTGCTCGCTCAACCCACCAGTAGATACATACAGAGTGTGATCTAGAGTA
GCAGAGCTAGACCTGCCAGCAAAATTAAAGAGGTGTGGCACACTTGGGAATTTCGATGAAGTAT
GCCGCAAGGCTATGGAACTGGATGGTTAACTTAACCACTGCACCATCTGAAATTCTTGGGAAAGTGA
C.  
GCATAAAATATGCAAGAGATGGTTTTAAACCGATTGAGGGGCAGAAAGATCGCTTTGTGCTAGTGCA
AAAACTCATTGCAACATTTGTGCCACATCTGCGACTATTGT GGACTGAAATCGACATTCAATCT
D.  
CTCGGGCAACCTCAATCACAGTTTGACTGGGTATGAGCAGGATACAGATTGGCTTATGGTTTGGTG
E.  
GCCGCTCAAGAAGAACTGGAGTTCCCCGAGGACGAGGCTCAAGCAGACATTCGTGTTTACAAAG
GACAACTCCTATGGGCAACCTCAGAAGGTTCCTTTCCTCGGAGCCGAGTCTATCAGACTGTTGCC
CCATACAGTGAATACCTGAGGCAAGTTCCTCTGAGATCTGGAGGACTCCCCATGGAAAGTATCAT
CCCTCAGTCTACGTCAAAGCGTCCAAATTACGA

Figure: 3.2. Different genes sequences of PCR amplified fragment. A: NTP-binding helicase of PVX; B: Coat Protein of PVX; C: Helper Component Protease of PVY; D: Coat Protein of PVY; E: Coat Protein of PLRV.
Table: 3.1. Virus isolates from different countries to which local genes sequences has the highest level of nucleotide sequence identity.

<table>
<thead>
<tr>
<th>Virus gene</th>
<th>Highest nucleotide identity (%)/strain</th>
<th>Country/ accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC-Pro (PVY)</td>
<td>98 N China EU182576</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 N France NC001616</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 N Japan AB270705</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 NTN USA EF026075</td>
<td></td>
</tr>
<tr>
<td></td>
<td>92 NTN Canada AY166866</td>
<td></td>
</tr>
<tr>
<td></td>
<td>97 NTN Germany AJ889866</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82 O USA EF026074</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82 O China AY547324</td>
<td></td>
</tr>
<tr>
<td></td>
<td>84 C Germany AJ890348</td>
<td></td>
</tr>
<tr>
<td>CP (PVY)</td>
<td>99 N Japan AB185831</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99 N UK EU161658</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 N Germany AY319647</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 NTN Japan AB331550</td>
<td></td>
</tr>
<tr>
<td></td>
<td>93 O China AB451182</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94 O USA EF026076</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94 O India AY061994</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94 O UK AJ390292</td>
<td></td>
</tr>
<tr>
<td>NTP (PVX)</td>
<td>95 China EF423572</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 USA NC001455</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95 Japan AB056719</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98 UK M95516</td>
<td></td>
</tr>
<tr>
<td>CP (PVX)</td>
<td>97 China EF423572</td>
<td></td>
</tr>
<tr>
<td></td>
<td>97 Japan D87962</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96 Canada AF202462</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95 UK AF111193</td>
<td></td>
</tr>
<tr>
<td>CP (PLRV)</td>
<td>99 USA NC001747</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 France AF453394</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99 China EF063711</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99 India AF539791</td>
<td></td>
</tr>
</tbody>
</table>
3.1.3 Amplification and cloning of genes using anti-sense primers
The five anti-sense fragments were also amplified from the cloned fragment using the specific primers synthesized for anti-sense cloning. Five amplified DNA fragments were purified and individually ligated in the pTZ57R plasmid (section 2.3.8).

3.1.4 Combining of the genes fragments by fusion PCR
The five individually cloned genes fragments were combined in two different combinations by second round of PCR reactions. Each fragment was taken out by restricting individual plasmid with their specific enzymes. The restriction reactions were run on 1 % agarose gel and the exact size fragments were eluted from the gel. In the first combination the chimeric gene fragment designated as SN1 was obtained by fusing three fragments, NTP (300 bp) of PVX, HC-Pro (365 bp) of PVY and CP (300 bp) of PLRV, by ligation and subsequently amplified by PCR (Fig. 2.1). The resulted 965 bp fragment were purified and ligated to pTZ57R. The second combination of CP genes of PVX (375 bp), PVY (340 bp) and PLRV (300) results in the 1015 long chimeric gene fragment designated as SN2 (Fig. 2.2). The ligated fragment was amplified by PCR, purified, ligated in pTZ57R and transformed into *E. coli* strain DH5α through electroporation. About 20 trans-conjugants of each clone were grown individually. The plasmid were isolated and screened for the presence of clone by restricting with their specific enzyme i.e., SN1 by Xhol/KpnI, SN2 by EcoRI/KpnI, and resolved on 1 % agarose gel (section 2.4.3). A fragment of 965 bp confirms the SN1 and 1015 bp confirms the SN2 fragment insertion. The positive clones were also confirmed by PCR amplification (Fig. 3.3). Further verification was done by cutting each internal fragment with their specific enzymes.
3.1.5 Combining of the anti-sense fragments by fusion PCR

The five genes fragments amplified by using anti-sense primers were also combined by ligation and PCR amplification. The strategy used for the fusion of anti-sense fragments was same as used for sense fragments fusion described in Fig. 2.4. The fusions of NTP, HC-Pro and CP resulted in 965 bp AN1 fragment. In second combination CP genes of PVX, PVY and PLRV resulted in 1065 bp AN2 gene fragment. These fragments were cloned individually in pTZ57R (section 2.4.4).

