Artificial microRNA mediated transgenic resistance against *Sugarcane mosaic virus* (SCMV) in *Nicotiana benthamiana*

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ABSTRACT

Sugarcane (*Saccharum* spp.) is a major sugar crop in Pakistan, cultivated on about 1.3 million hectares. Mosaic (caused by *Sugarcane mosaic virus* (SCMV)) is amongst the major diseases of sugarcane in the country, a decline due to mosaic was recorded in cane yield from 10-32% and in sugar yield from 6-10%. Developing transgenic resistance in monocotyledonous crops against pathogens remains a challenging area of research. The objectives of present study were: to determine the virus isolate from local sugarcane varieties; and to analyze transgenic expression of artificial microRNA (amiRNA) and hairpin RNA (hpRNA), targeting simultaneously *CP* (Coat Protein) and *Hc-Pro* (Helper Component-Proteinase) genes of SCMV, in *Nicotiana tobacum* and rice plant. Out of thirty-two symptomatic sugarcane leaf sample, collected from Punjab and Khyberpakhtunkhwa (KPK) provinces, Pakistan during 2013-2014, twenty samples were PCR amplified using CP gene universal primer pairs. These products were cloned and sequenced in both directions. These nucleotide sequences shared highest nucleotide sequence identity between 85-100% nt identity to an isolate of SCMV.

Conserved nucleotide sequences, exclusive for DAG and KITC motifs, derived from SCMV *CP* and *Hc-Pro* genes were used to develop amiRNA constructs (CP-amiR and Hc-Pro-amiR). These conserved nucleotide sequences were also fused together and assembled into the hpRNA cassette under maize ubiquitin and 35S promoters, to form hpRNA constructs (Ubi-hpCP:Hc-Pro and 35S-hpCP:Hc-Pro). The 35S-GUS:CP:Hc-Pro served as a target reporter gene construct. amiRNA constructs were mobilized into *Nicotiana tobacum* through Agrobacterium mediated transformation. Only two transgenic lines (CP-amiR-15 and Hc-Pro-amiR 7) showed highest gene expression (about 0.08% and 0.27%, respectively) amongst the other expressing lines. hpRNA constructs delivered into rice callus tissues and *N. benthamiana* through particle bombardment and Agro-infiltration, respectively, both Ubi-hpCP:Hc-Pro and 35ShpCP:Hc-Pro constructs induced strong silencing of 35S-GUS:CP:Hc-Pro transgene. Transgenic rice plants, containing Ubi-hpCP:Hc-Pro construct, expressed high level of 21-24 nt small interfering RNAs, which induced efficient silencing against the GUS:CP:Hc-Pro transgene delivered by particle bombardment and conferred strong silencing by mechanically inoculated SCMV. It is concluded that fusion hpRNA approach is an effective and affordable method for developing resistance against SCMV in model rice plant and it could confer SCMV resistance when transformed into sugarcane.

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LIST OF ABBREVIATIONS

aa	Amino acid
Ago	Agronuate
amiRNA	Artificial microRNA
APS	Ammonium per sulphate
BLAST	Basic local alignment search tool
bp	Base pair
BYV	*Blackberry virus* Y
CaMV	*Cauliflower mosaic virus*
CI	Cylindrical inclusion
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DPA	Days post Agro infiltration
dsRNA	Double stranded RNA
DTT	Dithiothreitol
g	Gram
GFP	Green florescent protein
gRNA	Genomic RNA
GUS	*β*-glucuronidase
Hc-Pro	Helper-component proteinase

hpRNA	Hairpin RNA
HSP	Heat shock protein
ICTV	International committee on taxonomy of viruses
IRES	Internal ribosome entry sites
kb	Kilo bases (nucleotides)
kDa	Kilodalton
LB	Luria-Bertani broth
M	Molar
MCS	Multiple cloning site
MEGA	Molecular evolutionary genetics analysis
mg	Milligram
miRNA	MicroRNA
mRNA	Messanger RNA
MS	Murashige and Skoog
MUG	Methylumbelliferyl β-D-glucuronide
NCBI	National center for biotechnology information
NIa	Nuclear Inclusion A
NJ	Neighbor joining
*npt*II	Neomycin phosphotranspherase II enzyme
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PABP	Poly (A) binding-protein

PCR	Polymerase chain reaction
PD	Plasmodesmata
PDK	Pyruvate dehydrogenase kinase
PIPO	Pretty interesting *Potyviridae* ORF
PPT	Phosphinothricin
PTGS	Post transcriptional gene silencing
PVLP	*Potyvirus* like particles
PVY	*Potato virus* Y
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RdRP	RNA dependent RNA polymerase
RH8	RNA helicase-like protein 8
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase – PCR
SCMV	*Sugarcane mosaic virus*
SDS	Sodium dodecylsulphate
SDT	Sequence demarcation tool
siRNA	Short interfering RNA
SrMV	*Sorghum mosaic virus*
TBE	Tris-borate EDTA
TE	Tris-EDTA

TEMED	NNN'N'-tetramethylethylenediamine
TEV	*Tobacco etch virus*
TGS	Transcriptional gene silencing
Ubi	Ubiquitin
UTR	Untranslated region
UV	Ultraviolet (light waves)
VIGS	Virus induced gene silencing
VPg	Virus genome linked protein
VRC	Viral replication complex
WMD	Web microRNA designer
µg	Microgram
µl	Microliter
µM	Micromolar

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Chapter 1

INTRODUCTION & REVIEW OF LITERATURE

1.1 Family *Potyviridae*

Family *Potyviridae* (including genus *Potyvirus* and several other genera) is the second largest family of plant viruses after family *Geminiviridae.* According to International committee on Taxonomy of viruses about 30% of total numbers of plant viruses are included in this family. Genus *Potyvirus* derived its name from the type species *Potato virus* Y (PVY), which along with other species such as *Potato virus* A and *Potato leaf roll virus* are the serious threat to potato crop all over the world and reducing crop production up to 90% (Salazar, 2003). This family is divided into seven other genera on the basis of amino acid sequence of coat protein: *Potyvirus*, *Ipomovirus*, *Rymovirus*, *Macluravirus, Bymovirus, Brambyvirus* and *Tritomovirus* (Brunt, 1992; Patil and fauquet, 2011) (Table 1.1). Potyviruses are divided into different groups on the basis of their mode of transmission through vector species i.e. they may be transmitted through aphid, fungus, mites and white flies (Matthews, 1982).

1.1.1 Classification of family *Potyviridae*

Taxonomic classification of family *Potyviridae* is always very challenging. For species and strains classification, data based on different approaches were used such as serological method, cross protection, vector or host specificity and molecular analysis. Currently, coat protein amino acid sequences analysis is the most appropriate species demarcation criteria. Amino acid sequences identity higher than 80 percent depicts that isolated viruses are related species of *Potyvirus*, as according to 9th report of ICTV. Similarly,

Table 1.1: Classification of family *Potyviridae* and their mode of transmission [(Patil and Fauquet., 2011). Ecology of plant viruses, with special reference to *Geminiviruses*)].

Family *Potyviridae*	**Number of species**	**Mode of transmission**	**Genome**
Genus *Potyvirus*	146 species	Aphids	Monopartite
Genus *Ipomovirus*	5 species	Whiteflies	Monopartite
Genus *Rymovirus*	3 species	Mites	Monopartite
Genus *Macluravirus*	6 species	Aphids	Monopartite
Genus *Bymovirus*	6 species	Fungus	Bipartite
Genus *Brambyvirus*	1 species	Unknown	Monopartite
Genus *Tritimovirus*	4 species	Mites	Monopartite

Nucleotide sequence identity higher than 76% demonstrates that they are isolates of same species (Adam *et al*., 2011).

1.1.1.1 Genus *Macluravirus*

Macluravirus genus contains long filamentous virus particle of about 700-750 nm in length. It is transmitted through aphid species in non-persistent manner (Liou *et al*., 2003).

1.1.1.2 Genus *Ipomovirus*

This is the small genus in the family consisting of four species having flexible linear single strand virus particle. Their genome size is 750-950 nm and they are transmitted through white flies in non-circulative and semi-persistent manner (Dombrovsky *et al*., 2014).

1.1.1.3 Genus *Tritimovirus*

Tritimoviruses are 690-700 nm in length and 12 nm in width. They are spread through eriophydid mites and also by mechanical means. They consist of linear positive strand RNA genome either monopartite or bipartite (Fauquet *et al*., 2005).

1.1.1.4 Genus *Rymovirus*

Genus *Rymovirus* is rod shaped virus about 680-750 nm in size and closely related to genus *Potyvirus*. It is also transmitted through eriphydid mites vector and have monopartite genome (Fauquet *et al*., 2005).

1.1.1.5 Genus *Bymovirus*

The *Bymovirus* genome consists of bipartite genome. Their genome sizes are 275 and 550 nm and they are transmitted by the fungus, *Polymyxa graminis* (Crane *et al*., 1995).

1.1.1.6 Genus *Brambyvirus*

Brambyvirus is the novel genus in the family *Potyviridae* as it shows minimum nucleotide identity with other members of family *Potyviridae*. Genus *Brambyvirus* contains only *Blackberry virus* Y (BYV) species which caused blackberry yellow vein diseased symptoms in blackberry. Virus particle is non-enveloped, long flexible particle with diameter of 12-15 nm and 800nm its length. BVY has about 11kb genome which is the largest genome in this family (Susaimuthu *et al*., 2008).

1.1.1.7 Genus *Potyvirus*

Genus *Potyvirus* is largest genus amongst the other genera in family *Potyviridae* and includes approximately 200 species. Virion size ranges from 700nm to 850nm in length and comprises of single stranded positive sense RNA genome. Its mode of transmission is both through insect vector (aphid) and also through mechanical inoculation (Matthews, 1991; Dolja and Carrinngton, 1992).

Genome organization of this family is very similar to certain plant as well as animal viruses such as como and nepovirus and picorna virus group. It has been suggested to place *Potyvirus*, *Comovirus* and *Nepovirus* in the picorna-like viruses supergroup (Goldbach *et al*., 1990). *Potyviridae* has distinct property of forming cylindrical shaped inclusion bodies in the cytoplasm of infected host cells but every genus has particular shape for it (Shukla, 1991).

1.1.2 Symptoms of family *Potyviridae*

Viral infected plants show typical mosaic symptoms having alternate light and dark regions. Symptoms are more obvious on younger leaves of plants. Apart from this, leaf curling, dwarfing of plants and fruits distortion are also some times appear on later stage of infection (Handley *et al*., 2001) (Figure 1.1).

1.2 Genus *Potyvirus*

1.2.1 *Potyvirus* genome

Potyvirus are positive sense RNA virus having single linear genome. They produced pinwheel shaped inclusions bodies in the cytoplasm of infected plant cells (Matthews, 1991; Dolja and Carrinngton, 1992). Approximately 9.8 kb genome contains only single open reading frame with a polyA tail (Hari *et al*., 1979; Riechmann *et al*., 1992). Three proteinases (P1, NIa and Hc-Pro) catalyzed the cleavage of single polyprotein either co- or post-transcriptionally and produced ten mature viral proteins (Verchot *et al*., 1991; Riechmann *et al*., 1992) (Figure 1.2). About 2000 copies of coat protein surround the genome for its protection. They are helically arranged around the genome (Shukla and Ward, 1989). *Potyvirus* genome is shown in Figure 1.2.

1.2.2 *Potyvirus* proteins and their functions

1.2.2.1 Helper component proteinase (Hc-Pro)

Hc-Pro is 50-53 KDa multifunctional protein of *Potyvirus*. Hc-Pro is involved in proteolytic activity of polyprotein, which was confirmed in the mutational study of *Tobacco etch virus* (TEV), mutated cleavage site was unable to cause infection and reduced virus stability (Kasschau and Carrington, I995). Hc-Pro is functional in dimer and multimers form and it has three conserved motifs, N terminus contains conserved tetrapeptide Lys-Ile/Leu-Thr/Ser-Cys (KITC) domain which is involved in the interaction with aphid's stylet. Alteration of aminoacid from Lysine to Glutamine determined the loss of binding capacity to the aphid stylet which suggests that Lysine (K) is essential aminoacid to perform its specific function while the rest amino acids conservation is not very crucial (Blanc *et al*., 1998; Sasaya *et al*., 2000). Hc-Pro has conserved cysteine and Histidine residue at C terminal end

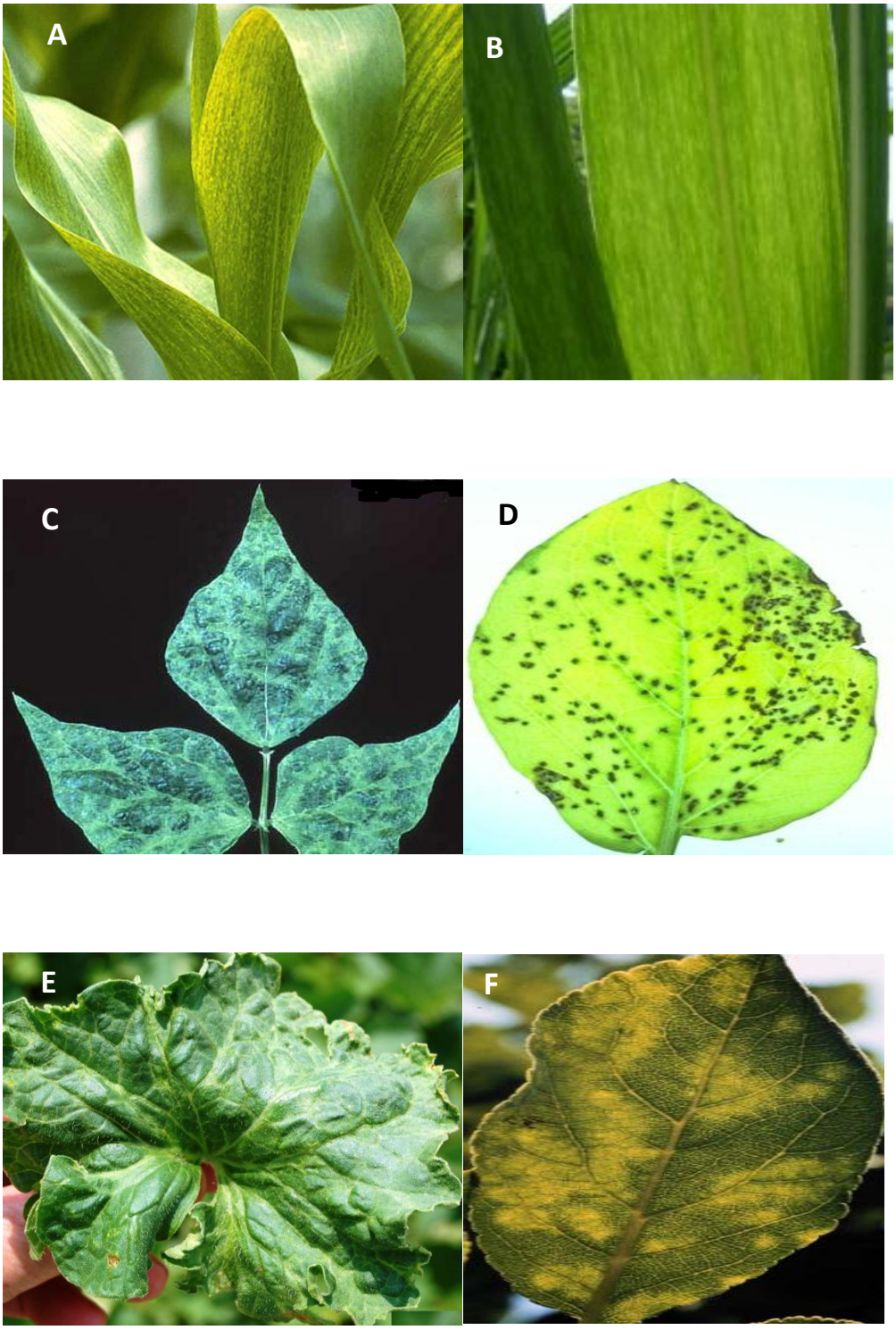

Figure 1.1: Symptoms caused by *Potyvirus* infection on various host plants. Mosaic symptoms on maize leaves caused by *Maize dwarf mosaic virus* (A); Mosaic symptoms on sugarcane leaves caused by *Sugarcane mosaic virus* (B); Green and yellow mosaic symptoms on bean leaves caused by *Bean yellow mosaic virus* (C) Leaf necrosis on potato leaves caused by *Potato virus* Y (D); Systematic mottling symptoms on cantaloupe leaves caused by *Watermelon mosaic virus* (E); *Butternut canker plum pox virus* sympotms on plum leaves (F)

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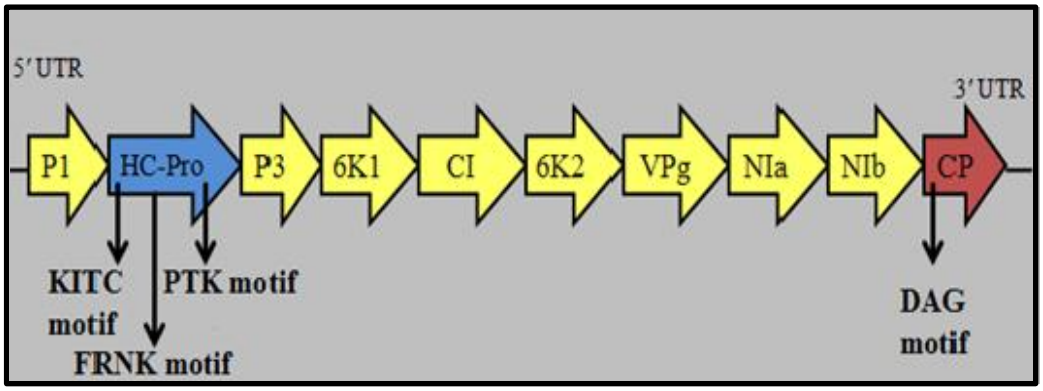

Figure 1.2: *Sugarcane mosaic virus* genome and conserved regions of *CP* and *Hc-Pro* genes (Hari *et al*., 1979; Riechmann *et al*., 1992).

which form zinc finger structure involved in virus transmission through interaction with conserved DAG motif of coat protein. Importance of this conserved PTK motif (Pro-Thr-Lys) was proved when alanine was substituted in place of threonine which resulted in total loss of gene function (Huet *et al*., 1994). Central domain is involved in multiple functions and contains various conserved motifs. One of its motifs is FRNK, which suppresses plant silencing machinery and causes severe infection in plants. Another central conserved motif is cys-cys-cys (CCC), which is essential for replication and cell to cell movement of virus. Apart from these, Hc-Pro also forms characteristics amorphous shape inclusion bodies in the cytoplasm of infected cell (Dolja *et al*., 1993).

1.2.2.2 Coat protein (CP)

Coat protein (36KDa) is responsible for protection through encapsidation and movement of virus. It is also involve in replication and translation of virus genome during early infection stages. Translation and replication of viral genome inter-related to CP concentration inside the cell. As less accumulated CP allows more viral replication while its high concentration result in the en-capsidation of virus genome and proteins (Yi, *et al*., 2009; Boni *et al*., 2005). Coat protein contains three domains; its N and C terminus is exposed on the virus surface containing DAG conserved motif on N terminus which is responsible for long distance virus transmission through interacting with Hc-Pro (Allison *et al*., 1985; Shukla *et al*., 1988), while central region is responsible for assembly of virus, genome multiplication and its movement from one cell to another (Dolja *et al*., 1994, 1995; Rojas *et al*., 1997).

1.2.2.3 Nuclear inclusion proteins

Equimolar ratios of 49 KDa (NIa) and 54KDa (NIb) *Potyvirus* nuclear inclusion proteins form the characteristics non-structural inclusion bodies inside the infected cells. NIa protein comprised of two domains, its N- terminus contains VPg domain while its C-terminus

contains NIa-pro domain. VPg protein has NTP binding domain which after uridylylation by viral RNA dependent RNA polymerase (NIb) attaches itself to the virus genome and acts as a primer to initiate the viral replication. C terminal proteinase domain involves in the non-specific DNase activity and protolytic activity, cleaving 430 aminoacids protein into 189 (VPg) and 241(NIa-pro) amino acid products (Dougherty and Parks, 1991). VPg protein also participates in translation as it interacts with eukaryotes translational machinery. This fact is supported by its studied interaction with RNA dependent RNA polymerase (NIb) and helicase proteins such as DEAD box which are necessary for translation (Dunoyer *et al*., 2004; Puustinen and Makinen, 2004; Huang *et al*., 2010). NIa (unprocessed form) of *Turnip mosaic virus* was also found to be localized in nucleus where it interacted with poly (A) binding protein and translational initiation factors such as eIF (iso) 4E.