3.1.6 Plant expression cassette construction in RNAi based vector pN6

Two plant expression cassettes were constructed in RNAi based vector pN6. For the construction of the expression cassette I the 965 bp SN1 gene fragment was excised out by restriction enzyme \textit{Xho I}, \textit{Kpn I} and ligated at the same \textit{Xho I}, \textit{Kpn I} site in pN6 vector (Fig. 2.1). In a second reaction the 1015 bp SN2 fragment was restricted with \textit{Kpn I}, \textit{EcoR I} enzymes and ligated at the same \textit{Kpn I}, \textit{EcoR I} site in pN6 downstream to CaMV 35S (Fig. 2.2). The ligation product was transformed separately into \textit{E.coli} through electroporation (section 2.5.1.3). The verification of recombinant pN6 clone was carried out by isolating the

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**Figure: 3.3.** PCR amplified products of SN1 and SN2 genes. Lane 1: 1kb DNA Ladder; Lane 2-4: SN1 gene fragment; Lane 3-4: SN2 gene fragment; Lane 8: Negative control.
plasmid of trans-conjugants colonies and digesting with *Xho* I, *Kpn* I for confirmation of SN1 fragment and with *Kpn* I, *EcoR* I for the confirmation of SN2 fragment. The clone having the exact size fragment was purified and used for anti-sense cloning.

### 3.1.7 Cloning of AN1 and AN2 fragments to complete expression cassette I and II

To complete the construction of expression cassette I, the AN1 965 bp anti-sense fragment cloned in the pTZ57R vector was restricted with *Cla* I, *BamH* I restriction enzymes and ligated in pN6 at the same site as an inverted repeat of SN1 fragment. Both the fragments SN1, AN1 were cloned between the CaMV 35S promoter and OCS terminator, separated by an untranslatable sequence intron, as shown in Fig. 2.1. The intron stabilizes the construct during cloning and enhances its effectiveness to induce hpRNA mediated silencing (Smith *et al.*, 2000). Similarly to complete the construction of cassette II, the 1015 bp anti-sense AN2 fragment was restricted with *Cla* I, *Hind* III from pTZ57R vector in separate restriction reaction and ligated in the pN6 vector as an inverted repeat to SN2 fragment downstream to the CaM 35S promoter (Fig. 2.2). The ligation product was transformed into *E.coli* through electroporation. The plasmid DNA was isolated from 15 trans-conjugant colonies and the individual clones were verified by restricting with *Cla* I, *BamH* I restriction enzymes for AN1 confirmation and by *Cla* I, *Hind* III for AN2 fragment confirmation (section 2.5.1.3). Fig. 3.4 shows the restriction pattern of 965 bp SN1 and AN1 genes fragments. Similarly the Fig. 3.5 shows the restriction pattern of 1015 bp SN2 and AN2 fragment cloned in pN6 vector as inverted repeat. Positive clone was marked and used for large plasmid preparation (section 2.5.1.4).
**Figure: 3.4.** Restriction pattern of the cloned chimeric SN1 gene of expression cassette I in RNAi vector pN6. Lane 1: 1kb DNA Ladder; Lane 2-4: SN1 gene fragment in pN6; Lane 5-7: AN1 gene fragment.

**Figure: 3.5.** Restriction pattern of the cloned chimeric SN2 gene of expression cassette II in pN6. Lane 1: 1kb DNA Ladder; Lane 2, 3: SN2 gene fragment; Lane 4, 5: AN2 gene fragment; Lane 6: Control plasmid.
3.1.8 Cloning of cassette I and II into a plant binary vector pGreen

The plant expression cassette I of about 4.96 kb, carrying partial fragments of NTP, HC-Pro and CP cloned in sense and antisense orientation between CaMV 35S promoter and OCS terminator (Fig. 2.1) were restricted with Not I restriction enzyme, purified and placed into pGreen at the same Not I site (Fig. 2.4). The ligation product was transformed into E.coli strain and 30 trans-conjugant colonies were screened for the presence of SN1 and AN1 gene fragments. The isolated plasmid was digested with Xho I, Kpn I and Cla I, BamH I restriction enzymes to verify the presence of SN1 and AN1 fragments respectively (section 2.6). Further the chimeric genes SN1 and AN1 were also verified by PCR and the Fig. 3.6 shows the PCR amplified pattern of the cloned gene fragments of cassette I in plant binary vector pGreen. Similarly the plant expression cassette II of about 5.0 kb were restricted with Not I (Fig. 2.2) and sub-cloned in the pGreen at the same Not I site (Fig. 2.5) as described for the cloning of expression cassette I. The cloned SN2 and AN2 fragments were verified by restricting with Kpn I, EcoR I and Cla I, Hind III respectively. The PCR amplified pattern of the cloned fragments of expression cassette II in pGreen is shown in Fig. 3.7. The positive clones were identified on the basis of fragments size released and used for the large plasmids preparation.
Figure: 3.6. PCR amplified fragments of chimeric gene SN1 and AN1 of expression cassette I cloned in binary vector pGreen plasmid containing expression cassette I; Lane 1: 1kb DNA Ladder; Lane 2-4: SN1 gene product; Lane 5, 6: AN1 gene products; Lane 7: Negative control; Lane 8: Positive control.

Figure: 3.7. PCR amplified fragments of chimeric gene SN2 and AN2 of expression cassette I cloned in binary vector pGreen plasmid containing expression cassette. Lane 1: 1kb DNA Ladder; Lane 2-7: SN2 gene product; Lane 8, 13: AN2 gene products; Lane 14: Negative control; Lane 15: Positive control.
3.1.9 Transformation of recombinant pGreen plasmid into \textit{Agrobacterium tumefaciens} strain LBA 4404

The pGreen plasmids carrying hairpin RNA (hpRNA) constructs were transformed into \textit{Agrobacterium tumefaciens} strain LBA4404 with the help of helper plasmid pSoup through electroporation. The diluted cells of each construct were spread on LB plates and incubated for 48 hour at 28 °C (section 2.8). Six colonies of trans-conjugants from each construct were grown overnight individually in LB liquid medium containing antibiotic tetracycline, kanamycine and streptomycine. Screening of cultures for the presence of recombinant plasmid was done through PCR (section 2.10.1). PCR amplification of the specific genes as well as nptII was performed for verification of the clone in \textit{Agrobacterium}. The correct and precise amplified product of SN1 and AN1 genes from \textit{Agrobacterium tumefaciens} strain LBA4404 are shown in Fig. 3.8. Similarly the genes of second construct SN2 and AN2 were confirmed by PCR as shown in Fig. 3.9.
Figure: 3.8. Confirmation of pGreen plasmid containing expression cassette I in *Agrobacterium tumefaciens* strain 4404. Lane 1: 1kb DNA Ladder; Lane 2-6: SN1 gene products; Lane 7-11: AN1 gene products; Lane 12: Negative control; Lane 13: Positive control.

Figure: 3.9. Confirmation of pGreen plasmid containing expression cassette II in *Agrobacterium tumefaciens* strain 4404. Lane 1: 1kb DNA Ladder; Lane 2-6: SN2 gene products; Lane 7-11: AN2 gene products; Lane 6: Negative control; Lane 7: Positive control.
3.2 Plant transformation

3.2.1 Transformation of *Solanum tuberosum* cv Desiree and Kuroda

Independent transformation experiments were carried out in four batches to introduce the hp-RNA constructs into potato cv Desiree and Kuroda through *Agrobacterium* (section 2.9.2). In each batch, 20 explants (stem cuttings) of each cultivar were agro-inoculated. The untransformed explants of potato were also included as control. The explants were transformed to selection medium CIM (Table 2.2) after 3 days of co-cultivation on CIM medium without antibiotics. Calluses were induced in both transformed and control explants after a week but with the passage of time the control untransformed callus turned black or died while the transformed callus remains greenish indicating the successful integration of transformation events (Fig. 3.8 C, D). The well developed callus transformed to shooting medium SIM selective medium (Table 2.2). After 4 weeks of culture on SIM, green buds were obtained and produced well defined shoots (Fig. 3.10 E). These buds were excised after attaining the length of 2 to 3 cm and transformed to large test tubes containing RIM selective medium (Table 2.2) for the development of roots (Fig. 3.10 H). The regenerated plantlets developed roots on the selective medium was used as the first indicator of the transgenic nature of regenerated plantlets. Since untransformed control plants were unable to generate roots on the same selective media. Multiplication of rooted plants were carried out by cutting auxiliary buds and cultured on MS selective rooting medium. The observed transformation efficiency was about 50 % (Table 3.2). About 20 independent transgenic plant lines of each construct and cultivar were produced in 4 to 6 months from four independent experiments in which phenotypically well performed 14 lines from each construct were selected for viral assay experiments in greenhouse. The plants were transferred to plastic pots and initially kept covered under plastic bags for retaining humidity. The bags were removed after a week to acclimatize them to the ambient temperature. Finally the plants were transferred to soil in large pots and shifted to containment for getting T₀ progeny plants. Transgenic plants produced were normal in their phenotype in every aspect. Correlating with the normal pattern of growth, there was no decrease in stem and leaves development and was indistinguishable in appearance from control potato plants.
Results

Figure: 3.10. **Transformation and regeneration of potato cv Desiree**; A: Inoculated stem cuttings cultured on callus induction medium; B: Callus formation (arrows) of the transformant cells on the selective callusing medium while the non-transformant explants become dead after 2-weeks; C: Control callus on selection medium after 3 -4 weeks; D: Transformed callus on selection medium after 3-4 weeks; E: Bud formation (arrows) (on shoot inducing selection medium); F: Well developed shoots (on shoot inducing selection medium); G: Shoot cuttings on RIM; H: Rooting (arrows) of a transgenic bud on RIM; I, J: Transgenic plants in growth chamber and tunnel for bio-assay.
Table of regeneration and transformation efficiency
3.3 Molecular analysis of transgenic plants

3.3.1 Detection of transgenes in transgenic plants by polymerase chain reaction

Genomic DNA was isolated from the leaves of putative transgenic potato plants (section 2.10.1). The presence of transgene of expression cassette I and II were determined by amplification of SN1 and SN1 fragments respectively by PCR amplification using their specific primers (section 2.13.2). The \textit{npt II} gene was also determined in the transgenic plant by PCR amplification by using \textit{npt II} specific forward and reverse primers. The Fig. 3.12 shows the PCR amplified products representative of SN1 (965 bp) genes product in transgenic plants. Similarly the Fig. 3.13 shows the PCR amplified fragments of SN2 (1015 bp) in transgenic plants. The 730 bp amplified fragments of \textit{npt II} gene representing the presence of transgene in transgenic plants in Fig. 3.11. The amplification of desired size products confirmed the successful transformation of transgene while no amplification was detected in the untransformed and negative controls.
Figure: 3.11. PCR product of npt II T₀ representative transgenic line of expression cassette I. Lane 1: 1kb DNA Ladder; Lane 2-7: npt II gene product of cv Desiree; Lane 8-13: npt II gene product from cv Kuroda; Lane 14: Negative control; Lane 15: Positive plasmid control.