NIb protein is RNA dependent RNA polymerase, as the sequence of NIb was found to be homologous to RNA polymerases of other viruses (Domier *et al*., 1987). It is separated from polyprotein by cis cleavage through proteinase domain of NIa. NIb forms the complex with cytoplasmic inclusion protein CI and VPg protein and aids in the replication of viral RNA. Analysis of NIb protein demonstrates that the important conserved motif GDD is involved in the polymerase activity of this protein (Hong and Hunt, 1996).

1.2.2.4 Cylindrical inclusion protein (CI)

Virus systemic infection is caused by the movement of virus and its associated protein from one cell to another cell through specialized proteins (Carrington *et al*., 1996). *Potyvirus* CI protein ultra-structural analysis indicates that it is an integral factor for intracellular transport of virus. Evidences provided proved that CI protein is necessary for replication of RNA genome by its distinctive RNA helicase activity (Eagles *et al*., 1994; Latin *et al*., 1991). CI protein creates particular laminate or cylindrical shape inclusion bodies in the cytoplasm

of infected cell (Lesemann *et al*., 1988). During the early infection stages, CI proteins associated with CP and RNA of virus and form cylindrical or cone shaped bodies near to plasmodesmata of cell membrane (Roberts *et al*., 1988). Thus, CI protein assists the cell to cell movement of virus either directly through interaction with its protein or by modifying the plasmodesmata (Carrington *et al*., 1998).

1.2.2.5 P1 and P3 protein

P1 and P3 proteins sequences are relatively least conserved so their role in virus life cycle is not clear. P1 and P3 interact with cytoplasmic inclusion bodies in the infected cell (Brantley and Hunt, 1993; Soumounou and Laliberte, 1994; Merits *et al*., 1998). P1 (34KDa) binds to viral RNA non-specifically and act as a trans-acting factor during viral replication (Verchot and Carrington, 1995). The exact function of P1 is not clear but literature suggests that it is involved in the movement of viral proteins, although mutations and alteration of P1 sequence has not drastic effect on movement of virus (Verchot and Carrington, 1995a, 1995b).

42KDa P3 protein was described to be involved in viral replication as mutation in this gene prevents the genome replication (Klein *et al*., 1994). A new reading frame was discovered in the P3 protein by Chung *et al*. and named as Pretty Interesting Potyviridae ORF (PIPO). This new ORF is present in P3 cistron region and translated though +2 frame-shift in the long original ORF. Its length is variable among different species and is involved in the movement of virus particles through its interaction with different host proteins such as hydrophilic cation PCaP1 (Lin *et al*., 2011). P3N-PIPO causes infection in the plants which contain recessive genes for resistance. It interacts with large and small subunit of RubisCO protein, resulting in the enhanced virus symptoms (Choi *et al*., 2013).

1.2.2.6 K1 and 6K2 protein

6K1 and 6K2 proteins both are responsible for severity of chlorosis symptoms in virus infected plants. It was studied that 6K2 protein attaches the viral replication machinery to the host endoplasmic reticulum membrane (Li *et al*. 1997; Schaad *et al*. 1997). 6K2 is 54 amino-acid protein, its amino-acids sequence from 22 to 44 forms a hydrophobic loop which encompasses the N and C terminal of CI and VPg proteins to cell cytoplasm during replication (Shaad *et al*., 1997).

1.2.3 *Potyvirus* life cycle

Potyvirus life cycle within the diseased cell is a strongly synchronized process, which comprises many pathways, such as inoculation, translation and replication, movement to adjacent cell and encapsidation. Viral route in infected cell through inoculation to vector acquisition is shown in the Figure 1.3.

1.2.3.1 Transmission of *Potyvirus* through aphid vector

Aphid vectors used non persistent and non-circulative modes for *Potyvirus* transmission. Aphid species are very specific in transmission of *Potyvirus*. Specificity not only depends upon aphids vectors but also depend upon host species as well as utilization of Helper component of virus. Studies proved that *Zucchini yellow mosaic Potyvirus* was efficiently transmitted by *Aphis gossypii* as compared to the other aphid species (Yuan and Ullman, 1996). Moreover, *Myzus persicae*, *Schizaphis graminum*, *Aphis gossypii*, *Rhopalosiphum maidis*, *Rhopalosiphum padi* and *Sitobion avenae* are responsible for the transmission of *Potyvirus* (Hasan *et al*., 2003).

Helper component proteinase (*Hc-Pro*) and coat protein (*CP*) are the two significant viral proteins involve in virus-vector interaction. Several different models were predicted to describe the viral transmission but most acceptable and applicable model was first presented

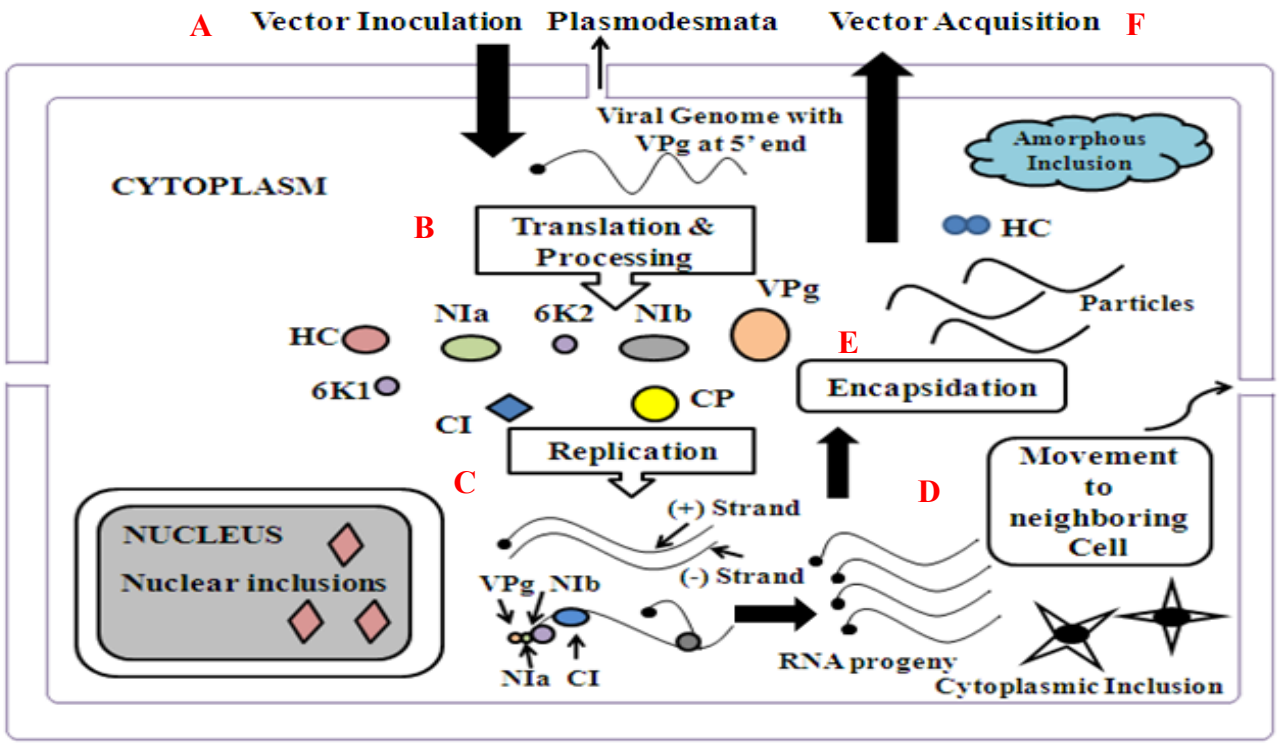

Figure 1.2: Life cycle of *Potyvirus* in infected plant cell (Makinen and Hafren, 2014). **A.** Virus inoculation through aphid vector. **B.** Translation of genome **C.** Replication of virus genome. **D.** Movement of viral RNA to the neighboring cells. **E.** Capsidation of viral genome. **F.** Virus acquisition through vector.

by Govier and kassanis named as bridge hypothesis model (Govier and Kassanis, 1971) (Figure 1.4). *Hc-Pro* N terminal domain contains KITC (Lysine-Isoleucine-Threonine-Cysteine) motif which specifically interacts with aphid stylet while its C-terminal domain has conserved PTK (Proline-Threonine-Lysine) motif which binds with coat protein N terminus DAG (Aspartic acid-Alanine-Glycine) motif. Thus, *Hc-Pro* acts as a bridge between virus capsid protein and aphid stylet (Kassanis and Govier, 1971). Mutational studies confirmed the importance of these conserved regions by the replacing the Lysine with glutamic acid which significantly reduced the transmission of virus as well as symptoms development (Atreya *et al*., 1992). *Hc-Pro* also seemed to involve in systemic spread of virus which was analyzed after alteration of central conserved region of triple cysteine by triplet Arginine-Proline-Alanine (Cronin *et al*., 1995).

1.2.3.2 Replication of virus in infected cell

Virus replication takes place in the cell chloroplast, which is facilitated by replication vesicles formed by endoplasmic reticulum (Wei *et al*., 2010). 6K2 protein spans the whole membrane, formed viral replication complex (VRC) and with the help of endoplasmic membrane protein Syp71 fused the VRC to chloroplast membrane (Schaad *et al*., 1997). Once the infection proceeds, VRC attaches with the tubular proteins which form complex structure with chloroplast, endoplasmic reticulum and Golgi bodies. Replication process form two types of RNA strands, (+) strand RNA enters into cytoplasm while (-) strand RNA stays in the VRC (den Boon and Ahlquist, 2010). Electron microscopy images showed that VPg protein form pores in the VRC which releases the (+) strand RNA obligatory for infection (Rantalainen *et al*., 2009). Multiple rounds of replication continue until all energy, replication factors and host membranes required for amplification are exhausted.

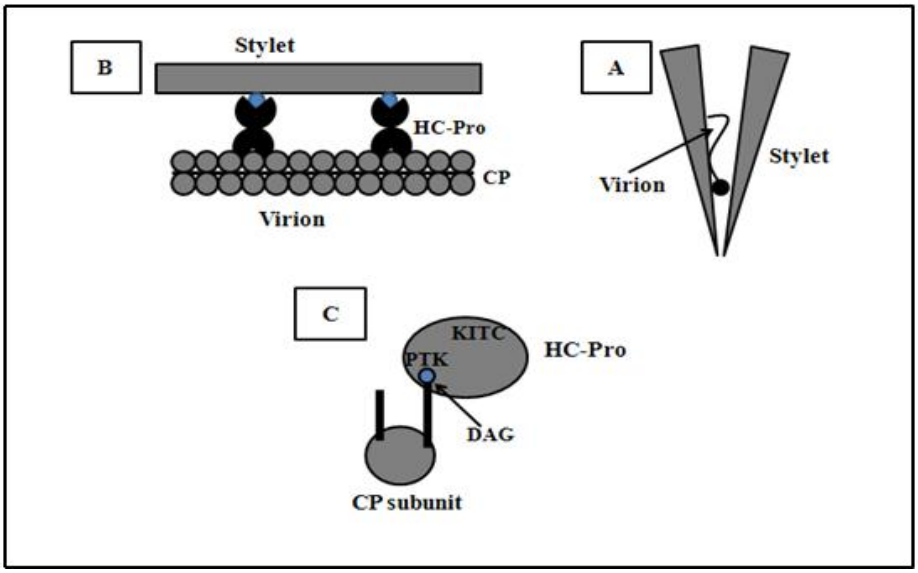

Figure 1.3: Aphid and virus interaction (Bridge Hypothesis) (Roudet-Tavert *et al*., 2002). **A.** Virus attachment to the aphid's stylet. **B.** Hc-Pro acts as a bridge by interacting with the virus CP on one side and receptors present on the aphid stylet on the other side. **C.** Interaction of CP and Hc-Pro through DAG and PTK conserved motifs.

1.2.3.3 Translation of genome

After virus inoculation, virus un-coating takes place and translation of viral genomic RNA occurs by utilizing the host machinery. Viral entry into cell occur through two ways either it is transmitted through aphid vector or by direct passage through neighboring infected cells. Virus infection is initiated in the cell through the translation of its polyprotein. Potyvirus (+) sense RNA is directly utilized for both translation and replication but the factors which are responsible for switching of translational pathway to replication is not clearly recognized. Although authors proposed that replication is not started until enough viral proteins are accumulated in cell cytoplasm after translation, which are necessary for genome amplification (Mahajan *et al*., 1996). Translation of genomic RNA takes place on endoplasmic reticulum membrane through a multifunctional protein i.e VPg (Wei *et al*., 2010). It acts as a primer for amplification of RNA by interacting with eukaryotic translational initiation factors. One of the translational initiation element eIF4E or its iso-form eIF (iso) 4E interacts with Virus protein genome linked VPg (Wittmann *et al*., 1997; Leonard *et al*., 2000). Eukaryotic translational initiation elements involved in binding with 5' end of genomic RNA thus down-regulating the translation of eukaryotic mRNA. Other eukaryotic proteins which participates in the translation process are eukaryotic elongation factor 1A (eEF1A), poly(A) binding-protein (PABP), RNA helicase-like protein RH8, and heat shock protein 70 (HSP70) (Beauchemin and Laliberte, 2007; Beauchemin *et al*., 2007; Dufresne *et al*., 2008; Thivierge *et al*., 2008; Cotton *et al*., 2009; Huang *et al*., 2010). VPg recruits PolyA binding proteins PABP2 to binds with poly A tail at 3' end of RNA genome. This facilitates the circularization of genome which is necessary for replication and translation of viral genome (Harold and Andino, 2001). Meanwhile, eIF4G protein initiates its interaction with 5' UTR region which also known as internal ribosome entry site (IRES) and initiate the movement of microtubules towards the complex. N terminal domain of VPg

comprised of important NTP binding region which utilize energy for removal of secondary structures of viral RNA and assists in translation (Cotton *et al*., 2006; Grzela *et al*., 2006). Thus, complete complex formation results in translation of genomic RNA with the aid of energy (Figure 1.5).

1.2.3.4 Movement to the neighboring cells

Cell to cell viral movement occur through three viral movement proteins i.e. cylindrical inclusions (CI), P3N-PIPO and CP and through plasmodesmata channels which forms connection between cytoplasm and endoplasmic reticulum (Dolja *et al*., 1994, 1995; Carrington *et al*., 1998; Wei *et al*., 2010; Wen and Hajimorad, 2010). These proteins transported the viral RNA to the neighboring cells by the formation of cone shaped complex by CI proteins which are attached to P3N-PIPO on one side and plasmodesmata on the other side (Wei *et al*., 2010). These cone-shaped CI were reported to be transient in nature and onlyactive during the translation and replication stages, suggesting its role only during early stages of virus infection (Roberts *et al*., 1998). CP also facilitates the virus transport from one cell to another once its assembly is complete. N terminal region of CP is required for viral assembly and it was stated that only assembled virus or RNA complexes attached to CP entered the plasmodesmata (Torrance *et al*., 2006). This complex is allowed to pass through plasmodesmata when it further attaches to P3N-PIPO, which has capacity for PD passage by attaching it through PCaP1-PD binding protein (Vijayapalani *et al*., 2012).

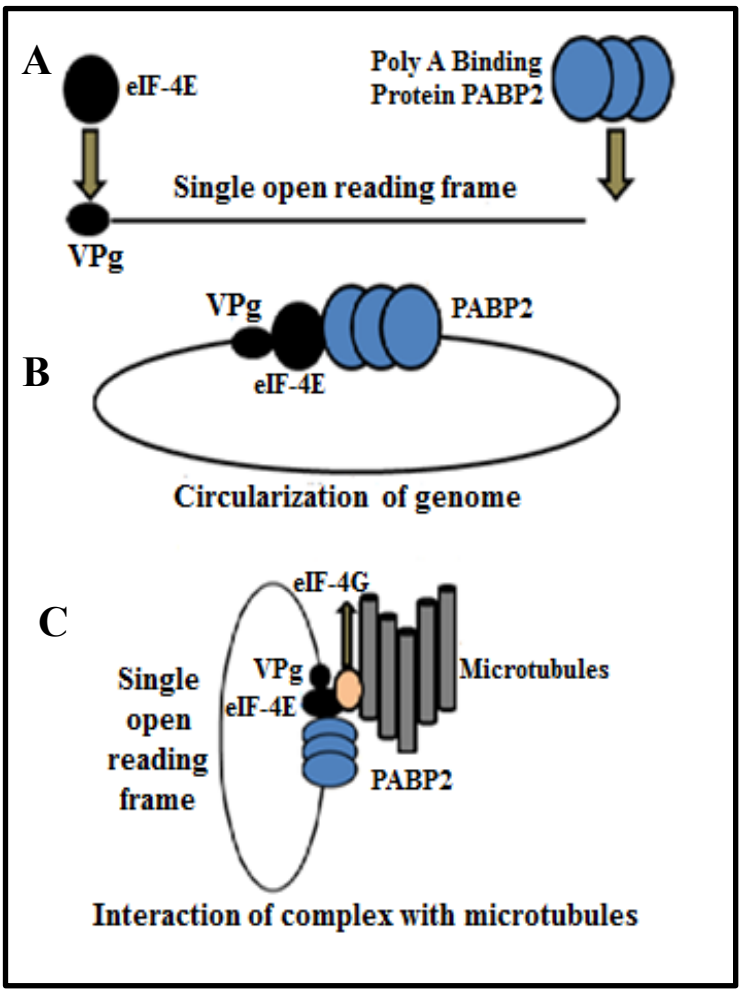

Figure 1.4: Translation mechanism of SCMV in infected plant cell. **A.** Binding of translation initiation complex and PolyA binding proteins to the single open reading frame. **B.** Circularization of genome. **C.** Interaction of complex with microtubule.

1.2.3.5 Viral encapsidation

Virions encapsidation is necessary for the systemic virus spread and their transmission to the other host species by means of aphid vector. Detail picture of how virus encapsidation occur and its mechanism is still missing. Early data recommended that virus assembly takes place in the globular structure formed near peri-nuclear area during viral infection (Grangeon *et al*., 2012a). Potyviruses coat protein form N and C terminus interaction with each other and stabilized them in the attached ring form (*CP-CP*), even in the absence of viral RNA (Anindya and Savithri, 2003). A model predicted the virions assembly, in which two host chaperones proteins, CPIP and HSP70 play a key role in virus encapsidation (Hofius *et al*., 2007; Hafren *et al*., 2010). These chaperones detect the high level of coat protein formed after viral translation and replication, sequester it from viral RNA and encapsidate the viral genome. Gene expression is inhibited once viral assembly started because of unavailability of free viral RNA (Nagy *et al*., 2011).

1.3 *Sugarcane mosaic virus*

Sugarcane mosaic virus (SCMV) belonging to family *Potyviridae*, is a significant sugarcane pathogen as it causes extensive yield loses when its infectivity rate reaches upto 50% (Husnnain and Afghan, 2004). It is prevalent in more than 70 countries causing significant crop reduction every year (Jeffrey *et al*., 1998). Sugarcane mosaic disease was first time reported in Louisiana, United States in 1919. It causes the $US 100 million loses per year (Brandes and Sartoris, 1936). However, it causes 6-10% sugar yield loses and 10-32% cane reduction in Pakistan every year (Anwar, 2005).

1.3.1 Strains of *Sugarcane mosaic virus*

At least, eight different strains of SCMV are reported which cause mild to severe virus infection, includes strain A, B, D, E, H, I, M and SC. However, mixed infection of these strains has also been reported. Strain A and H produced the mild symptoms of irregular mottling with stunted plant growth. However, strain B, D and E are differentiated from other strains by producing severe patterns of chlorosis, necrosis and reddening in the young plant leaves (Summers *et al*., 1948).

1.3.2 Host crops of SCMV

Naturally, SCMV infection is restricted to members of the family *Poaceae*. Sorghum, maize and some wild grasses growing near infected sugarcane fields may be infected naturally. However, cultivated cereals such as rye, barley and rice are rarely infected under natural conditions (Koike and Gillaspie, 1989).