Figure: 3.12. PCR product of SN1 genes in T₀ representative transgenic lines of expression cassette I. Lane 1: 1kb DNA Ladder; Lane 2-6: SN1 gene product in transgenic lines of cv Desiree; Lane 7-11: SN1 gene products in transgenic lines of cv Kuroda; Lane 12: Untransformed control plant; Lane 13: Positive control (Plasmid DNA).
Figure: 3.13. PCR product of SN2 genes of T₀ representative transgenic lines of expression cassette II. Lane1: 1kb DNA Ladder; Lane 2-6: SN2 gene product in transgenic lines cv Desiree; Lane 7-11: SN2 gene products in transgenic lines cv Kuroda; Lane 6: Untransformed control plant; Lane 7: Positive control (Plasmid DNA).

Figure: 3.14. PCR product of npt II T₀ representative transgenic line of expression cassette II. Lane1: 1kb DNA Ladder; Lane 2-7: npt II gene product of cv Desiree; Lane 8-12: npt II gene product from cv Kuroda; Lane 13: Negative control; Lane 14: Positive plasmid control.
3.3.2 Genomic Southern of transgenic potato lines for the estimation of copy number per genome

Southern analysis of the transgenic plants DNA was carried out in order to determine the copy number per genome. About 10 µg of total genomic DNA of the selected T₀ transgenic lines of expression cassette I (LD3, LD4, LD6, LK3, LK4, LK7) and expression cassette II (LD9, LD10, LD13, LK12, LK13, LK14) were digested with *Hind*III, size fractionated on 1 % agarose gel, transferred to nylon membrane and hybridized with random primed biotin labeled PCR amplified fragments of SN1 and SN2 genes respectively (section 2.5.1). Fig. 3.14 and 3.15 show the results of Southern analysis of representative transgenic lines of potato showing the integration of copy number per genome of transgenes while no hybridization was observed in untransformed control lines. The analysis reveals that all transgenic lines have low copy number (1 or 2) of integrated transgenes.
Figure: 3.15. *Hind* III-digested genomic Southern of representative transgenic Potato lines of expression cassette I. Equal amount of DNA (10 µg) were resolved on 0.8% agarose gel and blotted to nylone membrane and hybridized with PCR amplified SN1 fragment. WT is a wild type untransformed DNA; LD3, LD4, LD6 are transgenic lines of cv Desiree; LK3, LK4, LK7 are transgenic lines of cv Kuroda; M is control plasmid.

Figure: 3.16. *Hind* III-digested genomic Southern of representative transgenic Potato lines of expression cassette II. Equal amount of DNA (10 µg) were run on gel and transferred to nylon membrane. The membrane was hybridized with PCR amplified SN2 fragment. M is positive plasmid control; WT is untransformed wild type plant DNA; LD 9, LD10, LD13 are transgenic lines of cv Desiree; LK12, LK13, LK14 are transgenic lines of cv Kuroda.
3.4 In vitro assay of virus resistance

The T₀ generation transgenic lines of expression cassette I (14 lines) and II (14 lines) were challenged by PVX, PVY and PLRV through mechanical inoculation and viruliferous aphids respectively. The plant showed variable degrees of resistance/tolerance/susceptibility against the inoculated viruses compared to the non-transgenic control plants. On the basis of visible symptoms they were categorized in three groups: susceptible (early symptoms), tolerant (delayed symptoms) and resistant (no symptoms). Out of 14 lines of expression cassette I (LD1, LD2, LD3, LD4, LD5, LD6, LD7, LK1, LK2, LK3, LK4, LK5, LK6, LK7), 6 (42.8 %) (LD2, LD3, LD5, LK1, LK5, LK6) lines showed early symptoms within 15 days post inoculation (dpi), 6 (42.8 %) (LD1, LD4, LD7, LK2, LK4, LK7) lines showed delayed symptoms (30-35 dpi) and the remaining 2 (14.2 %) (LD6, LK3) lines were symptomless even after 60 dpi. In the expression cassette II out of 14 lines (LD8, LD9, LD10, LD11, LD12, LD13, LD14, LK8, LK9, LK10, LK11, LK12, LK13, LK14), 4 (28.5 %) (LD11, LD12, LK8, LK10) lines showed early symptoms, 7 (50 %) (LD8, LD10, LD13, LD14, LK9, LK11, LK12) lines showed delayed symptoms and 3 (21.4 %) (LD9, LK13, LK14) lines remained without symptoms until the maturity stage. The untransformed control plants 13/14 (92.3 %) showed severe symptoms within 14-20 dpi, while 1/14 (16.6 %) plant showed delayed symptoms.
Figure: 3.17. Symptoms development in control and transgenic lines after 20 days post inoculation. A: transgenic resistance line LK3 which shows no symptoms even after 30 dpi. B: non transgenic control plants showed symptoms after 15 dpi. C: transgenic lines LD3 showed delayed symptoms after 22 dpi. D: transgenic susceptible line LK1 showed early symptoms after 15 dpi.
Table: 3.2. Statistical analysis of phenotypes data of transgenic potato lines of expression cassette I

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LD: Desiree line
LK: Kuroda line
WT: Control lines
Figure: 3.18. Box plot presentation of virus resistance data of transgenic lines of expression cassette I.