Sugarcane (*Saccharum officinarum*) is one of the significant hosts of SCMV belonging to family *Poaceae*. It is an important crop of Pakistan and plays a chief role in economic development of country. Sugarcane is not only significant for sugar production but also utilized for the production of alcohol, cardboards, paper and many other commercial chemical by-products during sugar manufacturing. According to the sugarcane productivity and consumption all over the world, Pakistan is on 4^{th} number for the production of sugar and on forteen position for cane production. Sugarcane is widely grown crop in all provinces of Pakistan. Approximately, 1.5 million employees (either directly or indirectly) contribute to 1.9% share in GDP in about 88 sugar mills all over the country. On the basis of agriculture, sugar industry is the second largest industry in Pakistan which is grown on approximately 1.1 million hectares areas. The Punjab, Sindh and Khyberpakhtunkhwa (KPK) provinces are the major sugarcane growing regions of Pakistan with a total annual sugar production of

3,172,408 per tonn from Punjab, 1,547,547 per tonn from Sindh and 310,174 per tonn from KPK (http://edu.par.com.pk/wiki/sugarcane/#punjab). According to the official estimates, sugarcane production was slightly down in 2015 due to the monsoon floods. Farmers are growing those varieites of sugarcane which have high sugar contents and due to this 15 percent recovery rate is increased over the past decade. It is estimated that sugarcane production will be increased in 2016 as 3.8 percent area is increased for crop harvesting as compared to the last year (Sources: Provincial Agriculture Departments and FAS/Islamabad). Different varieties of sugarcane are grown in different areas depending upon environmental factors such as area temperature, moisture conditions and soil fertility. BL – 4 , L –116, BF – 162, CP 43-33,CP 72-2086, CP 77-400, SPSG-26, CPF-237 are early maturing varieties, TRITON, COL –54, SPF-213, are midseason varieties and L –118, COJ-84 are late maturing varieties in Punjab. CP-43-33, CP-81-1435, BF-162, BL-4, SPF-234 and T-10 are commonly used sugarcane varieties for tissue culture. Data mentioning the sugarcane production, yield and area used for its cultivation in all provinces of Pakistan are shown in the Table 1.2.

It is worth mentioning that Government of Pakistan established $ 124 per metric ton subsidy on sugar export which covered the export of about 500,000 metric tons through March, 2016. During the year 2014/15, approximately 579,526 metric ton of sugar was exported from Pakistan. However, exports during 2016/2017 are currently forecast at zero due to high price of sugar and absence of announced subsidy and quota (Rehman, 2016) (Table 1.3).

Table 1.2: Survey of sugarcane production, area and yield produced during 2011-15.

Year	Area (000 hectare)	Production (000 Tonnes)	Yield (Kg/Hec.)
2010-11	988	55,309	55,981
2011-12	1,058	58,397	55,196
2012-13	1,129	63,750	56,466
2013-14	1,173	67,460	57,511
2014-15	1,141	62,652	54,910

Table 1.3: Sugar Import and Export in Pakistan (**Source:** Pakistan Bureau of Statistics).

	Sugar Imports		**Sugar Exports**	
Months	**MY 2014-15**	**MY 2015-16**	**MY 2014-15**	**MY 2015-16**
October	1,246	1,458	51,657	0
November	2,638	1023	4,158	0
December	830	852	75	0
January	1,180	1,054	49,342	27,344
Febuary	288	865	99,330	119,845
March	1,254	650	99,676	125,033
April	652	--	98,167	--
May	375	--	123,002	--
June	499	--	35,003	--
July	1,720	--	19,116	--
August	1,775	--	0	--
Septmeber	3,856	--	0	--
Total	**16,313**	**5,902**	**579,526**	**272,222**

1.3.3 *Sugarcane mosaic virus* diversity in Pakistan

However, to identify the genetic diversity of SCMV in Pakistan symptomatic and asymptomatic sugarcane leaves were collected from different areas. Coat protein gene was amplified by using the *CP* detection primers. Amplified products were sequenced and aligned with multiple SCMV *CP* sequences collected from Genbank database. Studied virus isolates showed that they are the variants of SCMV with about 96-97% identity with Australian Bundaberg isolate (Haider *et al.*, 2011).

Further, the phylogenetic analysis was done to report the variations in the *CP* gene sequences of SCMV from the isolates of Pakistan. One hundred and two sugarcane samples were collected from various districts of Pakistan. Phylogenetic tree was constructed between 12 isolates of Pakistan and 38 isolates from other countries. All Pakistan isolates showed 82-100% nucleotide sequence identity and formed a single clade in the tree. It was reported that no co-relation found between isolates of Pakistan and the isolates from the rest of world. They also reported the existence of novel population of SCMV in Pakistan, named it as SCMV-PAK (Ali *et al*., 2014).

1.4 Strategies for *Potyvirus* control

1.4.1 Non-engineered resistance

1.4.1.1 Vaccination of plants

Plant vaccination is a noteworthy phenomenon to control the plants damage from severe virus strains. This approach is based on the infection of the weak symptomless primary virus that has potential to activate virus-induced gene silencing (VIGS) and to target primary as well as challenging viruses. Primary virus act as vaccines that provides cross-

protection against same virus isolates or some closely related viruses (Ziebell and Carr, 2010; Nishiguchi and Kobayashi, 2011). A reported example of this method is the utilization of *Zucchini yellow mosaic potyvirus* isolate that has been approved and register as the pesticide (CUBIO ZY-02) and efficiently used to control virus disease in Japan (Kosaka and Fukunishi, 1997; Kosaka *et al*., 2009).

1.4.1.2 Detection of resistant varieties

Breeding of natural resistant varieties is the possible approach to control the virus spread. Molecular basis of SCMV resistance in European and U.S. inbred strain was screened. In glass house and under field trials, U.S. Pa405, B68, Oh7B, Mp339, GA209.strains were found to be completely resistant against SCMV (Roane *et al*., 1989). RFLP analysis proved that resistance against *Maize dwarf mosaic virus* was developed due to location of single major gene Mdm1 near the centromeric region on the chromosome number 6 is responsible (McMullen and Louie 1989; Louie *et al*., 1991). Allelic test and segregation analysis also specified that (FAP1360A, D21 and D32) Eurpeon inbreds were not only resistant to SCMV but also to MDMV, *Sorghum mosaic virus* (SrMV) and *Johnsongrass mosaic virus* (JGMV) (Kuntze *et al*., 1995). However, it was suggested that Scm1 gene which is tightly linked to Mdm1 supposed to be responsible for three Europeon inbrebs resistance (D21, D32 and FAP1360A) (Melchinger *et al*., 1998). However, in China Huangzaosi, Siyi, X178, and Hai9-21 showed complete resistance to SCMV (Chen *et al*., 2004; Wu *et al*., 2002).

1.4.1.3 Chemicals means for viral resistance

Mineral oil reduces the transmission and retention of virus in aphid stylet which is confirmed by autoradiography and transmission electron microscopy evidences. The proposed hypothesis was proved on *Nicotiana tobacum* seedlings, their leaves were sprayed

with oil emulsion. Every test plant was kept with single aphid overnight. Leaves covered with mineral oil didn't allow aphids to transmit virus. Autoradigraphy and transmission percentage values of oil sprayed leaves were considerably lower as compare to the control samples. Similarly, retention period of virions in the aphid's stylet was also very short. Preacquisition and postacquisition probing clearly confirmed that mineral oil significantly reduce the retention and virus transmission in insect vector (Wang and Pirone, 1996).

Subsequently, exogenous applications of plant hormones initiate broad-spectrum resistance to viral as well as non-viral pathogens. Benzothiadiazole (BTH) (common name-acibenzolar-*S*-methyl) was reported to be safe and most effective activator of systemic acquired resistance (SAR) that protects the plant against many viruses (Mandal *et al*., 2008;Takeshita *et al*., 2013;Trejo-Saavedra *et al*., 2013).

1.4.2 Engineered Resistance

1.4.2.1 Gene silencing in plants

Gene silencing is the natural defense system in eukaryotes that provide resistance against virus infection and regulates endogenous gene expression (Baulcombe, 2005; Voinnet, 2005). Two types of gene silencing are known which ultimately down regulate the transcript level. First occurs at transcriptional level known as transcriptional gene silencing (TGS) while second type of silencing is known as post transcriptional gene silencing (PTGS) which degrades the accumulated mRNA in cell (Vaucheret and Fagard, 2000).

1.4.2.1.1 Transcriptional gene silencing (TGS)

TGS is the form of epigenetics gene silencing that causes the methylation of promoter DNA sequences producing heterochromatin area around the gene resulting in transcription repression of gene (Waterhouse *et al*., 2001; Eckardt, 2002).

1.4.2.1.2 Post transcriptional gene silencing (PTGS)

Post transcriptional gene silencing or RNA interference (RNAi) phenomenon triggered silencing by two types of small RNA that is microRNAs (miRNAs) and small interfering RNAs (siRNAs) which are generated by precursor of stem loop structure or through long double stranded RNA (Baulcombe, 2005; Tomari and Zamore, 2005). siRNA and miRNA silencing phenomenon follow the same biochemical pathway inside cell.

1.4.2.1.2.1 Mechanism of PTGS/ RNAi

Small interfering RNA triggers gene silencing which are derived from transposons (natural mobile genomic element) or from viruses. However, microRNAs (miRNAs) constitute a large group of endogenous single stranded small RNAs (21-24 nucleotides). Both siRNA and miRNA have negative regulatory function on gene expression (Mishra and Mukherjee, 2007). Silencing of target genes by hpRNAi and amiRNAs is very similar and is based on cleavage-mediated transcript degradation. However, hpRNAs target perfectly complementary mRNAs, while amiRNA preferentially avoid perfect complementarities to easily predict the off-targets (Schwab *et al*., 2006; Ossowski *et al*., 2008) (Figure 1.6).

Engineered amiRNA and hpRNA strategy mimics the natural silencing phenomenon. Hairpin RNA (hpRNA) constructs is designed by cloning the targeted gene fragment in hairpin vector in both sense and antisense orientation separated by spacer region. While pre-miRNA backbone is used to develop the amiRNA construct (Boden *et al*., 2004; Zeng *et al*., 2002) which can be design by replacing endogenous miRNA and its complementary sequences. Stemloop structure will be recognized as natural pre-miRNA structure and undergo its cleavage through dicer and degrades the targeted mRNA (Niu *et al*., 2006; Schwab *et al*., 2006; Mathieu *et al*., 2007).

RNA polymerase III transcribed the long dsRNA from the expression plasmids (Eamens *et al*., 2008), conversely RNA polymerase II transcribes the miRNA gene which also contains intron. The transcripts generated have the polyadenylated tail and methylated cap (Griffiths-Jones *et al*., 2005; Kim, 2005). Immediate product of transcription is pri-miRNA or siRNA which further processed into mature miRNA or siRNA through atleast two steps mediated by Dicer proteins (Bartel, 2004; Kim, 2005). Dicer comprises of two RNase III domains (RNase III a, RNase III b) and a PAZ domain which are linked through long α- helix. PAZ domain is responsible for recognizing the end of dsRNA while RNase III domains catalyze the cleavage of dsRNA (Zhang *et al*., 2004). After cleavage, dicer generates 2 nts overhang at 3' region in each strand (Knight and Bass, 2001; Zhang *et al*., 2004).

Methylation occurs on the ribose of last nucleotide on each strand of duplex either at 2' or 3' hydroxyl group by HUA Enhancer 1 (HEN1). HEN1 protein mediates this step which comprises of two domains; dsRNA binding domain and methyltransferase domain at C-terminal end (Boutet, 2003; Chen *et al*; 2002). This methylation facilitates the export of duplex (miRNA/miRNA*, siRNA) outside the nucleus into cytoplasm in association with HASTY (HST) protein (Bartel, 2004). In cytoplasm RNA induced silencing complex (RISC) is assembled which incorporate the guide strand of (miRNA/miRNA*, siRNA) duplex. Thermodynamic stability determines the polarity of strands as the 5' end of less stable strand is selected as guide strand and the second strand is passanger strand which is discarded (Khvorova *et al*., 2003; Schwarz *et al*., 2003). Guide strand integrate into RISC, whose essential member is argonaute (Ago) protein. Argonaute protein is characterized by three domains; PAZ, PIWI and MID (Bohmert *et al*., 1998). PAZ domain contain specific pocket that binds the 2 nt overhang at 3' region of miRNA or siRNA while PIWI domain is analogous to RNase H (Parker *et al*., 2004; Rashid *et al*., 2007). MID domain is responsible

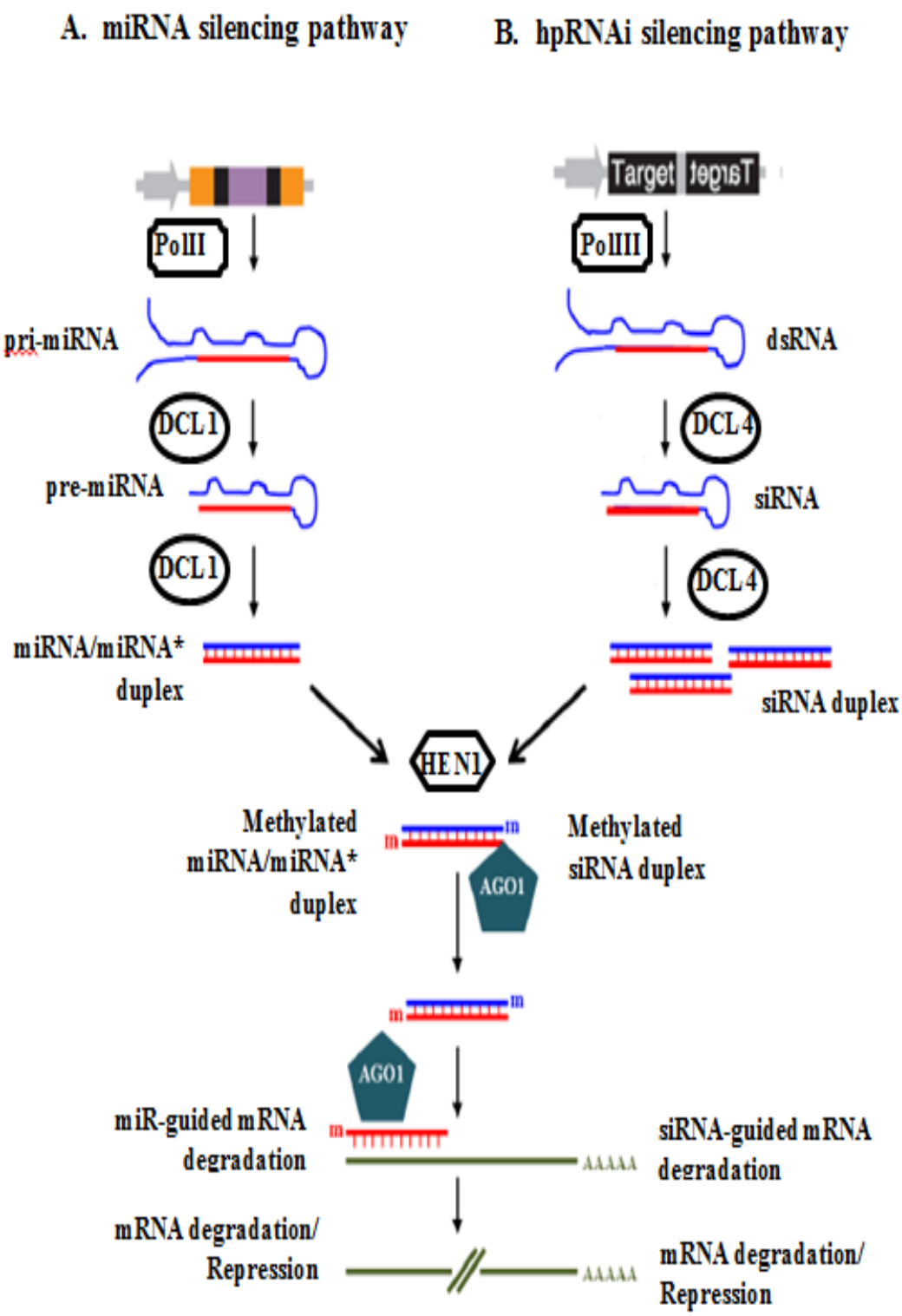

Figure 1.5: Diagrammatic representation of mechanism of RNAi (Agrawal *et al*., 2003).

for binding of 5′ phosphate of miRNA or siRNA and helps its anchorage with Ago (Till *et al*., 2007). After attachment with miRNA or siRNA, Ago protein by its endonucleolytic action mediate repression or degradation of targeted mRNA which are further removed by exonucleases (Haley and Zamore, 2004; Martinez and Tuschl, 2004).

For designing and analysis of amiRNA following are the key points: Substitution of 5′ U is essential in pre-amiRNA, as it contributes to thermodynamic instability of duplex and loading of guide strand into RISC. GC contents should not be greater than 70%. Position 2 to 5 should avoid three or consecutive G/Cs. Sequences chosen for amiRNA constructs should not include off targets and homology. Previous studies suggested that backbone of six *Arabidopsis* pri-miRNA which encodes miR171a, miR319a, miR172a, miR164b and miR159a are mostly used for amiRNA construction (Alvarez *et al*., 2006). For hairpin RNA construction, suitable restriction sites insertion in 5’ end of primers are mandatory for the cloning into conventional hairpin (pKannibal) restriction site vectors.

1.4.2.1.2.2 Applications of RNAi

RNAi technology is useful for quick study of pathways of large number of genes in different organisms. RNAi technology has been used in *D. melanogaster* to identify and analyze the genes which are involved in various cellular mechanisms such as in signaling pathways and developmental stages of embryo (Clemens *et al*., 2000). In plants this technique described the functions of gene by its knockdown. Several plant endotoxins are removed by targeting the toxin gene by hairpin constructs. Similarly, decaffeinated coffee plants are produced by the knock down of theobromine gene in coffee plants by RNAi constructs (Ogita *et al*., 2003). Virus-induced gene silencing (VIGS) has also been verified to be an effective approach for plant genetics (Baulcombe, 2001).

In the area of drugs development, RNAi assists in the screening and synthesis of drugs by finding the genes or novel compounds which provides drug resistance. It is also beneficial to study the functions of large number of genes as multiple genes can be silenced together. This technology also offers benefits in the field of therapeutics as siRNA down regulates the expression of mutant genes in the infected cells. Down-regulation through siRNA is fruitful as no side effects observed due to perfect hybridization with targeted mutant genes. It is stated that even single mismatch within 19-21 nucleotides of siRNA leads to mismatch and disruption in cleavage of targeted mRNA (Elbashir *et al*., 2001).

Using RNAi technology, CSIRO scientist developed barley transgenic varieties against *Barley yellow dwarf virus* (BYDV) that showed complete resistance to BYDV as compared to control plants (Wang *et al*., 2000). Similarly, Kusaba and his collegues contributed in the reduction of glutenin in the rice varieties. These low glutenin content (LGC-1) rice varieties were relief for those patients who have difficulty in glutenin digestion (Kusaba *et al*., 2003; Williams *et al*., 2004). Another significant contribution of RNAi is the development of *Banana bract mosaic virus* resistant banana varieties through silencing of coat protein gene of BBrMV (Williams *et al*., 2004). Moreover, RNAi technology was also used to regulate the level of gossypol in cotton seeds. Naturally, gossypol in vegetative parts of cotton plant is beneficial for insect repellent. However, its production in seeds reduced the utilization of surplus proteins stored in seeds. RNAi construct was developed by subsequently fusing the d-cadinene synthase gene (gossypol synthesis gene) with the seed specific promoter sequence (Sunilkumar *et al*., 2006). These transgenic cotton plants proved to be excellent insect repellent along with seeds producing high protein contents (Tang *et al*., 2007). Another application of RNAi is the down regulation of a vital enzyme (named as Corchorus ssps: Corchorus capsularis and Corchorus olitorius) involved in the lignin

biosynthetic pathway RNAi constructs targeted the 4-coumarate: CoA ligase (4-Cl) key enzyme to down-regulate the lignin synthesis. It leads to the production of low lignin content jute varieties (Williams *et al*., 2004).