These lines were inoculated separately with PVX, PVY, PLRV and also with the MIX inoculums of these three viruses. Resistance data was calculated 35 days post inoculation. Different transgenic and non-transgenic wild type (WT1, WT2) lines are shown at x-axis and their percentage (% is the mean of three readings) resistance on y-axis.
Table: 3.3. Statistical analysis of phenotypes data of transgenic potato lines of expression cassette II

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LD: Desiree line
LK: Kuroda line
WT: Control lines
Figure: 3.19. Box plot presentation of virus resistance data of transgenic lines of expression cassett II. These lines were inoculated separately with PVX, PVY, PLRV and also with the MIX inoculums of these three viruses. Resistance data was calculated 35 days post inoculation. Different transgenic and non-transgenic wild type (C1, C2) lines are shown at x-axis and their percentage (% is the mean of three readings) resistance on y-axis.
3.5 Detection of virus through DAS-ELISA

To exclude symptomless infection the presence of virus was evaluated by DAS-ELISA in 5 resistance (LD6, LD4 from construct I and LK3, LK13, LK14 from construct II) and one susceptible line (LD3), using four times the background value as a cut-off point. The ELISA readings confirmed the phenotypic symptoms and showed that virus accumulation in systemically infected leaves of these resistance lines was inhibited at 15 (Fig. 3.15), 30 (Fig. 3.16) and 50 (Fig. 3.17) days post inoculation (dpi) as compared to the susceptible line LD3 and non-transformed (NT). Amongst these resistance lines, the two lines (LD6, LK14) showed very lower viral accumulation as an indicator of high resistance (Fig 3.15, 3.16 and 3.17).

![Figure: 3.20. Elisa plate readings (O.D at 405 nm) of leaf samples of susceptible (LD 3) and resistant (LD 6, LK 3, LD 4, LK 4, LK 7) lines 15 days post inoculation (DPI). NT: non-transgenic control plant. + Ve: positive control. PVX: potato virus X. PVY: potato virus Y. PLRV: potato leaf roll virus.]
Figure: 3.21. Elisa plate readings (O.D at 405 nm) of leaf samples of susceptible (LD 3) and resistant (LD 6, LD 4, LK 3, LK 4, LK 7) lines 30 days post inoculation (DPI). NT: non-transgenic control plant. +Ve: positive control. PVX: potato virus X. PVY: potato virus Y. PLRV: potato leaf roll virus.

Figure: 3.22. Elisa plate readings (O.D at 405 nm) of leaf samples of susceptible (LD 3) and resistant (LD 6, LK 3, LD 4, LK 13, LK 14) lines 50 days post inoculation (DPI). NT: non-transgenic control plant. +Ve: positive control readings. PVX: potato virus X. PVY: potato virus Y. PLRV: potato leaf roll virus.
3.6 Detection of small interfering (si) RNAs

After the transcription and pre-mRNA processing of the inverted repeat transgene, hairpin structures were expected to form and processes into small interfering RNA (siRNA) by the DICER activity. To confirm that the observed resistance was due to RNA silencing and correlate with the abundance of transgene-specific siRNAs, RNA fractions derived from the three segments of the transgene cassettes were examined in one transgenic susceptible (LD 3), one non-transgenic (NT) and 4 transgenic resistance lines (LD 6, LD4, LD3, LK 3, LK4) of expression cassette I. To this end, total nucleic acids of these transgenic lines were extracted prior to virus inoculation and enriched for small RNAs. Twenty micrograms of total small RNAs from each sample were separated on 15 % poly acrylamide gel (PAGE) and analyzed by Northern blotting using specific double-stranded DNA probes, labeled with digoxigenin (Roch), from each of the three fragments of expression cassette I (HC-Pro, NTP, CP). Double-stranded RNA expressing transgenic potato plants contain greatly varying amounts of siRNA and could readily be detected, but exclusively in virus resistance transgenic lines (Fig. 3.18). However, there is no accumulation of siRNA detected in non-transgenic control and susceptible transgenic lines even the transgene presence was confirmed by PCR in the susceptible lines.
Figure: 3.23. siRNA analysis of three transgenic non-inoculated lines. Enriched siRNA were analyzed with three different probes of cassette I. LD 3, LD4, LD6: Expression cassette I resistant lines of cv Desiree. LK 3, LK 4, LK7: Expression cassette I lines of cv Kuroda. LD 3: susceptible line of Desiree. The control was total siRNA from non-transgenic, non-infected potato plant.
Discussion

Significant advances have been made in our understanding of the plant-virus interaction and the plant defense mechanisms against different invading viruses, over the recent years. Phytopathogenic viruses are significant cause of increasing threats to the stable agricultural production around over the world. However, high losses caused tremendous economic disturbance in the tropical and subtropical regions. The fact is that, diversity and the incidence of plant-infecting viruses, which are higher in these regions, are due to favorable environmental conditions for the vectors of the viruses. The chemicals for controlling virus vectors are very expensive as compared to poor economic condition of farmers.

About more than 80% of plant-infecting viruses are RNA viruses and unfortunately Pakistan, with all other countries of Southern Asia, is home to members of many taxonomic groups of plant-infecting viruses (Verma et al., 2004; Ali et al., 2004; Naidu et al., 1998; Mandal et al., 2004; Lauren et al., 2006; Amin et al., 2007) which is one of the reasons in low agriculture productivity in the country.

The development of resistant varieties can provide a long-term approach for the control of many plant virus diseases. However, for many commercial crop varieties, very few sources of natural resistance genes have been identified or resistance may be overcome by new emerging virus strains. Genetic engineering has the potential to provide increased and durable resistance to plant viruses using transgenic approaches which are a major focus in plant virus research. Pathogen-derived resistance (PDR), initially proposed by Sanford and Johnston (1985), is a type of transgenic resistance in which plant express certain virus genes or part of genes against which the resistance is desired (Beachy, 1997; Prins, 2003). PDR is mediated either by the transgene protein (protein mediated resistance) or by the transgene RNA (RNA mediated resistance). A most accepted model for the protein-mediated resistance, suggests
that the transgenically expressed protein prevents the virions from disassembly, which is an important event of infection (Wu et al., 1990). RNA mediated resistance or homology sensing resistance mechanism not required the expression of transgene protein. It is an evolutionary conserved surveillance process that occurs in almost all eukaryotic organisms. It is well known that post-transcriptional gene silencing (PTGS) or RNA interference in which an RNA dependent RNA polymerase (RdRp) produces double-stranded RNAs (dsRNA) from an mRNA template. These dsRNAs are processed by RNAs III like enzyme DICER into 21-25 nucleotide long short interfering RNA (siRNA) that directs RNAses to target homologous transgenes and viral RNAs (Berstein et al., 2001; Hamilton and Baulcombe 1999; Lipardi et al., 2001; Cerutti 2003; Tang et al., 2003). The siRNAs also have a role in promoting transcriptional gene silencing (TGS), by promoter methylation (Matzke et al., 2001; Sijen et al., 2001; Mette et al., 2001; Beclin et al., 2002; Cerutti, 2003).

The work presented here, deals with the stable transformation of potato, an economically very important crop that is severely affected by numerouse viruses, with single construct which could induce a broad-spectrum resistance against three important viruses, potato virus X (PVX), potato virus Y (PVY) and potato leaf roll virus (PLRV), of potato. This phenomenon of broad-spectrum resistance is based on using a single transgene construct of multiple virus sequences to obtain high frequency RNA silencing simultaneously, against multiple viruses. The pathogen-derived resistance strategy was utilized to develop multiple genes hairpin RNA (hpRNA) constructs and transformed into potato. This silencing is operated by sequence-specific RNA degradation, a particularly effective method of silencing an endogenous gene in plants. In this case the plants were transformed with genes constructs encoding an hpRNA, consisting of an inverted repeats of fragments of different genes sequences separated by spacer (intron) to allow easier hairpin loop construction after transcription. The frequency of silencing is increased when the spacer of hpRNA is replaced by an intron (Smith et al., 2000; Wesley et al., 2001). It has been shown in the number of experiments that the expression of virus-derived dsRNA from transgenes can efficiently suppress the viral infection through RNA silencing (Smith et al., 2000; Kalantidis et al., 2002; Wang et al., 2000). Engineering resistance through RNA silencing has been becoming a method of choice due to several
reasons. The induced resistance is very effective and in most of the cases attained immunity (Kalantidis et al., 2002; Schul et al., 2006). Although the frequency of resistance is variable amongst the transgenic lines, a high proportion of them are strongly resistance or immune.

Previously in most of the reports, potato was engineered for inducing resistance against single or double viruses (Kaniewski et al., 1990; Lawson et al., 2001; Thomas et al., 2000; Thomas et al., 2004; Missiou et al., 2004). In the work presented here, the multiple viruses were targeted and showed that by using triple partial genes hairpin RNA constructs, maximum resistance to viruses of most of the transformed lines was achieved by multiple gene knock-downs through homology dependent RNA silencing mechanism. It is demonstrated by showing that the plants became resistant to three different viruses at once, which was also observed by the detection of siRNA originating from each segment of the cassette. It has been shown earlier that the virus resistance has usually been correlated with the detection of specific siRNA in transgenic plant lines expressing an hpRNA construct (Kalantidis et al., 2002).

Five important genes, NTP and CP genes of PVX, HC-Pro and CP genes of PVY and CP gene of PLRV, were selected as potential RNA silencing target, based on the following considerations.

These genes play very important role in replication, cell-to-cell and long distant movement, aphid transmission, suppressor of host gene silencing and infectivity in plant cell replication. Disruption or altered expression of these genes can inhibit viral RNA replication and dysfunction pathogenicity. The cDNA of each gene fragment was amplified and cloned individually in PCR cloning vector. Custom sequencing of the five cloned gene fragments were performed and amino acid sequences were compared with the sequences of viruses isolated from different regions of the world, present in the NCBI Genbank Data Base. The highest sequence similarities were found with different isolates (Table 3.1). Ghosh et al. (2002) cloned and sequenced the CP gene of PVY from an Indian isolate. Coat protein gene
of an Indian PLRV isolate was sequenced and its amino acids sequence was compared with other member of *Luteoviruses* (Mukherjee *et al.*, 2003).

Two constructs of three genes, each were proposed here in which all the three genes act together or independently to resist the replication of the three viruses. The targeted cDNA amplified regions of NTP (PVX), HC-Pro (PVY) and CP (PLRV) were combined through fusion PCR to form a single N gene and cloned in an RNAi vector as inverted repeat. Similarly the cDNA amplified regions of three CP sequences of PVX, PVY and PLRV were fused to form a single gene and cloned in sense and anti-sense orientation in RNAi vector under the control of enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter. Bucher *et al.* (2006) used the same procedure and combined four different genes fragments of *Tospoviruses* through fusion PCR under CaMV 35S promoter. The purpose of the development and validation of two vectors containing multiple genes is to maximize the efficiency and versatility of the vector based gene silencing approach. This approach is considered to be an important step forward in the development of more efficient and broad virus resistance in crop plants, based on RNA silencing.