Furthermore, blue color rose and carnations were produced for the ornamental purposes by downregulating the biosynthetic pathway of cyanidin gene (responsible for the production of red pigment in flowers) and introduced the Delphinidin gene responsible for blue pigment synthesis (Van Uyen, 2006). Beside this, the best oil for human health is the one which has high level of oleic acid content in it such as olive oil. But number of genes are involved which naturally convert oleic acid into different fatty acids. Therefore, RNAi approach silenced these genes which are involved in the degradation of oleic acid (Waterhouse *et al*., 1998). Apart from these, wood and fruit quality improvement, pests control and regulation of flavonoids and carotenoid in tomato has also been successfully achieved (Hu *et al*., 1999; Levin *et al*., 2003; Amancio *et al*., 2007).

1.5 Importance of present study

Sugarcane is significant and highly valueable cash crop growing in 105 countries of the world. Brazil ranks 1st position for growing sugarcane in the world. However, Pakistan is on 4th in terms of area, 14th for its production and 60th on the basis of its yield. Although, Pakistan is on world's 4th largest grower of sugarcane but it accounts the lowest yield in the world. World's average yield for sugarcane is about 65 metric tonnes per hectare. However, Asia 65.4 while China 77.1, Pakistan 46.0, India 70.6, Philippines 92.6, Australia 75.5, Thailand 92.6, and Egypt 105 tonnes per hectare production, respectively. However, Pakistan produces about 99% of the sugar from sugarcane. Sugarcane is an important cash crop of Pakistan which plays a pivotal role in the economy of our country. Sugarcane added up 3.4

percent increase in agriculture and 0.7 percent in GDP. (www.http://edu.par.com.pk/wiki/sugarcane).

Pakistan has all the resources to produce high yield of sugarcane, but still it has not accomplished to export sugar to the international market. This is because of reduction of estimated yield due to numerous biotic and abiotic factors. Biotic factors mainly include bacteria, fungi, phytoplasma and viruses (Bock and Baily, 1989). Approximately, 50 diseases of sugarcane have been reported to be cause by phytoplasma and viruses. Among them, *Sugarcane mosaic virus* caused 6-10% sugar yield loses and 10-32% cane reduction in Pakistan every year (Anwar, 2005). However, in the year 2008, 2009 and 2010 about 38% of sugarcane loses were observed in Punjab province and approximately 32% of sugarcane loses were recorded in KPK province of Pakistan (Yasmin *et al*., 2011). SCMV disease has been reported in more than 70 countries (Jeffrey *et al*., 1998). High occurrence of disease incidence is due to cultivation of susceptible sugarcane germplasm and presence of variety of insect pests which are responsible for the transmission of virus. In recent years, lot of molecular work was done relevant to understanding the basic mechanism of viral infection, their life cycle and strategies to control virus spread. Various techniques have been developed to control the virus infection but due to high rate of mutation and recombination viruses have potency to skip the barriers. Although a lot of efforts have been done in this field but still there is capacity to do more work to develop appropriate control measures. Present study is conducted to understand the molecular diversity of virus in various regions of Pakistan along with the efforts to develop model resistance crops against the SCMV.

1.6 Objectives of present study

Mosaic, caused by *Sugarcane mosaic virus*, is amongst the major diseases of sugarcane in the world including Pakistan. It causes extensive yield loses in the country when its infection rate reaches upto 50%. Following are the main objectives of present study.

1. Determination of *Sugarcane mosaic virus* in different areas of Pakistan.
2. Development of artificial microRNA/hairpin RNA based binary constructs.
    a. Development of amiRNA/hpRNA constructs targeting conserved regions of *CP* and *Hc-Pro* genes of SCMV.
    b. Agrobacterium mediated *N. benthamiana/N. tobaccum/ O. sativa* transformation.
    c. Expression analysis of amiRNA/hpRNA constructs.
    d. Screening transgenes for resistance against SCMV.
3. Comparison of amiRNA and hpRNA strategies.

Chapter 2

MATERIALS & METHODS

2.1 Nucleic acid isolation

2.1.1 Total RNA extraction

Total ribonucleic acid (RNA) extraction was done using TRIzol® Reagent. Approximately 0.1 gram of leaf sample was grinded into fine powder with pestle and mortar by using liquid nitrogen. Powdered sample was mixed with 1mL TRIzol reagent (Guinidium isothiocynate and phenol solution) (Thermo Fisher Scientific) to form suspension. Sample mixture was then incubated at 4 ^{o}C for 5 min during which cell disruption occurred while maintaining RNA integrity. Phase separation was initiated after the addition of 200 µL chloroform. Mixture was shaked well and then incubating it for 10 min on ice. After incubation, slurry was spinned at 12,000 g for 15 min in chilled conditions (4^{o}C) to avoid degradation of RNA. Two distinct phases, upper aqueous phase and lower organic phase were appeared after centrifugation. Total RNA was present in upper aqueous phase while DNA and proteins were suspended in interphase and organic phase, respectively. Upper clear phase containing RNA was transferred into a new centrifuge tube. RNA precipitation was started with the addition of chilled isopropanol (500 µL), followed by its incubation at 4 ^{o}C for 15 min. Pellet was obtained by centrifugation of precipitated RNA. Supernatant was discarded and pellet was washed with 1 mL of 75% ethanol to remove contamination. After washing, pellet was dried and dissolved in 40 µL of 1X TE (Tris-EDTA) buffer. Finally, extracted RNA was visualized on 1.5% agarose gel and stored at -80 ^{o}C for further use.

2.1.2 Extraction of genomic DNA

Genomic DNA was extracted from rice transgenic plants for Southern blotting and PCR analysis using Doyle and Doyle (1990) protocol with minor modifications. Fresh leaf sample (1g) was grinded with pestle and mortar using liquid nitrogen. CTAB buffer (50 mM Tris HCl pH 8.0, 0.7 M NaCl, 10 mM EDTA, 20 mM 2-Merceptoethanol and 1% CTAB (cetyl triethyl ammonium bromide) was heated at 65 ^{o}C in water bath. Then, about 25 mL of heated CTAB buffer was mixed in the powder and incubated for 30 min at 65 ^{o}C. After incubation, heated slurry was centrifuged at 3000 g for 20 min. Centrifugation separated the mixture into two distinct layers. Upper aqueous layer was collected in to a new falcon tube and chilled isopropanol was added in equivalent amount it. After gentle mixing, tubes were left overnight at room temperature for precipitation of DNA from mixture. Overnight precipitated mixture was then centrifuged at 3000 g for 15 min. Precipitated DNA threads formed a pellet at the bottom of tube which was then washed with 70% ethanol for removal of impurities. Purified DNA pellet was dissolved in 1X TE (1 mM EDTA, 10 mM Tris HCl) buffer and stored at -20 ^{o}C for further use.

2.2 Quantification of nucleic acids

Total nucleic acid concentration was calculated through spectrophotometer at 260 nm and 280 nm wavelength absorbance. 260 nm and 280 nm ratio is used for the calculation of DNA and RNA concentration. Good quality DNA and RNA prep have 260/280 ratio about 1.8 and 2, respectively.

2.3 Analysis of DNA and RNA

2.3.1 Agarose gel electrophoresis

To visualized DNA, 1% agarose gel was prepared. 0.4 grams of agarose was added into 40 mL 1X TAE [0.5 mM EDTA (pH 8.0) and 20 mM Tris Acetate]. Suspension was heated till the clear solution obtained. 0.01% ethidium bromide was added to gel solution, once it cooled. Suitable caster with required comb was filled with precooled gel mixture. Subsequently, solidified gel was loaded with DNA samples and run at 90 volts for 40 min. Dolphin Doc transilluminator was used for visualization of RNA bands.

2.3.2 Formaldehyde agarose gel for RNA

For RNA samples, formaldehyde gel was prepared by weighing 0.6 grams agarose and 31 mL of distilled water. Mixture was heated until agarose was completely dissolved in water. Then, 10 mL of 1X TBE (Tris-Borate EDTA) and 9 mL of 37% deinonized formaldehyde was added to the gel mixture whose temperature was lowered at about 70 ^{o}C. The gel was allowed to set at room temperature for 1 hour in laminar hood. Once the gel was solidified, it was pre-run in 1X TBE running buffer for about 15 min. For loading on gel, 15 µg of RNA sample was mixed with 3.5 µL formamide, 10 µL formaldehyde and 2 µL of 1X TBE. Samples were heated at 65 ^{o}C for 15 min and then loaded on gel along with gel loading buffer (0.25% xylene cyanol FF, 1 mM EDTA pH 8.0, 50% glycerol and 0.25% BPB). Gel was allowed to run for 3 hours at 85V.

2.4 PCR amplification and cloning of PCR product

2.4.1 Reverse transcriptase PCR (RT-PCR)

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is used for qualitative detection of gene of interest by reverse transcribing RNA in to complementary DNA (cDNA). cDNA synthesized was further amplified through traditional PCR technique.

2.4.1.1 Complementary DNA (cDNA) synthesis

RNA to complementary DNA synthesis was occurred through two incubation steps. Following components were used in the step one; 1 µL of gene specific reverse primer (5 µM) and 8 µL of RNA (600 ng/µL). Total reaction mixture was incubated in PCR machine for 10 min at 70 ^{o}C. After completion of incubation, tubes were placed on ice for 5 min.

In the step 2, 200 U/ µL U of M-MLV (Moloney-Murine Leukemia Virus) reverse transcriptase enzyme (1 µL), 40U/ µL RNase inhibitor (0.5 µL), 10 mM dNTPs (2 µL) and 5X reverse transcriptase buffer (4 µL) was used. Total 8 µL reaction mixture from step 2 was added to 12 µL cooled mixture of step one. Overall 20 µL mixture was heated at 42 ^{o}C for an hour. Synthesized cDNA was used for amplification of targeted gene and stored at -20 ^{o}C.

2.4.1.2 PCR amplification

Total 50 µL reaction for amplification of targeted gene contained 10X *Taq* buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) 100 pM primers, 1 Unit of *Taq* polymerase and 5 µL cDNA. PCR reaction mixture was placed in thermal cycler which was set (according to the primer used) (Appendix A) at following conditions: Initialization step was at 94 ^{o}C for 1 min, continued by 40 cycles of denaturation at 94 ^{o}C for 30s, annealing at 51 ^{o}C for 30s and extension for 1 min at 68 ^{o}C. However, final extension was done at 68 ^{o}C for 7 min. PCR product was run on 1% agarose gel.

2.4.2 Ligation of PCR product in to pTZ57R/T vector

Purified DNA was then ligated with pTZ57R/T vector according to manufacturer's protocol (InsTAclone^{TM} PCR cloning kit, Fermentas). Total 30 µL ligation reaction contained, 6 µL of 5X ligation buffer (250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000), 350-400 ng of DNA, 3 µL of pTZ57R/T vector and 1 Unit of T4 DNA ligase (1 µL). Ligation mixture was kept overnight at 4 ^{o}C in centrifuge tubes which was then utilized for *DH5α* competent cells transformation.

2.4.3 Plasmid isolation from *Escherichia coli* (*E. coli*)

Single white colony of *E.coli* was selected and added into LB (Luria Bertani) (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) broth media (Sigma-Aldrich) (10 mL) containing appropriate antibiotic. 16 hours incubation was done at 37 ^{o}C (130 rpm) for growth of bacteria. Media color and odor was changed due to bacterial cells multiplication. Culture was then centrifuged by shifting it into 1.5 mL eppendrof tubes at 12000 g for 15 min. Bacterial cells were clumped to form pellet which was used to extract plasmid by using Gene Jet Plasmid Miniprep kit (Thermo Scientific^{TM}; Cat # K0502) according to manufacturer's protocol. Supernatant was discarded and re-suspension solution (about 250 µL) was added to dissolve the pellet. Next, lysis solution (250 µL) was incorporated which lysed the bacterial cells by gentle mixing. Then, 350 µL of neutralization solution was added in suspension. Suspension was briefly mixed and centrifuged at maximum speed for 5 min. Pellet was settled down and clear solution containing bacterial plasmids started floating above. Supernatant was added into column provided into kit and spin at maximum speed for 2 min. Flow through was discarded and column was washed twice through washing buffer (500 µL per wash). Superfluous washing buffer was removed through an additional centrifugation step. Finally, elution buffer was added into column, left it for 2 min and again

centrifuge for 2 min at 12,000 g. Purified plasmids were collected in eppendrof tubes which were run on 1% agarose gel to analyze it further.

2.4.4 Glycerol stock preparation

Liquid cultures of *E.coli* and Agrobacterium were preserved in glycerol solution. 800 µL of grown cultures were added in 200 µL of autoclaved 100% glycerol and preserved in -80 ^{o}C.

2.4.5 Restriction digestion

Desired insert was cloned in binary vector by restriction digestion. Restriction digestion was performed on both insert and vector, separately. 1 U of enzyme was used in accordance with its suitable buffers for digesting 1 µg of DNA. Reaction conditions were adjusted according to NEB Cutter V2.0 (www.tools.neb.com/NEBcutter2/index.php).

2.4.6 Phosphatase treatment of vector

Linear vector digested with same enzyme as used for insert, was subjected to phosphatase treatment to avoid self-ligation of vector. Dephosphorylation of vector was done at 37 ^{o}C for half an hour and then enzyme inactivation at 75 ^{o}C for 10 min.

2.4.7 Cloning into binary vectors

Expression cassette was ligated into digested dicot and monocot binary vectors which were then transformed into *E.coli DH5α* electrocompetent cells. Colonies appeared were screened through restriction digestion for confirmation of positive clones. Plasmids were then transformed into *Agrobacterium tumefaciens* AGL1 strain by electroporation method.

2.4.8 DNA sequencing

Sequencing reaction was performed on the purified plasmids. Total 20 µL sequencing reaction consisted of following components: Plasmid DNA (600-800 ng/µL), 5X sequencing

buffer (400 mM Tris HCl pH 9.0 and 10 mM $MgCl_2$) 3.5 µL, 1 µL big dye, 1 µL of each primer (forward and reverse) (5µM). The reaction conditions were as follow: Initial denaturation at 96 ^oC for 2 min, proceeded by 30 cycles of denaturation at 96 ^oC for 5s, annealing at 50 ^oC for 15s and extension at 60 ^oC for 4 min and final extension at 60 ^oC for 10 min. PCR products were purified before submitted to sequencing. For purification, $1/10^{th}$ volume of sodium acetate and 2.5 vol of 100% ethanol was added in PCR product. Mixture was incubated at room temperature for 20 min and centrifuged for 30 min at maximum speed. Pellet formed, was washed with 70% ethanol by centrifuging the tube again at 12,000 g for 20 min. Supernatant was discarded and pellet was dried using speed vacuum for 3 min and submitted for sequencing.

2.5 Vector manipulations and development of constructs

2.5.1 Fusion PCR strategy for artificial microRNA based binary construct

In this study, amiRNA constructs were designed to target DAG and KITC conserved region of *CP* and *Hc-Pro* genes of *Sugarcane mosaic virus*. Vector template used for synthesis of amiRNA constructs contained naturally occurring miR319a Arabidopsis (*Arabidopsis thaliana*) stem loop in pRS300 vector (Figure 2.1). Web microRNA designer (WMD: http://wmd3.Weigelworld.org) predicted the suitable gene sequences and design primers for amiRNA construction which allows gene silencing in plant species. Fusion PCR scheme was designed which mutate the stem portion of miR*/miR with amiR*/amiR (Table 2.1) (Figure 2.2). 40-mer oligonucleotide primers were used in four PCRs. Primers sequences are mentioned in the above mentioned (Appendix A).

PCR (a), (b) and (c) amplified the stem loop portion resulting in the product in which PCR (b) product overlapped at one end to PCR (a) product and other end to PCR (c)

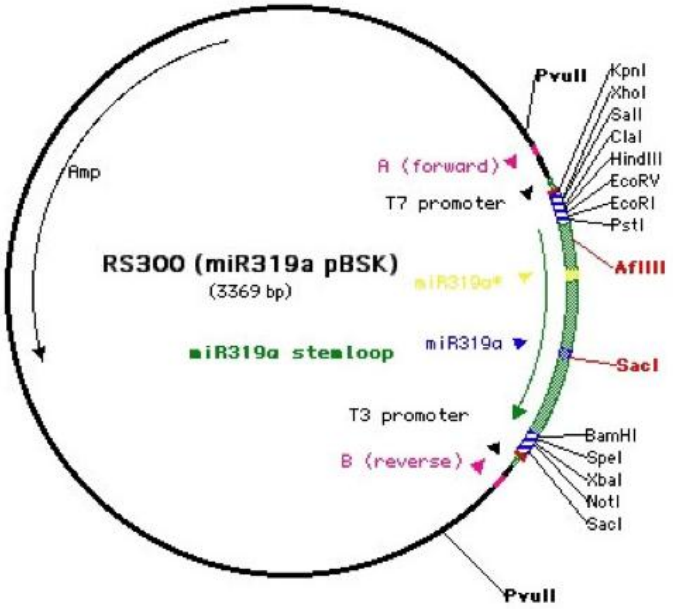

Figure 2.1: Map of RS300 vector with naturally occurring miR319a stem loop portion. A and B are forward and reverse primers specific to vector; Unique restriction sites are shown.

Table 2.1: Fusion PCR scheme for the development of amiRNA construct.

PCR Reactions	Forward Primer	Reverse Primer	Template	Product size
(a)	A	IV	pRS300	272 bp
(b)	III	II	pRS300	171 bp
(c)	I	B	pRS300	298 bp
(d)	A	B	(a)+(b)+(c)	701 bp

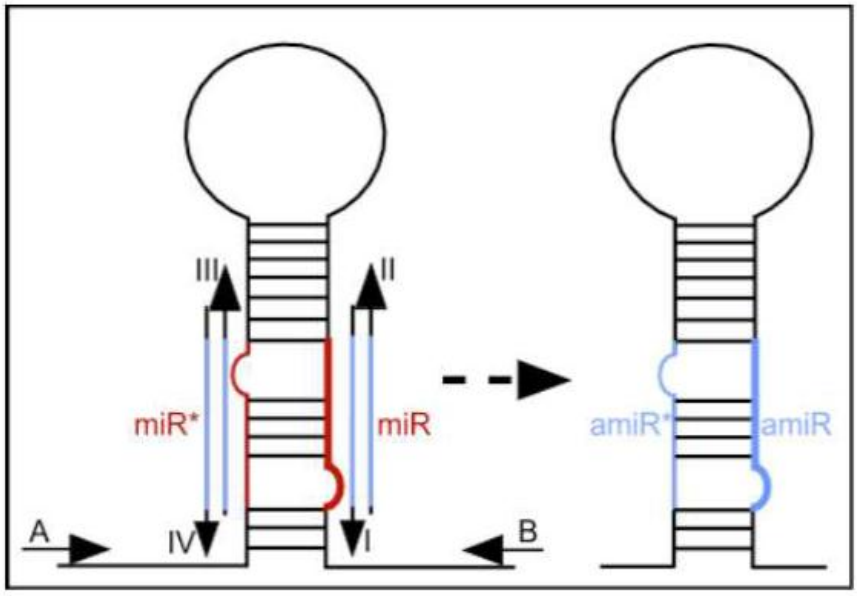

Figure 2.2: Strategy for fusion PCR scheme for the replacement of miR319a backbone (miR*/miR) with amiR*/amiR using primers specially designed through WMD3 software, targeting the sequences of *CP* and *Hc-Pro* (SCMV).

amplified product. Following PCR reagents were used in PCR (a), (b) and (c). pRS300 template (300 ng/µL) 0.5 µL, 2.5 mM $MgCl_2$ 1.5 µL, 5X GC buffer 5 µL, 2mM dNTPs 2 µL, 1U Phusion polymerase 0.5 µL and 10 µM oligos 2 µL. PCR conditions used for amplification were: Initial denaturation 98 ^{o}C for 30s, followed by 25 cycles of denaturation for 10s at 98 ^{o}C, annealing temperature for PCR (a) and (c) was 55 ^{o}C for 30s while for PCR (b) was 52 ^{o}C for 30s, extension occurred at 72 ^{o}C for 30s and final extension for 10 min at 72 ^{o}C.

Overlapped PCR products were then fused using primers A and B in PCR (d). PCR (d) reagents and conditions were same as described above in PCR (a) and (c). Fused product obtained was run on 1% agarose gel, eluted from gel and ligated with binary vector (Figure 2.3).