The virus induced gene silencing strategy using CP sequences in sense and anti-sense orientation as inverted repeat has been used in many crops against different viruses. Missiou *et al.* (2004) introduced CP gene of PVY in potato as inverted repeat under the CaMV 35S promoter and separated by intron. Park *et al.* (2005) reported the induced resistance in watermelon against cucumber green mottle mosaic virus (CGMMV). They introduced cDNA of CP gene of CGMMV under the control of CaMV 35S promoter as an inverted repeat. Although the detailed silencing mechanism was uncertain in their transgenic plants, their strategy based on the concept of PDR, was considered to induce RNA silencing through the formation of dsRNA (Lindbo and Dougherty 2005). Kamachi *et al.* (2007) also developed *N. benthamiana* lines, containing a transgene that encoded for dsRNA, derived from a CGMMV-CP sequence, highly resistance to CGMMV. CP-specific siRNA detection in the resistance lines suggests that dsRNA expression of the transgene induced the virus-specific RNA silencing.
From the previous research work it is obvious that the transgenic plants expressing the CP of PVX were highly resistant to PVX and did not show disease symptoms (Hemenway et al., 1988; Spillane et al., 1997). It is also known that the plants doubly infected with PVX and PVY develop a synergistic disease symptoms that is characterized by sever vein-clearing followed by necrosis of the first systemic leaf, accompanied by 3 to 10 fold accumulation of PVX as compared to plants infected with only PVX (Pruss et al., 1997). However, transgenic plants expressing CP of PVX did not show severe disease symptoms when infected with both viruses (Bazzini et al., 2006).

The HC-Pro gene is known as suppressor of RNA silencing, and play important role in viral pathogenesis. Kasschau and Carrington, (1998) has motivated us to illustrate the efficiency of hpRNA-mediated virus-specific resistance strategy by targeting this suppressor-encoding sequences of PVY. Previously Nicola-Negri et al. (2005) introduced the 35S promoter driven P1/HC-Pro hairpin sequence of plum pox virus in N. benthamiana plants showed more than 90 % resistance to PPV infection.

The reduction in PLRV titer for a transgene CP that is expressed only at the RNA level also has been recorded in our transgenic potato plants. Similar results of reduced PLRV accumulation in transgenic potato were obtained by expression CP RNA of PLRV (van der wilk et al., 1991). Palucha et al. (1998) reported that the strategy of anti-sense cloning of CP of PLRV seems to be more effective (Palucha et al., 1998). However, induced resistance to PLRV is confirmed equally, efficiently by both sense and anti-sense RNA expression of the CP gene (Kawchuk et al., 1991). On the other hand introducing inverted repeat transgenes, resulting in double-stranded RNA construct, instead of using originally (single) sense or anti-sense transgene constructs displayed more efficient higher frequency gene knock-down (Waterhouse and Helliwell 2003). Therefore, our strategy of inducing RNA silencing by cloning the gene fragments as inverted repeat in sense and anti-sense orientation was very successful.
Agrobacterium mediated transformation of potato using leave discs and potato tuber discs are the most common approaches for the development of transgenic plants resistance, to herbicides insect and diseases (De Block, 1988; Imai et al., 1993; Benchekroun et al., 1995). However, all these procedures have limitations, such as low frequency of transformation, and more importantly, the high rate of somaclonal variation occurrence (mainly due to changes in ploidy level) mainly due to harsh and lengthy tissue culture (Ooms et al., 1987; Ishida et al., 1989). An improved and efficient method of genetic transformation of two commercially important potato cultivars Desiree and Kuroda, using zeatin riboside (ZR) and longitudinally cut internodal explants, have been used. Desiree is the most frequently used cultivar for transformation procedure due to its high response to in vitro regeneration (Beaujean et al., 1998). The culture media was supplemented with ZR as a cytokinin which reduces the duration of culture on callus-inducing medium and initiate a large number of buds quickly before the induction of somaclonal variation (Beujean et al., 1998). More than 53 % transformation efficiency was obtained in both Desiree and Kuroda by using internodal explants in these varieties. Moreover, the efficiency of this protocol is likely to be independent of the variety used as nearly similar rate of regeneration of transgenic plants has been observed in both varieties. Newell et al. (1991) generated Russet Burbank plants expressing the CP gene of PVX and PVY in a transformation system with low efficiency. On average 0.1-0.3 shoots per explant were obtained as compared to our results of 2-4 regenerated shoots per explant.

Regenerated plants of each variety and construct were selected for molecular analysis. Phonotypically normal lines of T_0-generation of each construct (14 lines each) were selected for molecular analyses and in vitro viral assays. The regenerated plants were transferred to containment after initial analysis through PCR, Southern and siRNA detection to check the integration, copy number and expression of transgenes in these plants. PCR results indicated that almost all independent lines contained both specific and npt II genes. The transgene analysis using PCR is a routine practice and has been done previously by the number of scientists (Bazzini et al., 2006; Nicola-Negri et al., 2005; Missiou et al., 2004; Thomas et al., 2004).
The Southern analysis of the selected transgenic lines demonstrated that all the lines have low copies of transgene inserted (Fig. 3.12). Waterhouse *et al.* (1998); Ingelbresht *et al.* (1999) reported the association of the transgenic resistance with high transgene copy number and low steady state expression level of the transgene, while susceptibility correlates with the low copy number and high steady state transcription level. In our experiment the resistant did not correlate directly with the copy numbers as the resistant lines had different copy number, whereas some lines had the same copy number but different resistance phenotypes. These results are in agreement with the other results which showed that in most of the cases the frequency of resistance shown by a particular transgenic line is independent of the number of transgene copy inserted in that particular line and a single copy is sufficient to confer resistance (Missiou *et al.,* 2004; Smith *et al.,* 1995). Rovere *et al.* (2001) also have reported the similar findings. Therefore, no conclusion can be made, whether the presence of more than one copy of the transgene would increase or decrease the occurrence of resistance.

Out of 6 transgenic lines analyzed, only two did not produced detectible level of siRNAs, though the presence of transgene was confirmed by PCR and Southern. The remaining 4 transgenic potato lines produced comparable levels of siRNA. These differences in the level of siRNAs biosynthesis could be due to the integration of the transgene during transformation at different chromosomal loci which significantly influenced the level of its expression due to position effects and gene silencing (Taylor *et al.,* 2004) or due to the differences in the plant species (tetraploidy verses allotetraploidy). In addition, the reduced growth rate of potato compared with the fast growing crops like tobacco may also affect the siRNA generation.