2.5.2 Hairpin vectors construction

Hairpin constructs with specific promoter for dicots as well as monocot targeting conserved regions of *CP* and *Hc-Pro* genes of SCMV were synthesized. Conserved regions of *CP* and *Hc-Pro* genes of SCMV, 240 bp each, were selected and fused into a chimeric fragment for use as the trigger DNA sequence in the hpRNA cassette. These targeted regions were selected by retrieving the multiple sequences of *CP* and *Hc-Pro* genes from NCBI database and aligned by using ClustalW (Thompson *et al*., 1994). A conserved sequence (240 bp) from each gene was selected to form the fusion sequence. This CP:Hc-Pro fusion sequence was synthesized by Gene-Art-^{TM} Gene Synthesis (Thermo_Fisher Scientific, Waltham, MA, USA), and assembled into the hpRNA cassette in both sense and antisense gene orientations. For directional cloning into the pKannibal and pStarling vector (http://www.plantindustry.csiro.au/RNAi/vectors.htm), restriction sites of *Kpn*I/*Xho*I or *Bam*HI/ *Hind*III and *Kpn*I /*Spe*I or *Bam*HI/ *Sma*I were incorporated at 5’ and 3’ end of fusion

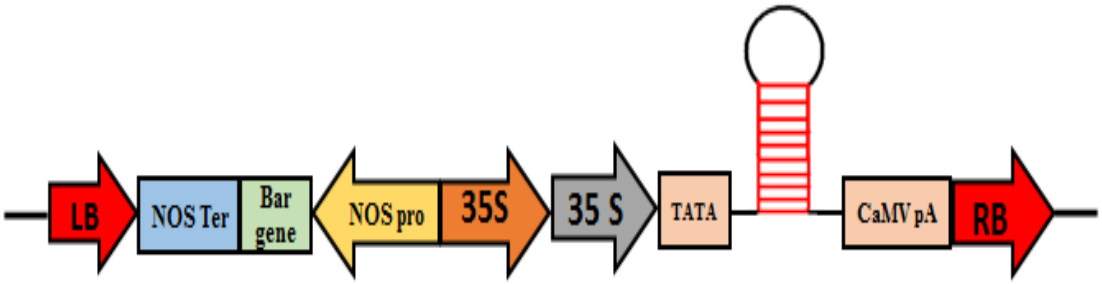

Figure 2.3: Schematic representation of pGreen0229 binary vector containing CP/Hc-Pro amiRNA construct (0.5 kb). Double 35S CaMV promoter transcribes the amiRNA gene, followed by TATA box and CaMV PolyA terminator signal.

fragment, respectively. The expression cassette in pKannibal and pStarling were excised with *Not*I and inserted into pWBVec2a and pWBVec8 binary plant expression vector (Wang *et al*., 1998) (Figure 2.4). The vector was transformed into *Agrobacterium tumefaciens* AGL1 strain by electroporation method for plant transformation. pWBVec2a and pWBVec8 binary vector was also transformed as an empty vector control.

2.5.3 Preparation of 35S-GUS:CP:Hc-Pro fusion target construct

The synthesized CP:Hc-Pro fusion fragment was transcriptionally fused with coding sequence of *E. coli β*-glucuronidase gene (GUS) which was sub-cloned into pART7 (Gleave, 1992) at the *Bam*HI and *Xho*I sites between the CaMV 35S promoter and the Octopine synthase (OCS) terminator. The expression cassette was then excised with *Not*I and cloned into pWBVec2a to form the binary vector suitable for Agrobacterium-mediated transformation (Figure 2.5).

2.5.4 Preparation of 35S-GUS:Y-Sat fusion constructs

Binary vector containing GUS construct (35S-GUS:Y-Sat), with a 3' fusion of the *Cucumber mosaic virus* Y-satellite RNA sequence (Zahid *et al*., 2015), was included as a control target (Figure 2.6).

2.5.5 Preparation of 35S-hairpin GUS vector

Hairpin GUS vectors used to analyze the efficiency of GUS vectors. Preparation of hpGUS vector was described in (Schumann *et al*., 2013) (Figure 2.7).

2.5.6 Preparation of 35S-GFP Vector

Green florescent protein (GFP) was cloned between 35S promoter and OCS terminator was used as control in Agro-infiltration experiments in dicot (Figure 2.8).

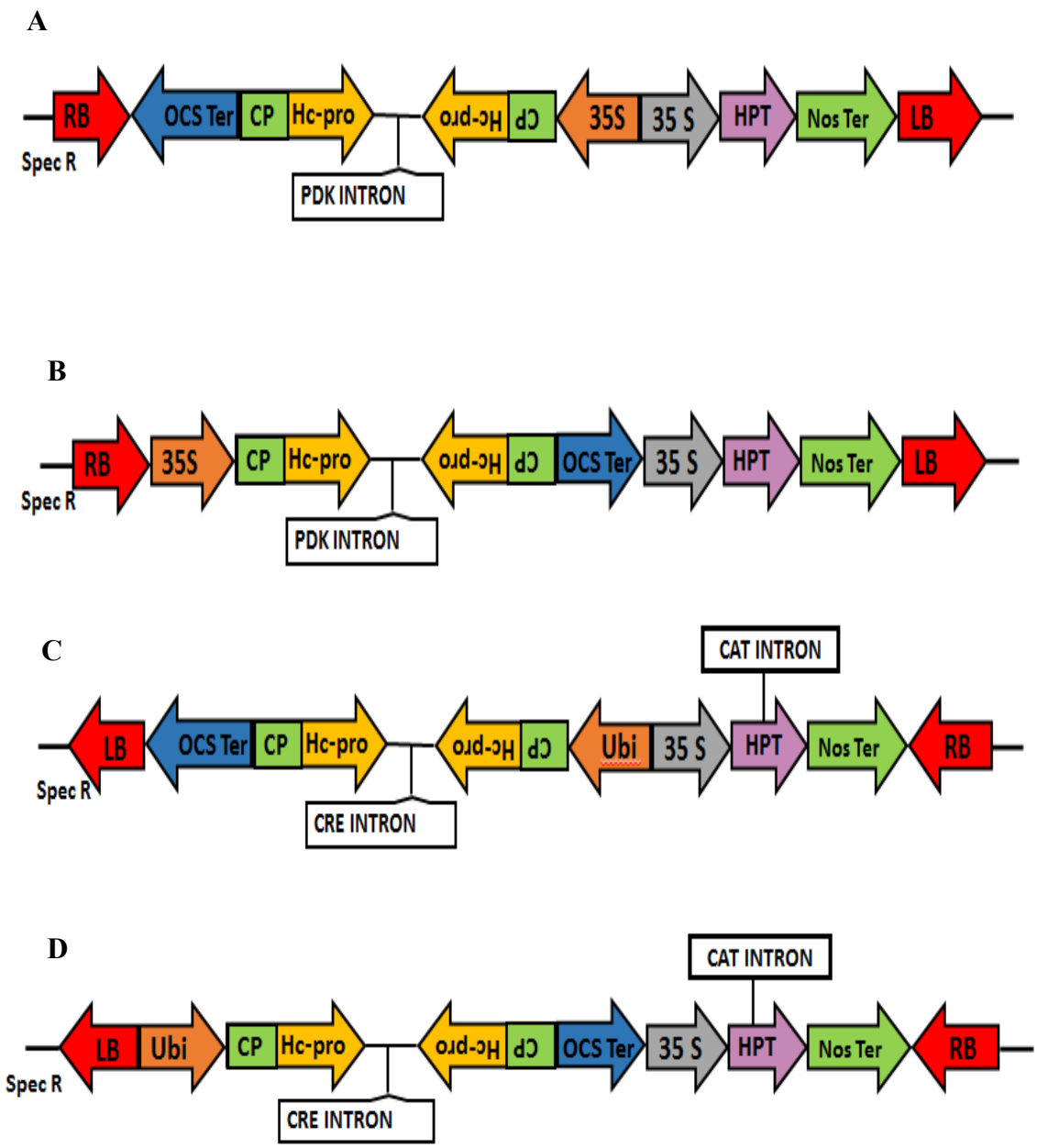

Figure 2.4: Schematic representation of dicot and monocot hairpin RNA binary constructs. (A, B) Hairpin RNA dicot binary vector (35S-hpCP:Hc-Pro) with expression cassette in sense and antisense orientations a, b. Inverted repeat (CP: Hc-Pro) fused sequence was directed by 35S *Cauliflower mosaic virus* (CaMV) promoter spaced by PDK (Pyruvate dehydrogenase kinase) intron and terminated by Agrobacterium octopine synthase gene terminator sequence (OCS). (C, D) Hairpin RNA monocot binary vector (Ubi-hpCP:Hc-Pro) with expression cassette in sense and antisense orientations a, b. Inverted repeat (CP: Hc-Pro) fused sequence was driven by maize Ubiquitin promoter spaced by CRE intron and terminated by OCS terminator. Binary vectors contained hygromycin phosphate transferase gene for hygromycin resistance (HPT) as a plant selectable marker. Nos Terminator (Agrobacterium nopaline synthase gene terminator); CAT INTRON, first intron of castor bean catalase-1 gene.

Figure 2.5: Reporter gene by cloning (CP:Hc-Pro) fused sequence in GUS binary vector. GUS fused gene (GUS:CP:Hc-Pro) transcribed by 35S (CaMV) promoter and terminated by OCS terminator. A hygromycin (HPT) antibiotic was used as plant selection marker.

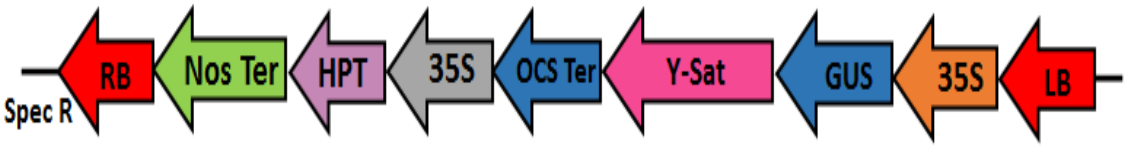

Figure 2.6: 35S-GUS:Y-Sat gene cloned between 35S promoter and OCS terminator. Plant selection marker is hygromycin.

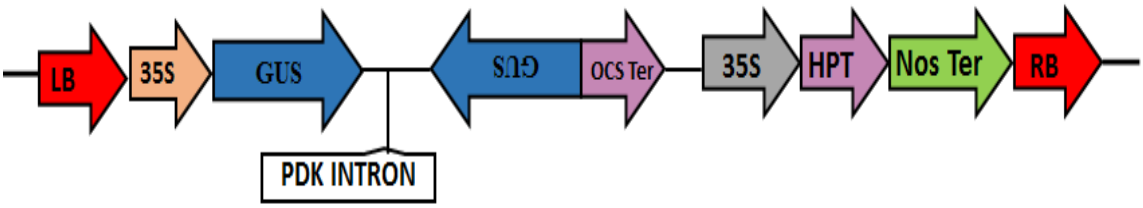

Figure 2.7: 35S-Hairpin GUS vector with 35S (CaMV) promoter, OCS terminator and hygromycin (HPT) as plant selection.

Figure 2.8: Green florescent protein (GFP) vector with 35S promoter and OCS terminator.

2.6 Microbiology techniques

2.6.1 Preparation of *DH5α* competent cells

Competent cells of *DH5*α strain were prepared by inoculating single *DH5*α colony from streaked plate into 10 mL of LB broth without antibiotics. Cultures were incubated at 37 ^{o}C in shaking incubator until O.D. reached at 0.3-0.4. Cells at their exponential stage were then transferred to 50 mL falcon tube and spinned at 3000 g for 20 min. Pellet formed was dissolved in 10 mL of ice cold 80 mM $MgCl_2$+ 20 mM $CaCl_2$. Again centrifugation at 3000 g for 10 min and re-suspension of pellet was done in ice cold 0.1 M $CaCl_2$ + 15% glycerol. Competent cells were distributed into small aliquots and kept at -80 ^{o}C.

2.6.2 Transformation of *DH5α* competent cells

Transformation of *E. coli* competent cells were done through heat shock method. Ligation mixture was added into thawed competent cells in laminar hood. Competent cells were then chilled on ice for half an hour. These cells were further given heat shock at 42 ^{o}C for 2 min through which foreign DNA entered into competent cells. Again 2 min chilling on ice was carried out. 1 mL L.B broth was added in centrifuge tubes for bacterial cells growth and left in incubator at 37 ^{o}C for 2 hours. Transformed cells were spread on agar plates containing appropriate antibiotics as a selectable marker while for blue white screening IPTG (Isopropyl *β*-D-1-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside) was added as a screening marker. Plates were left for 16 hours incubation at 37 ^{o}C which allow appearance of colonies.

2.6.3 Preparation of *Agrobacterium tumefaciens* competent cells

For the preparation of Agrobacterium competent cells, isolated colony of Agrobacterium was picked from the Agrobacterium streaked LB plate, inoculated into LB media and incubated at 28 ^{o}C overnight. Overnight culture was refreshed by adding 100 µL

of this starter culture into 200 mL of fresh LB media and again incubated at moderate shaking at 28 ^{o}C for few hours until its O.D. reached at 0.6. Once required O.D. was obtained, culture was incubated in ice for 30 min before proceeding to centrifugation. Centrifugation of Agro cells was done at 3000 g for 15 min to form cells pellet. Pellet was washed with 1 mL of ice cold 10% glycerol solution and spin again. Pellet formed was again washed as mentioned above and dissolve in 1 mL of 10% glycerol solution. 50 µL of competent cells were divided into eppendorfs and stored at -80 ^{o}C.

2.6.4 Transformation into Agrobacterium

Binary vectors were transformed into *Agrobacterium tumefaciens* AGL1 strain through electroporation method. 2 µL of plasmid was added into the competent cells and incubated for 5 min on ice. Mixture was then injected into the chilled cuvettes and set in the electroporation chamber for electric shock. Apparatus was set at 1.44 KV and start button was pressed to discharge the current. Immediately after the electric impulse LB media was added in the cuvette, mixed gently and whole culture was shifted to the sterile centrifuge tubes. Culture was incubated for 4-5 hours at 28 ^{o}C, followed by spreading on LB media plates with appropriate antibiotics.

2.7 Histochemical analysis

2.7.1 Agro-infiltration of *Nicotiana benthamiana* leaves

Agrobacterium cultures were incubated overnight in the shaker at 28 ^{o}C in 2 mL LB liquid media with 50 mg/mL spectinomycin and 50 mg/mL rifampicin as selection antibiotics. To refresh the cultures, 20 µL of grown cultures was added into 10 mL of LB media with the antibiotics used in the starter cultures and incubated for 24 hours at 28 ^{o}C. Then, 100 µM acetosyringone was added into overnight Agrobacterium cultures and again

kept in 28 ^{o}C shaking incubator for one hour. After incubation, centrifugation of cultures was done at 3000 g for 5 min. Pellet was formed which then dissolved in infiltration media (50 mM MES + 2mM MgSO4) with acetosyringone (100 µM) to make final $O.D_{600}$ of 0.3. Agrobacterium suspension was infiltrated into *N. benthamiana* leaves using 10 mL syringe. After infiltration, plants were again kept in growth room at 25 ^{o}C for three to four days.

2.7.2 GUS (*β*-Glucuronidase) histochemical assays

GUS histochemical assay was performed according to standard published protocol (Jefferson, 1987). Following the protocol, experimental leaves samples were immersed in the GUS staining solution (50 mM $NaPO_4$ (pH 7.2), 0.5% Triton X-100, 10 mM EDTA, 0.5 mM Potassium Ferrocyanide ($K_4F_3(CN)_6$), 0.5 mM Potassium Ferricyanide ($K_3Fe_3(CN)_6$:H_2O) and 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) dissolved in 100mM dimethyl sulfoxide) and vacuum infiltrated for 5-10 min. Leaves samples were incubated with staining solution for 3-4 hours at 37 ^{o}C. Samples were then washed with 70% ethanol until all chlorophyll contents was removed. *β*-glucuronidase staining pattern was captured using florescent microscope.

2.7.3 Fluorescent β galactosidase (MUG) assay

Agro-infiltrated leaf samples were grinded and powder sample was transferred to pre-cold centrifuge tube. About 120 µL of protein extraction buffer (150 mM Sodium Phosphate pH 7.0, 140 µM PMSF, 10 mM EDTA, 0.1% Triton X-100, 10 mM *β*-mercaptoethanol and 0.1% sarcosyl) was added to the powder and re-suspended. Centrifugation of mixture was done at maximum speed for 5 min. Clear phase appears on the top which was transferred to the 96 well plates. Plate reader temperature was already set according to standard protocol at 37 ^{o}C. MUG solution (1.2 mM 4-methylumbelliferyl *β*-D-glucuronide) about 100 µL was added into plate, mixed well and incubated at 37 ^{o}C for 2 min on water bath and placed in

plate reader. Protein concentration was estimated by standard Bradford assay. 40 µL of Bio-Rad protein assay reagent was dissolved in water and 0.5 µg/µL of BSA was used to make the dilution series. Samples volume was adjusted according to the color of BSA from serial dilutions. GUS activity was estimated using relative ratios of protein amount in cell extract using Microsoft excel software.

2.7.4 Particle bombardment using gene gun

Plasmid DNA about 1 µg concentration was mixed with 0.6 micron size of gold particles that was vortexed briefly for homogenization. 2.5 M $CaCl_2$ (16 µL) was added later to the homogenized mixture, which aids in binding of negatively charged DNA molecules to gold particles. After brief vortexing, 0.1 M spermidine solution (6.4 µL) was added into the mixture that provides protection to the suspension from endonucleases. Master mix prepared was bombarded with the help of PDS 1000-He Biolistic particle delivery system at 1100 Psi with vacuum pressure of 26 inch Hg at 6 cm optimized target distance.

2.8 Agro-bacterium mediated plant transformation

2.8.1 *Nicotiana Tobacum* transformation

3-4 weeks small plantlets of *N. tobaccum* were ready to use for transformation through leaf disc method (Horsch *et al*., 1985). Agrobacterium was streaked on LB plates containing required antibiotics for selection and incubated for 2 days at 28 ^{o}C in dark. Substantial bacterial growth on plates was scratched and mixed gently through pipetting by adding liquid MS media. Leaf discs about 1 cm in width and length were prepared by cutting the tobacco leaves with scalpel blade in the bacterial suspension. Agrobacterium started infecting the cutting edges of leaves. Leaf discs were first washed with autoclaved water, then

blotted on filter paper to get rid of excess Agrobacterium and placed on MSO plates for 2 days in dark. Media composition is mentioned in the appendix B.

After co-cultivation, discs were shifted into shoot inducing media (MS9) containing MSO plus 150 mg/mL timentin and desirable plant selectable marker. Plates were sealed with parafilm and kept them in 24 ^{o}C growth room for 3-4 weeks until shoots started appearing. Shoots with apical buds trimmed from the callus and transferred to the small glass bottles containing root inducing media with antibiotics. Glass bottles were left in growth room (24 ^{o}C) until small roots originated from shoots. Rooted plantlets were shifted to compost soil in glass house and covered with small glass pots to evade desiccation and removed them once plants were hardened.

2.8.2 Rice transformation

Agrobacterium glycerol stocks were streaked on LB plates (Rifampicin + spectinomycin) and kept them in 28 ^{o}C incubator for bacterial growth. When bacterial lawn was appeared on LB plates, liquid NB media containing 100 µM acetosyringone was poured on to bacterial lawn and transferred to new plate after scraping the Agrobacterium. Rice calli were then dipped in suspension for 10-15 min for Agrobacterium infection. Rice calli were drained and blotted on to filter paper to remove excess bacterium. Calli were then transferred onto NBA plates (NB plus 100 µM acetosyringone) and incubate them for 2-3 days in dark at 28 ^{o}C. After co-cultivation, calli were washed with sterile water for 3-4 times containing 150 µg/mL timentin. Washed calli were shifted on NBHT50 media plates after drying them on filter paper. Plates were sealed with parafilm to avoid the contaminants and kept in the darkness for 3-4 weeks. New resistant calli started appearing after 24-30 days of infection. Healthy growing calli were then shifted to the PRHT50 media plates and plates were left in half-darkness for 8-12 days. Each growing callus was marked as a separate transformation

event. Potential growing calli were transferred to the next media (RHT50) plates and kept under light at 28 ^{o}C for 30 days. Once roots and shoots were appeared, young plantlets were shifted to the half MS pots and left them under light for 10-14 days. Fortnightly grown plantlets were planted in soil in glass house for further growth. Pots were dipped in high water level for proper growth of rice plants at 28 ^{o}C. Media composition is mentioned in the appendix C.