Transgenic potato lines were mechanically inoculated and exposed simultaneously to viruliferous aphids for assessing virus resistance. It is well known that the success of artificial virus inoculations is variable and the viruses may not be transferred with 100 % efficiency. Considering the number of replicates in each line that have been assayed and the success in infecting the un-transformed control as well as most of the susceptible transgenic lines, we believe that the line found as resistant are probably to be truly resistance. The degree of resistance expressed in our different transformant lines ranged from near immunity to full
susceptibility. In comparison with control, 6 lines out of 14 transgenic lines of expression cassette I showed delayed symptoms development and 2 lines showed resistance while the rest 6 lines are susceptible and showed symptoms with in 10 to 15 days post inoculation. Similarly out of 14 transgenic lines of expression cassette II, 4 lines showed early symptoms, 7 lines showed delayed symptoms development and the three were symptomless until the flowering stage. Similar results of variable range of resistance exhibited by transformants have been reported by many scientists previously. Schubert et al. (2004) reported that transgenic potato plants expressing truncated NIb gene of PVY showed either recovery type or extreme resistance against PVY. Thomas et al. (2000) expressed a truncated replicase gene of PLRV in potato and reported susceptible to extreme resistance in transgenic plants against PLRV. Nicola-Negri et al. (2005) also observed variable degree of local and systemic resistance when he expressed Plum pox virus P1/HC-Pro genes in N. benthamiana as inverted repeat hairpin RNA and challenged the transgenic plants with PPV. Missiou et al. (2004) expressed double-stranded RNA derived from the 3’ terminal part of the CP gene of PVY in transgenic potato and reported that the lines generating transgene-derived short interfering RNAs were highly resistance, while other are susceptible, to all strains of PVY. The phenomenon of lower resistance in transgenic plants induced by inwardly oriented hairpin construct was also observed by Chen et al. (2004).

The mechanism of observed variable resistance operates in transformants of our experiments may be due to many reasons like; (i) it is generally recognized that not all siRNA species are equally effective against a given mRNA and due to extensive positional effects along the mRNA, some siRNA shows limited efficiency, for example, secondary structure (Overhoff et al., 2005); (ii) the accumulation of siRNAs was known to inhibit by low temperature and both virus and transgene-triggered RNA silencing become attenuated (Szittya et al., 2003). It is, therefore, not surprising that siRNA-mediated resistance break down at low temperature (Szittya et al., 2003); (iii) all the plants may not be homogenous in their transgenic content, and may not represent the same behavior or the resistance in some lines may be in a fragile balance and overcome sometimes by virus due to variation in inoculum doze; (iv) one possible mechanism of low resistance in some lines is that ribosome scanning or shunting of
the sense RNA prevents proper folding of the RNA into perfect dsRNA and thereby suppresses effective dicer function (Bucher et al., 2006).

It is very uncommon under field conditions that multiple virus infections occur simultaneously, which frequently results in synergistic effects, i.e. stronger disease symptoms. This phenomenon of viruses can be at least in part explained by the simultaneous presence of suppressor of silencing of more than one virus. This is very common in PVX and PVY as they carry the silencing suppressor p25 and HC-Pro respectively (Brigneti et al., 1998; Davies et al., 1993; Voinnet et al., 2000). We have not study the synergistic effect of PVX and PVY in our transgenic potato but the possibility that they play a role in the suppression of RNA silencing in the transgenic plants can not be ruled out in this study. Resistance to other strains of PVY has not been studies. Since the majority of PVY strain found in the database have sequence homology greater than 90 % within the part of the PVY sequences used in our studies. So the range of resistance of the transgenic plants presented here is likely to be very high.

A number of environmental concerns have been raised regarding the large scale use of transgenic virus resistance crops (Tepfer, 2002) regardless of whether the resistance is mediated by RNA or protein. The potential risks related with the virus resistance include: (i) recombination may occur between the virus-derived transgene and non-target viruses; (ii) possible synergy with unrelated viruses; (iii) the transmission of unrelated viruses may be possible through heterologous encapsidation or enhanced pollen or seeds transmission; (iv) production of new allergens or toxic proteins may be possible in transgenic plants; (v) possible horizontal gene transfer from transgenic pollen to weedy relatives. With the exception of v, these potential risks can be minimized by using hpRNA-mediated approach because the transgenic viral sequence is not translated and the transgenic viral transcript is almost undetectable, probably, because it gets cleaved rapidly in short fragments. Moreover, potato cultivars may have male sterility, incapability in pollen production, premature flower abortion, infertility, or self-fertility that reduces the chances of horizontal gene transfer. Overall, the work presented here demonstrates simple procedure to obtain a broad virus resistance by RNA silencing in potato, using a single transgene construct of multiple viruses.
The broadness of resistance can be extended by adding more viral sequences to the transgene construct. Due to high efficiency of this multiple virus resistance, this approach can be applied to several other susceptible plant species to protect them from multiple viruses. Some other advantages of this method are; the cloned cDNA copies of genes can be obtained with relative ease, the strategy allow the knocking-down of several host components and the strength can be increased. Moreover, the analysis of transgenic plants that knock-down the different stages in the infection process is simplified because the knock-down is conferred by the known sequences, in comparison with the naturally existing types of resistance resulted in decreased cost and save time.
References


Bazzini, A. A., Asurmendi, S., Hopp, H. E. and Beachy, R. N. 2006. Tobacco mosaic virus (TMV) and potato virus X (PVX) coat proteins confer heterologous interference to PVX and TMV infection, respectively. Journal of General Virology. 87: 1005–1012.


References


References


References


References