2.9 Screening of transgenic plants

2.9.1 Northern blotting

2.9.1.1 Preparation of polyacrylamide gel

For preparation of 30 mL polyacrylamide gel, 12.6 grams urea was weighed in the weighing paper. 12.75 mL of 40% acrylamide/bis (19:1), 3 mL of 10X TBE and 5.25 mL of distill water was mixed with urea. Suspension was heated in 70 ^{o}C water bath until urea crystal dissolved in buffer and clear solution obtained. Then, gel loading apparatus was assembled. 15 µL of TEMED (NNN'N'-tetramethylethylenediamine) and 180 µL of 10% APS was added into warmed gel before pouring into plates. Polyacrylamide gel was allowed to solidify for one hour; meanwhile samples preparation was done for loading into gel.

2.9.1.2 Samples preparation

High quality total RNA (20-40 µg) extracted from samples was mixed with 12 µL of deinonized formamide/ BPB/ XCFF (10 mL formamide + 10 mg Bromophenol blue + 10 mg XCFF) and denatured for 2 min at 95 ^{o}C. Samples were loaded and gel was allowed to run at 25-30 mA in 1X TBE buffer for 3-4 hours.

2.9.1.3 Blotting to filter paper

Once gel run, it was ready to transfer to Hybond-N^+ membrane (GE Healthcare Amershamm, Rydalmere, NSW, Australia). Transfer cassette was assembled by placing the acrylamide gel on Hybond-N^+ membrane, sandwiched by two filter papers of same size as gel and whole cassette was dipped in 0.5X TBE buffer in electrophoresis tank. Complete electrical transfer of RNA to blotting paper took one hour. The membrane was then UV crosslink in UV strata crosslinker and stored in sheets of paper for further hybridization with probe.

2.9.1.4 Preparation and labeling of probe

Digested TOPO cloned fragment was used for the labelling of probe. T7 polymerase was used for in-vitro transcription of probe which consisted of following reaction components: transcription buffer (5X) 4 µL, 0.1 M DTT 2 µL, RNase block II 1 µL, ATP/GTP/CTP (2.5 mM each) 4 µL, 1 mM UTP 0.24 µL, oligonucleotide for labeling (~0.2 µg) 4 µL, radiolabelled ^{32}P-UTP 4 µL and T7 polymerase for transcription 1µL. Total 20 µL reaction was given incubation at 37 ^{o}C for an hour, then 1 µL of DNase was added with additional ten min of incubation at 37 ^{o}C. Precipitation of probe was done by adding ammonium acetate and 100% ethanol, incubated at 4 ^{o}C for ten min and then spin at 12,000 g for 15 min. Precipitation step was done twice and air dried RNA was finally re-suspended in 20 µL of 1X TE buffer.

2.9.1.5 Hybridization

Probe was hybridized with RNA samples on Hybond N^+ membrane using hybridization solution (125 mM Na_2PO_4 pH 7.2, 7% SDS, 250 mM $NaCl_2$, 50% formamide). Membrane was first pre-hybridized to avoid the non-specific binding of probe and then hybridized overnight at 42 ^{o}C. After hybridization, membrane was washed twofold with 2X

SSC/0.2% SDS at 42 ^{o}C. Hybridized membranes were visualized and recorded with phosphorimager (FLA-5000, Fuji Photo Film, Tokyo, Japan) after overnight exposure.

2.9.2 Southern blotting

Genomic DNA from transgenic rice plants were digested overnight with *Spe*I unique restriction site. Samples were run on 0.8% agarose gel at 41V for 16 hours. Gel was then soaked in 0.25M HCl for 30 min. Acid treated gel was washed twice with distill water for 15 min. Hybond N^{+} membrane (GE Healthcare Amersham) was placed on the gel which was sandwiched with filter papers to transfer the DNA on to the membrane. Membrane was first pre-hybridized with 6X SSPE and 0.5% SDS solution at 42 ^{o}C for about 4 hours. Probe was prepared by excising the CP:Hc-Pro fragment from the TOPO vector with *Spe*I and *Xho*I enzyme followed by gel purification. Purified fragment was labeled with ^{32}P radioactive dCTP using Deca label-^{TM} DNA labeling kit (Thermo Scientific, St. Louis, MO, USA), and hybridized with membrane at 65 ^{o}C in hybridization buffer (20X SSPE 30 mL, 10% SDS 10mL, dH_2O 60mL) at 42 ^{o}C overnight. DNA blots were washed with 2X SSC buffer and 0.1% SDS solution three times for 15 min, with decreasing the SDS percentage in each washing step. Blots were visualized and recorded by FLA-5000 phosphorimager (Fuji Photo Film) after overnight exposure.

2.9.3 Quantitative real-time PCR (qRT-PCR)

Real time PCR analysis determined the expression and abundance of transcript gene. Primers used for real time analysis were generated by using online free software Primer3 (http://simgene.com/Primer3). Stringent conditions were adjusted with the product size range between 50-150 bp and primer Tm was adjusted between 59-65 ^{o}C. House keeping genes (actin, tubulin and elongation factor-1alpha) primers were synthesized along with gene specific primers (Appendix A).

Dilution series of RNA samples were used to optimize the RNA concentration and to detect the primer efficiency. Standard curve was generated using series of RNA dilutions with the range of 1000 ng/µL to 1 ng/µL. Both house-keeping gene and gene specific primers were tested on each sample. Three replicates of each sample were run along with its multiple biological replicates. Total 10 µL reaction was run in PikoReal real time PCR machine which consisted of 5µL of (SensiMix^{TM} SYBR® No-ROX) SYBR green with fluorescein, 0.4 µL of both forward and reverse gene specific primer (10µM), 1 µL of RNA were used. 0.2 µL of 1U RNAse inhibitor and 0.1 µL of 1U Reverse transcriptase was used which was also available in SensiMix^{TM} SYBR® No-ROX kit for cDNA synthesis. A standard protocol for Sensimix was as followed: cDNA synthesis at 45 ^{o}C for 10 min, Initial denaturation 95 ^{o}C for 2 min, denaturation 95 ^{o}C for 5 s, annealing temperature 60 ^{o}C for 20 s and extension for 60 ^{o}C for 30 s .

To determine the efficiency of reference gene primers, a standard curve was designed by diluting the RNA concentration of control sample from 1000 ng to 1 ng. Each concentration was run in triplicate. RNA concentration of 10 ng was proved to be efficiently producing the maximum fluorescence (Figure 2.9; A, B). Standard curve was obtained which showed the linear relationship between RNA concentration and fluorescence produced by SYBR green (Figure 2.9; C, D).

The qPCR results were manually analysed using the Microsoft® Office Excel® 2007 software. The analysis was based on mathematical model for relative quantification of target transcript levels relative to a reference gene, as detailed by Mieog *et al*., 2013. The standard deviation for each set of replicates was also calculated using the Excel function. Results were then plotted as a column graph using Excel.

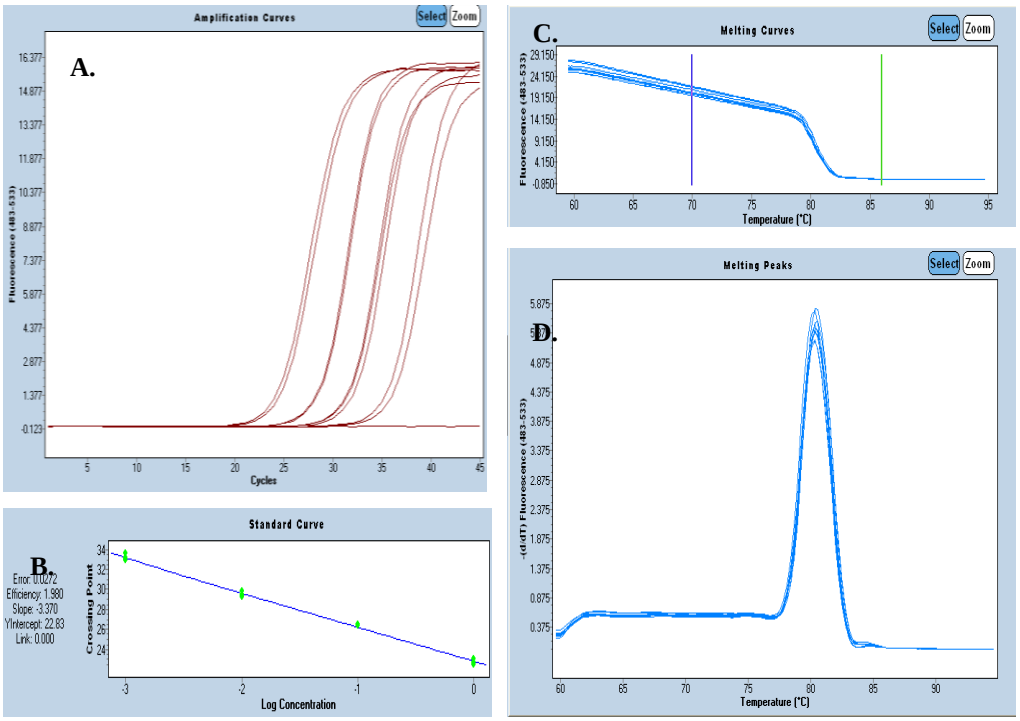

Figure 2.9: Evaluation of primer set for qPCR. **(A)** Fluorescence produced by using series of diluted RNA **(B)** Standard curve produced through Actin (Reference) gene primers. **(C)** Single curve peak obtained from the optimized concentration of RNA using reference gene primers. **(D)** The melt curve was obtained to ensure that only single product was produced and there is no unspecific binding of primers and formation of primer dimers.

2.10 Infectivity assays

Transgenic rice plants at 5-10 leaf stage were inoculated with SCMV by rubbing carborundum-dusted leaves with extract of SCMV-infected sugarcane leaves in 0.1 M sodium phosphate buffer (pH 7.0). Plants were again kept in the insect-proof container in the cabinet after inoculation. Symptoms were kept monitoring few weeks of post infection which were verified by RNA Northern blot hybridization, reverse transcriptase PCR and Real-Time PCR. Infected leaves were harvested at 25 days post inoculation (DPI), and total RNA was extracted using TRIzol reagent (ThermoFisher Scientific). The presence of SCMV was analyzed using RT-qPCR using a pair of CP and Hc-Pro specific primers (Appendix A).

Chapter 3

RESULTS

3.1 Determination of *Sugarcane mosaic virus* (SCMV) in different areas of Pakistan

3.1.1 Sample collection

Sugarcane leaf symptomatic and asymptomatic samples were collected from diverse sugarcane growing areas of Pakistan (Punjab, Khyber Pakhtoon Khwa (KPK), and Islamabad) during the year 2013-2014 (Figure 3.1). Map of Pakistan displaying the major sugarcane growing areas are highlighted (Figure 3.2). One of the sugarcane leaf samples which displayed the streak symptom was also collected from area of Islamabad (Table 3.1). Samples were well labeled in plastic zippers and stored at -80 ^{o}C for further processing.

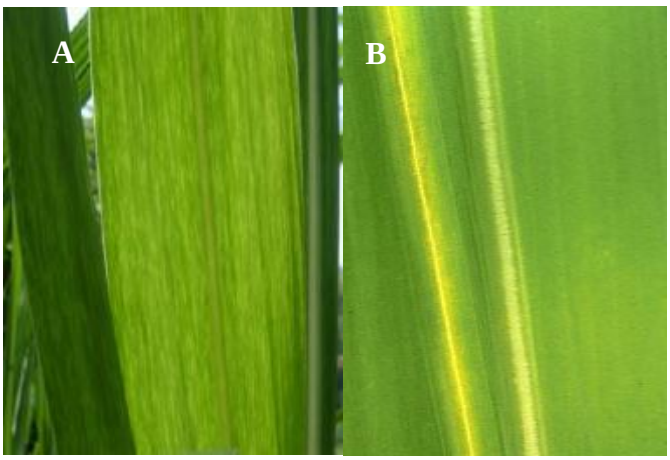

Figure 3.1: Sugarcane leaves samples showing typical mosaic symptoms (A) and asymptomatic leaves samples (B).

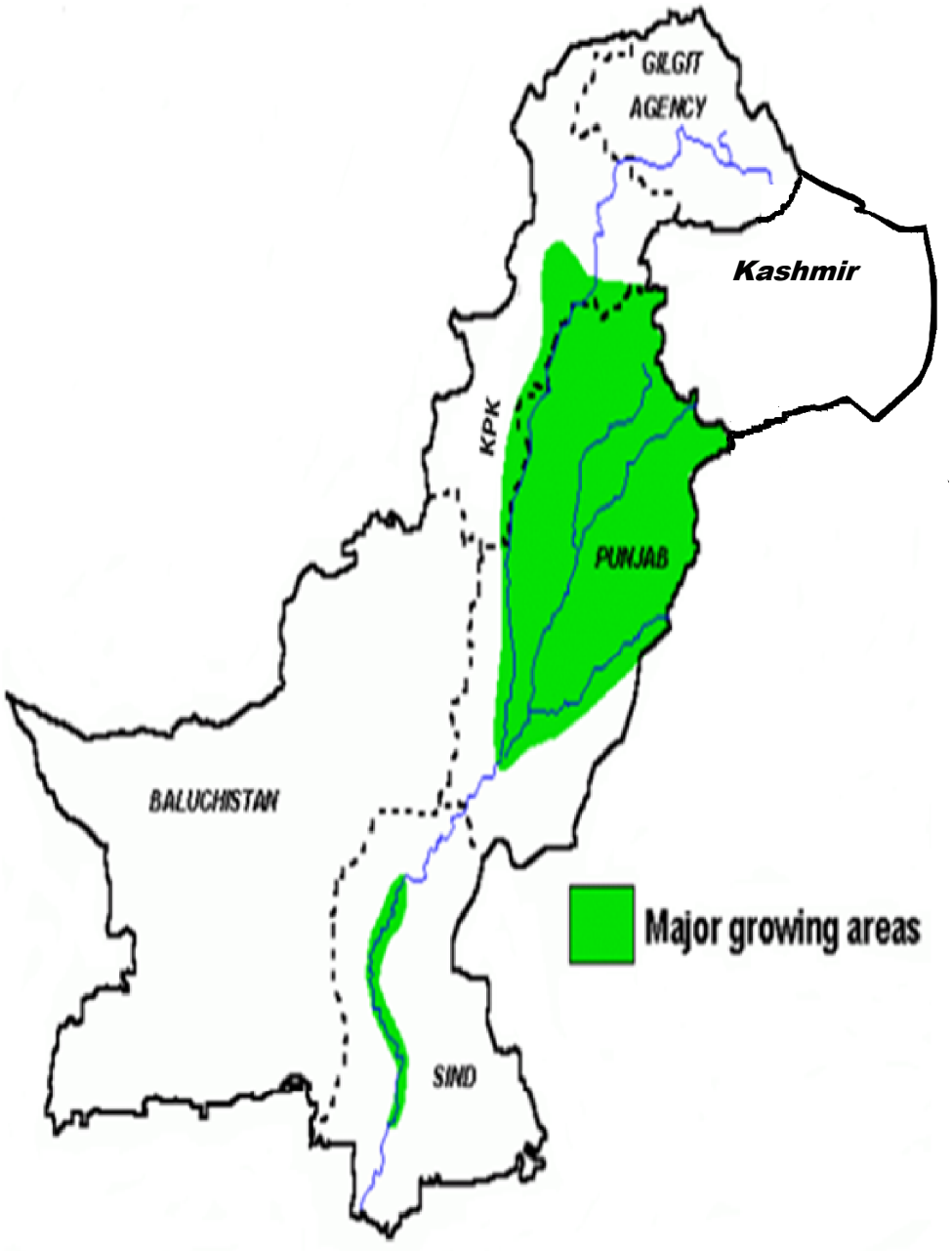

Figure 3.2: Map of Pakistan displaying sugarcane growing areas in green color.

Table 3.1: List of infected sugarcane leaves samples, sample codes, area of collection, accession no. and symptoms. *** NARC (National Agriculture Research Center)**

No.	Sample Code	Sample Name	Area of collection	Accession No.	Symptoms
1.	8ShA	US-640	*NARC (Faisalabad)	LM645013	Mosaic
2.	9ShA	CSSG-212	NARC (Jhang)	LM645014	Mosaic
3.	10ShA	CPSG-27	NARC (Jhang)	KU557287	Streak
4.	12ShA	HSF-240	NARC (D.I. Khan)	KU557291	Mosaic
5.	15ShA	AUS-375	NARC (Australia)	KU557288	Mosaic
6.	16ShA	HOSG-33	NARC	KU557289	Mosaic
7.	17ShA	NARC-I	NARC	KU557290	Mosaic
8.	18ShA	US-104	NARC (USA)	KU557292	Mosaic
9.	20ShA	CP-77-400	NARC (KPK)	KU557293	Mosaic
10.	21ShA	US-469	NARC (Faisalabad)	KU557294	Mosaic
11.	22ShA	MARDAN-1	Mardan	KU557295	Mosaic
12.	23ShA	MARDAN-2	Mardan	KU557296	Mosaic
13.	24ShA	MARDAN-3	Mardan	KU557297	Mosaic
14.	26ShA	CSSG-239	Shakarganj (Jhang)	KU557298	Mosaic
15.	27ShA	SPF-213	Shakarganj	KU557299	Mosaic
16.	28ShA	NSG-19	Shakarganj	KU557300	Mosaic
17.	29ShA	CPSG-2923	Shakarganj(Jhang)	KU557301	Mosaic
18.	30ShA	CPSG-437	Shakarganj(Jhang)	KU557302	Mosaic
19.	31ShA	US-127	Shakarganj (Faisalabad)	KU557303	Mosaic
20.	32ShA	CSSG-2453	Shakarganj (Jhang)	KU557304	Mosaic

3.1.2 Detection of *Sugarcane mosaic virus* through RT-PCR

Sugarcane mosaic virus CP gene was amplified by using universal primers. Primer sequence used for detection and amplification of coat protein gene is mentioned in the Appendix A.

Symptomatic leaf samples produced ~1.2 kb product of *CP* gene while asymptomatic leaf samples did not amplify any product. All of the collected symptomatic leaves samples were positive for SCMV. A predicted size band of ~1.2 kb was also amplified from one of the sample of sugarcane leaf (CPSG-27-NARC) which showed streak symptoms. The amplified products were T/A cloned (Fermentas) in pTZ57R/T vector according to the manufacturer instructions. Potential clones were double digested with *Hind*III and *Eco*RI to confirm the successful clonning of *CP* gene. Digested product was analyzed on 1% gel, which separated the 1.2 kb insert from 3 kb vector.

3.1.3 DNA sequencing and bioinformatics analysis

Positive clones were sequenced in forward and reverse both orientations using M13 universal primers. DNA sequences peaks were first read through Sequencher (version 4.9) software. To investigate whether cleaned sequences were similar to SCMV *CP* sequences, they were analyzed initially through BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) which determined the percentage identity with the relevant nucleotide bases already stored in data base (Table 3.2).

3.1.4 Phylogenetic analysis of SCMV isolates

Sequences were aligned using ClustalW (Thompson *et al*., 1994) program and phylogenetic tree was built with MEGA6 (Tamura *et al*., 2013) software using

Table 3.2: Pairwise nucleotide sequence identity (in percentage) for the comparative study between isolates of different countries and reported sequences from Pakistan (In Red).

	Spain (3)	**Germany (1)**	**Mexico (2)**	**South Africa (2)**	**Iran (2)**	**Australia (6)**	**France (8)**	**Thailand (4)**	**China (6)**	**Argentina (13)**	**Pakistan (22)**	**USA (2)**	**India (6)**
India	87-88	88-89	88-90	95-97	95-97	96-97	95-97	87-100	87-97	95-98	94-96	87-94	95-100
USA	87-94	88-92	89-92	89-94	89-94	89-95	88-94	86-90	87-94	89-94	87-94	90-100	
Pakistan	87-88	87-89	88-89	95-97	95-96	95-97	95-97	85-87	86-96	95-97	**95-100**		
Argentina	87-88	88-89	89-90	97-98	96-97	97-99	96-97	86-88	87-97	97-100			
China	88-97	89-94	90-94	87-98	88-98	87-99	87-97	86-97	86-100				
Thailand	90-91	89-91	89-90	86-88	87-88	86-88	87-89	92-100					
France	88-89	89-90	90	96-97	96-97	97-98	98-100						
Australia	88	89	89-90	97-98	97-98	98-100							
Iran	89	89	90	97	97-100								
South Africa	88	89	89-90	98-100									
Mexico	94	96	100										
Germany	93	100											
Spain	100												

Neighbor joining method. 1000 Bootstrap replicates were applied on the tree for the reliability of tree topology. Each percentage value of bootstrap test was mentioned next to the branches of closely clustered taxa. However, Pairwise distance values between sequences were estimated with Species Demarcation Tool (SDT) software. Scale is drawn at the base of tree (0.05) which is the measure of number of per site base substitution rate. Phylogenetic distances were measured with p-distance method, using branch lengths units as same as used to infer the evolutionary tree.

A phylogenetic tree was constructed using DNA sequences of present study and sequences retrieved from databases based on high sequence identity in BLAST (Basic Local Alignment Search Tool) search (Figure 3.3). These sequences were aligned using clustalW and phylogenetic tree was constructed using Neighbor joining method of MEGA6 software. The tree was rooted on coat protein gene sequence of *Potato virus* Y (Argentina; accession no. X14136). All *CP* sequences analysis of the present study formed a single clade, depicting that virus infecting sugarcane crop in different areas of Pakistan have identical sequence and may have been originated from same source. Phylogenetic analysis of SCMV *CP* sequences of present study isolates with the selected sequences available in the database divided them into three clusters. SCMV *CP* sequences from Pakistan, Australia, China, France, Argentina, South Africa and Iran grouped together in cluster I; while SCMV *CP* sequence from India make a distinct cluster II and is occupied its position between cluster I and III. Cluster III contains SCMV *CP* sequences obtained from China, Spain, Germany, Mexico and Thailand and showed divergence from the clusters I and II.

The results obtained indicate the 85-100% nucleotide identity among the sequences analyzed in this study (Table 3.2) and 95-100% nucleotide identities between all isolates

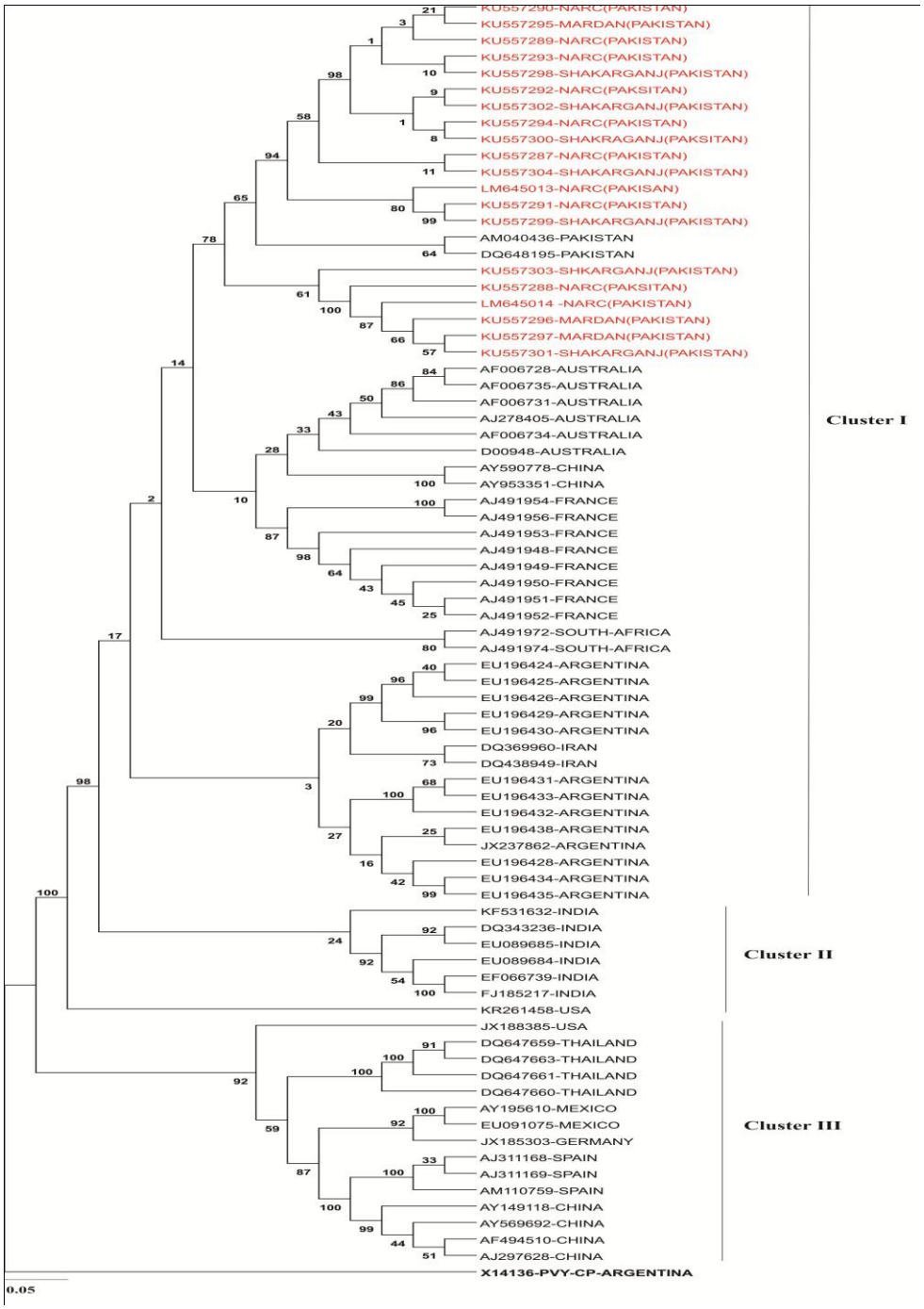

Figure 3.3: Phylogenetic relationship based on *CP* sequences of SCMV detected in the leaves of *Saccharium officinarium* collected from KPK and Punjab (Pakistan). Dendrogram was built through multiple sequence alignment (using ClustalW) of current study (In red) with the sequences retrieved from GenBank database. Phylogenetic tree was inferred through Neighbour joining method using 1000 Bootstrap value. Branch length is drawn to the scale; indicating the 0.05 rate of nucleotide substitution per site. As outgroup, *Potato virus* Y (PVY) isolate was used (Accession number X14136).

studied from Pakistan (Table 3.3). Studied sequences showed maximum nucleotide similarity (98%) with the accession number DQ648195 while minimum nucleotide similarity (~ 91%) was recorded with the accession number JX237869. According to species demarcation criteria of *Potyviridae* published in 9Th report of International Committee on Taxonomy of Virus (ICTV), nucleotide sequence identity either in coat protein or with in entire genome should be less than 76% and *CP* amino-acid sequence identity must be less than 80% (Adam *et al*., 2011). Since all the twenty *CP* sequences of present study shared a maximum nucleotide sequence identity between 95-100% hence these are the isolates of same species i.e. SCMV.

Pairwise nucleotide sequence identity was calculated by using Species Demarcation Tool (SDT) version 1.2 software. Estimated nucleotide identity between 22 isolates from Pakistan (20 sequences from the current study) showed 95-100 percent sequences similarity. Pairwise distances of all geographically studied sequences distributed into respective group are mentioned in the (Table 3.2). Similarly, pairwise sequence identities between the twenty studied isolates are shown in the (Table 3.3). Nucleotide sequences were aligned which showed the conservation of base sequences (showed in blocked) translated into conserved DAG amino-acids which is required for virus transmission (Figure 3.4)

3.1.5 Analysis of conserved amino-acids sequences of *CP*

Aligned nucleotide sequences were translated into amino-acid through MEGA6 software. Deduced amino-acids sequences contain conserved DAG (Asp-Ala-Gly) motif near N terminus of coat protein (Figure 3.5). Characteristics features of protein encoded by *CP* gene are mentioned below (Table 3.4).

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Table 3.3: Pairwise nucleotide sequence identity (in percentage) for the comparative study between the isolates from Pakistan.

	KU557287	KU557291	KU557288	KU557289	KU557290	KU557292	KU557293	KU557294	KU557295	KU557296	KU557297	KU557298	KU557299	KU557300	KU557301	KU557302	KU557303	KU557304	LM645013	LM645014
KU557287		98	96	99	99	99	99	99	99	99	96	96	99	98	99	96	99	98	99	99	96
KU557291	98		95	98	98	98	98	98	98	98	95	95	98	100	98	95	98	97	99	99	95
KU557288	96	95		95	95	95	95	95	95	95	99	100	95	95	95	100	95	97	96	96	100
KU557289	99	98	95		100	100	100	100	100	100	95	95	100	98	100	95	100	98	99	99	96
KU557290	99	98	95	100		100	100	100	100	100	95	95	100	98	100	95	100	97	99	99	95
KU557292	99	98	95	100	100		100	100	100	100	95	95	100	98	100	95	100	98	99	99	96
KU557293	99	98	95	100	100	100		100	100	100	95	95	100	98	100	95	100	98	99	99	96
KU557294	99	98	95	100	100	100	100		100	100	95	95	100	98	100	95	100	98	99	99	96
KU557295	99	98	95	100	100	100	100	100		100	95	95	100	98	100	95	100	97	99	99	95
KU557296	96	95	99	95	95	95	95	95	95		100	95	95	95	100	95	97	96	96	96	100
KU557297	96	95	100	95	95	95	95	95	95	100		95	95	95	100	95	97	96	96	96	100
KU557298	99	98	95	100	100	100	100	100	100	95	95		98	100	95	100	97	99	99	99	95
KU557299	98	100	95	98	98	98	98	98	98	95	95	98		98	95	98	97	98	99	99	95
KU557300	99	98	95	100	100	100	100	100	100	95	95	100	98		95	100	98	99	99	99	96
KU557301	96	95	100	95	95	95	95	95	95	100	100	95	95	95		95	97	96	96	96	100
KU557302	99	98	95	100	100	100	100	100	100	95	95	100	98	100	95		98	99	99	99	96
KU557303	98	97	97	98	97	98	98	98	97	97	97	97	97	98	97	98		98	98	98	98
KU557304	99	99	96	99	99	99	99	99	99	96	96	99	98	99	96	99	98		99	99	96
LM645013	99	99	96	99	99	99	99	99	99	96	96	99	99	99	96	99	98	99		99	96
LM645014	96	95	100	96	95	96	96	96	95	100	100	95	95	96	100	96	98	96	96		

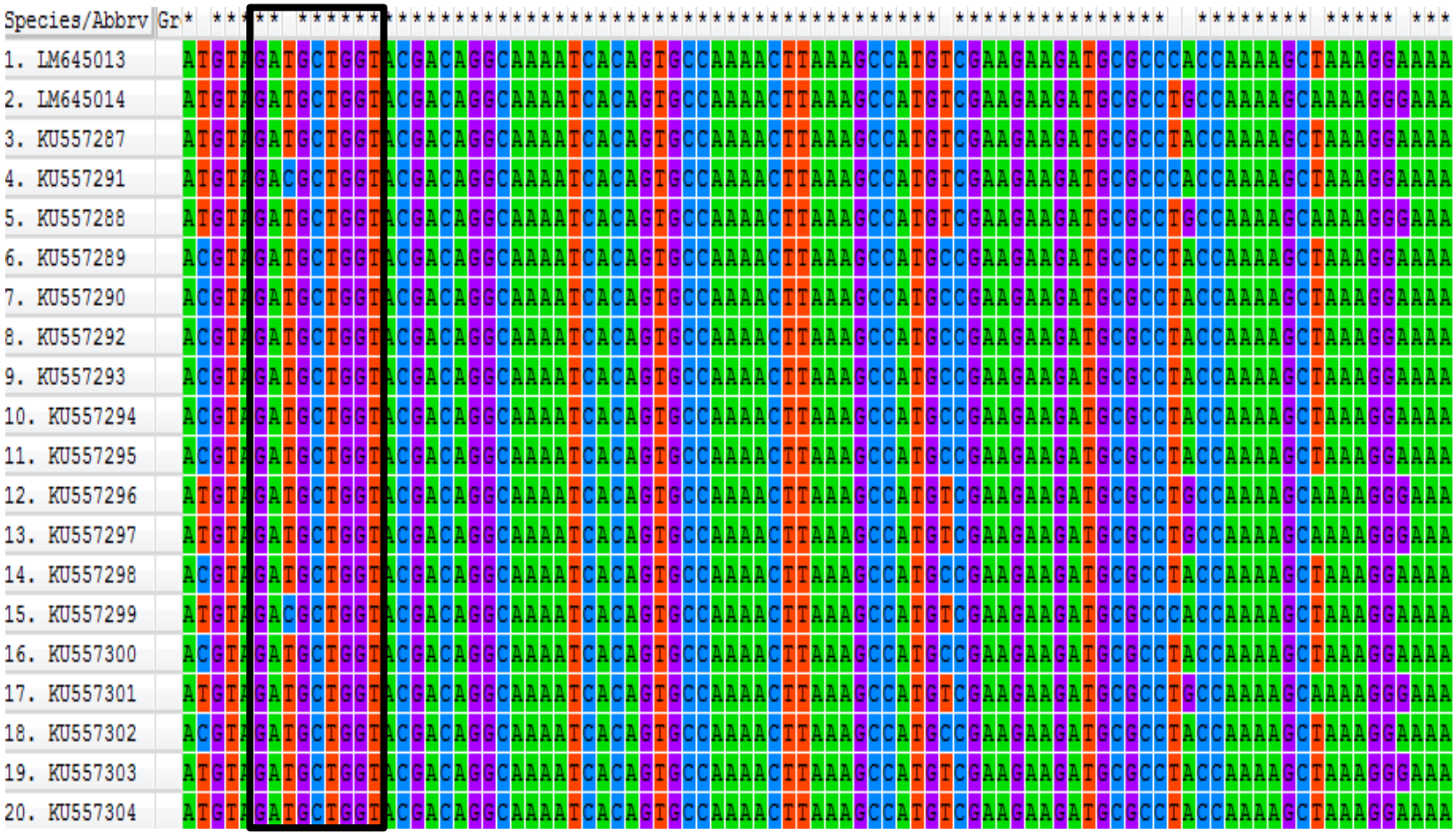

Figure 3.4: Multiple sequences alignment showing the conserved nucleotide sequence (in block) in the isolates of current study.

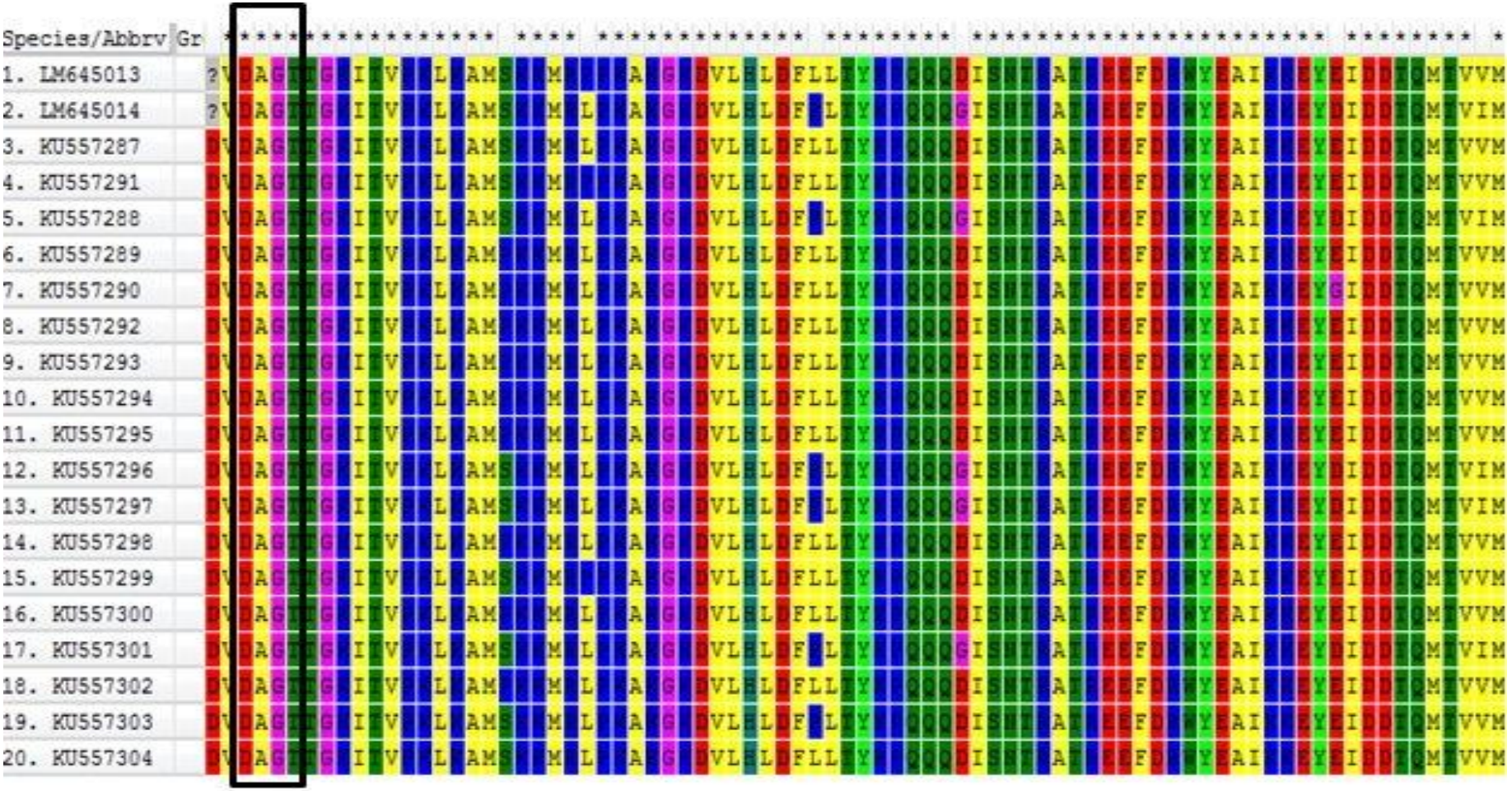

Figure 3.5: Multiple amino-acids alignment showing conserved DAG motif near N-terminus of studied isolates.

Table 3.4: Characteristics features of coat protein of studied isolates of SCMV

No.	Accession No.	Start Codon	Stop Codon	Predicted Size (No. of amino acids)	Predicted Molecular weight (KDa)
1.	LM645013	283	939	209	240
2.	LM645014	283	939	209	239
3.	KU557287	280	915	305	329
4.	KU557291	283	918	305	329
5.	KU557288	295	897	299	322
6.	KU557289	283	914	305	328
7.	KU557290	283	917	306	329
8.	KU557292	295	899	300	323
9.	KU557293	283	917	306	329
10.	KU557294	283	918	306	329
11.	KU557295	283	924	308	331
12.	KU557296	295	900	300	323
13.	KU557297	292	894	299	322
14.	KU557298	292	828	276	297
15.	KU557299	283	918	306	331
16.	KU557300	283	936	312	336
17.	KU557301	295	899	300	323
18.	KU557302	295	927	310	334
19.	KU557303	295	950	317	342
20.	KU557304	295	950	317	342

3.2 Developing resistance against SCMV

3.2.1 Selection of targeted genes

Sugarcane mosaic virus is the member of genus *Potyvirus*. Its genome size is approximately 10 kb which comprises of ten genes; with each gene conservation differs from each other. SCMV genome encodes single large polyprotein, which after translation processed into ten proteins by three virus-encoded proteases (Gell *et al*., 2015; Urcuqui-Inchima, 2001). These proteins have different roles in the life cycle of SCMV (Wu *et al*., 2013; Zhang *et al*., 2008). It is reported that nucleotide sequence identity is highest in the *CP* gene, followed by *CI*, *Hc-Pro*, and *P3*, with lowest nucleotide identity detected in *P1*. However, recombination events were found to be highest in *6K2*, *NIa-Pro*, *NIa-VPg* and *CI* gene, while *P1* and *CP* genes have very few recombination events detected. Thus, the genes which are near 5′ and 3′ termini were noted to be more conserved (Xie *et al*., 2016). Futhermore, *CP* gene has conserved region (DAG motif) near 5' terminus of gene. *CP* gene is not only involved in protection and encapsidation of virus but virus replication, translation and movement of genome also depends upon *CP* gene (Dolja *et al*., 1994, 1995; Rojas *et al*., 1997). The second most important gene of SCMV is *Hc-Pro;* as it comprises of three conserved regions from 5' to 3' direction (KITC, FRNK and PTK). Apart from these conserved regions, the other two impotant functions of *Hc-Pro* are (i) involvement of KITC and PTK conserved motifs in the transmission of virus (ii) it also acts as RNA silencing suppressor (Dolja *et al*., 1993).

In the present study, *CP* and *Hc-Pro* genes were selected to target for RNAi approach. Multiple sequences of each gene were selected from NCBI database and aligned together. The DAG and KITC conserved motifs of *CP* and *Hc-Pro* genes were targeted to develop the resistance through RNAi strategy in transgenic model plants.

3.3 Development of Artificial microRNA based binary constructs

3.3.1 Coat protein and Helper component proteinase gene (SCMV) based amiRNA constructs

A 22 bp sequence of Coat protein (*CP*) and Helper component proteinase genes (*Hc-Pro*) of SCMV were selected which contain the conserved DAG motif and KITC motif, respectively. Template RS300 contained naturally occurring stem loop precursor of Arabidopsis 319a , which was exploited to replace mi319a sequence with 22-nt sequences of targeted gene sequences using specific primers designed through Web MicroRNA Designer 3 (Weigel world) software. WMD3 software generated 4 oligos of 40 basepairs specific for *CP* and *Hc-Pro* targeted sequences. *CP* and *Hc-Pro* designed amiRNA-oligos together with the two primers (A and B) specific for adjacent vector sequence are mentioned in Appendix A.

Overlapped PCR scheme was used (see section 2.5.1) which generated approximately 700 bp product. The expression cassette containing mutated stem loop portion was cloned in to pGreen 0229 binary vector which is directed by double 35S CaMV promoter and contained polyA termination signal. Both cassette and vector was double digested by *Hin*dIII and *Xba*I restriction enzymes. About 500 bp digested insert fragment was ligated with dephosphorylated binary vector and transformed into *DH5*α cells. Potential clones were screened with restriction enzyme digestion (Figure 3.6) and then DNA sequencing ensured the successful cloning.

Sequences obtained were analyzed using the vector NTI® software which determined the 100% sequence identities to the *CP* and *Hc-Pro* designed oligos (Figure 3.7). Positive clones were transformed into GV3101 strain of Agrobacterium with rifampicin and kanamycin antibiotic selection. Binary constructs harboring *CP* and *Hc-Pro* amiRNA

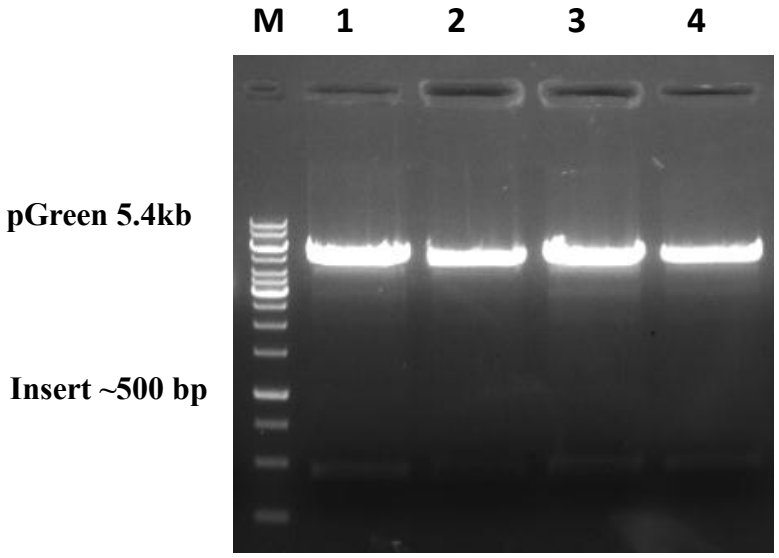

Figure 3.6: Restriction digestion of *CP* and *Hc-Pro* amiRNA constructs with *Hin*dIII and *Xba*I restriction enzymes. Lane M represents the 1 kb marker, Lane 1 and 2 are digested products of CP-amiRNA and lane 3 and 4 are the digested products of Hc-Pro amiRNA constructs.

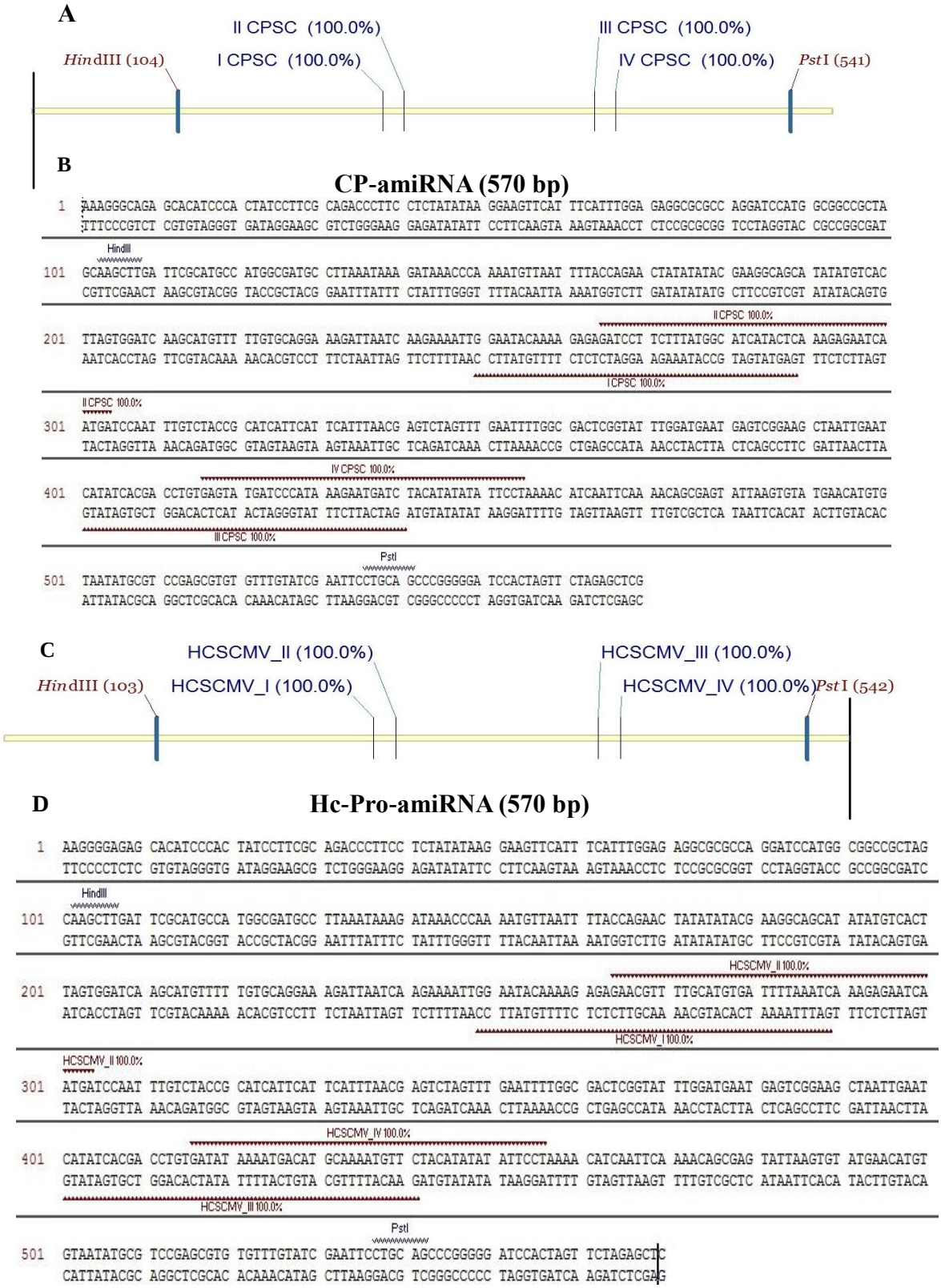

Figure 3.7: Sequence analysis of CP and Hc-Pro amiRNA binary constructs. **A, B**. Diagrammatic representation of sequence specificity of amiRNA oligos to the CP and Hc-Pro-amiRNA insert. **C, D.** Oligos (I, II, III, IV) showing 100% nucleotide sequence identity to the CP and Hc-Pro amiRNA insert.

transgene in Agrobacterium was used for *Nicotiana tobacum* transformation using phosphinothricin (PPT) as a plant selection marker.

3.3.2 Agrobacterium mediated *Nicotiana tobacum* transformation

N. tobaccum transformation is performed through many different procedures but Agrobacterium mediated transformation protocol is highly established and efficient protocol (Horsch *et al*., 1985) for the development of transgenic plants. *N. tobaccum* model plant was preferably selected for transformation because of its high transformation efficiency as compared to *N. benthamiana* model plant (Chetty *et al*., 2013). Brief summary of *N. tobaccum* tissue culture is mentioned below (Table 3.5).

3.3.2.1 Preparation of Agrobacterium cultures

Single colony of Agrobacterium containing binary construct was streaked on the LB media plate containing rifampicin and kanamycin. RS300 glycerol stock of Agrobacterium was also streaked on plates to be used as a positive control. Plates were ready for transformation after 48 hours of incubation at 28 ^{o}C in dark.

3.3.2.2 Co-cultivation of leaf discs with Agrobacterium

CP-amiRNA, Hc-Pro-amiRNA and control vectors leaf discs were prepared by cutting the wild type tobaccum leaves in the Agrobacterium suspension. Then, discs were placed on the MS plates (without antibiotics) for co-cultivation (Figure 3.8A).

3.3.2.3 Development of shoots on shooting media

Leaf discs when shifted to shooting media, callus started developing on phosphinothricin (ppt) antibiotic which further generated small shoots after 3-4 weeks as shown in (Figure 3.8B). CP-amiRNA, Hc-Pro-amiRNA and control leaf discs all of them showed same growth rate of development of shoots on shoot regeneration media.

Table 3.5: Summary of *N. tobaccum* tissue culture mentioning amiRNA constructs, components, duration on media.

Experiment Components	**Co-cultivation medium**	**Callus Induction medium**	**Shooting Medium**	**Rooting medium**
	48 hours (dark)	2-4 weeks (light)	2-4 weeks (light)	3 weeks (light)
CP-amiRNA + PPT	Growth of Agrobacterium	Callus formation	Green shoots develop	Roots develop
Hc-Pro-amiRNA + PPT	-do-	-do-	-do-	-do-
amiR71+PPT (Empty vector)	-do-	-do-	-do-	-do-

3.3.2.4 Development of Roots

After appropriate proliferation of shoots, roots were started emerging in all of the transformants on the ppt selection media (Figure 3.8C). Two weeks were sufficient for the plantlets to generate enough strong roots which can survive the plantlets in soil.

3.3.2.5 Transferring to the soil

Healthy growing plantlets were shifted in to soil in the glass house. About 20-25 independent lines of each construct were shifted into glass house under proper conditions. Plants were able to screen subsequently 10-15 days of soil transfer (Figure 3.8D).

3.3.3 Screening of transgene in transformed plants

3.3.3.1 PCR detection of transgene using gene specific primers

DNA was extracted from the young leaves of transformed plants (see section 2.1.2) and PCR (sequences mentioned in the Appendix A) was performed which amplified the sequence (about 800 bp) only from plants transformed with CP and Hc-Pro amiRNA constructs but no amplification was observed from control wild type plants (Figure 3.9).

The PCR products were cleaned through gel and sequenced in both orientations which confirmed the sequence of transgene in transformed plants. miR319a DNA sequence was compared with CP-amiRNA and Hc-Pro-amiRNA to determine the PCR directed mutagenesis of *CP* and *Hc-Pro* in the miR319a backbone. Blocked region clearly showed the difference in the number of bases from naturally occurring Arabidopsis to viral Coat protein and Helper component proteinase gene of SCMV (Figure 3.10).

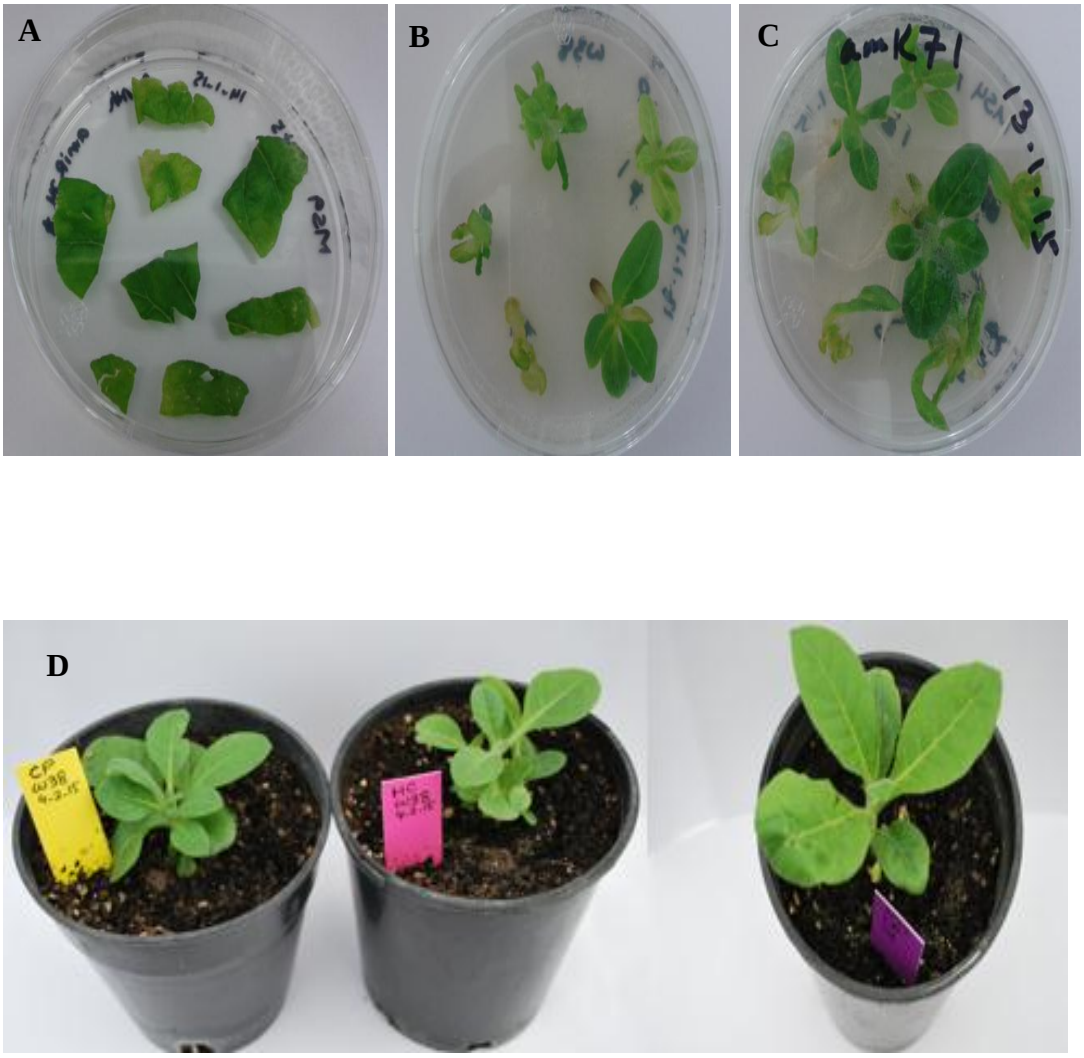

Figure 3.8: Representative picture of tissue culture of *Nicotiana tobacum* through Agrobacterium using amiRNA constructs. **(A)** Co-cultivation of leaf discs with CP and Hc-Pro amiRNA binary constructs in Agrobacterium on MS media without antibiotics **(B)** Development of shoots from the callus growing on the shoot regeneration media **(C)** Roots regeneration from the stem of developing plantlets on rooting media **(D)** Transfer of regenerated plantlets in soil in glass house.

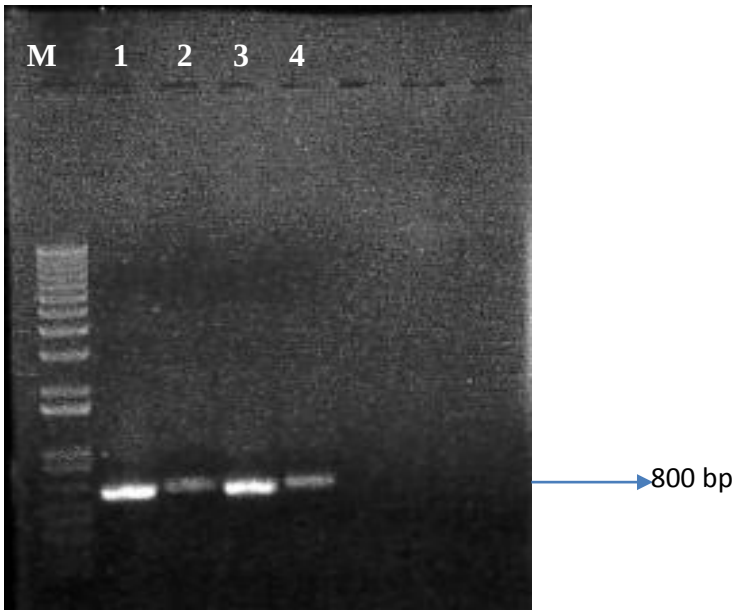

Figure 3.9: Representative agarose gel of *CP* and *Hc-Pro* transgene bands from transgenic *N. tobaccum* plants through PCR. Lane M is the 1kb marker, Lane 1 and 2, are the CP-amiRNA transgenes and 3 and 4 are Hc-Pro-amiRNA transgenes.

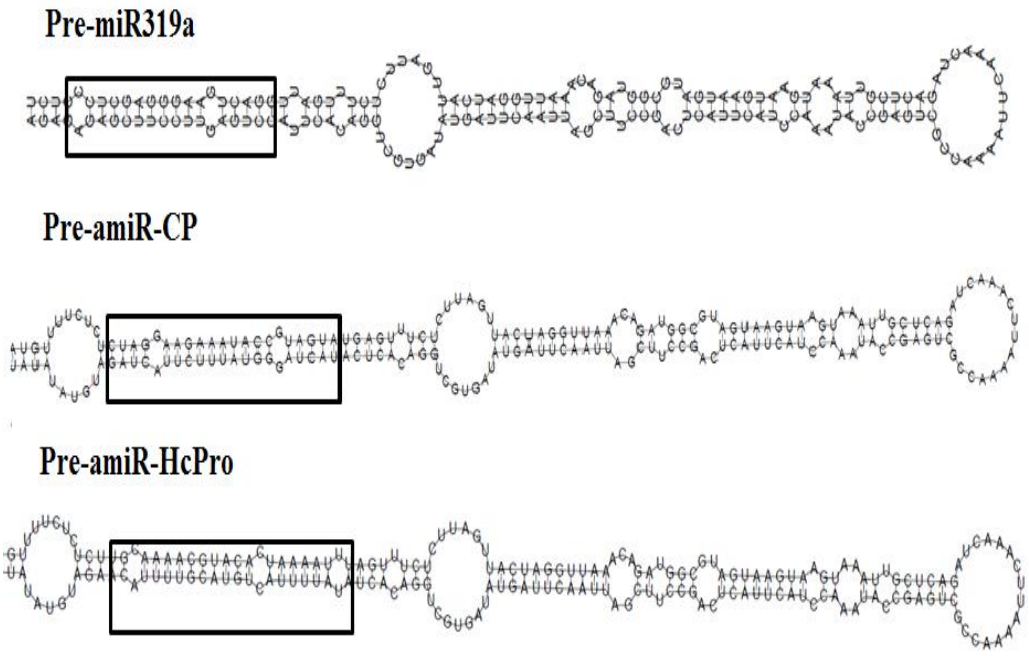

Figure 3.10: Formation of precursor stem loop structure of Arabidopsis miR319a, amiR-CP and amiR-Hc-Pro.Box region shows the nucleotide sequences of miR319a, amiR-CP and amiR-Hc-Pro.

3.3.3.2 Real time PCR for the expression analysis of transgene

Transcript abundance of CP-amiR and Hc-Pro-amiR was quantified by qPCR in all the 20 independent lines of each construct. Most of the lines showed higher expression of transgene as compared to wild type and two lines (CP-amiR-15 and Hc-Pro-amiR 7) exhibiting higher gene expression (about 0.08% and 0.27%, respectively) amongst the other expressing lines. qPCR data from each construct expressing the transgene are shown in the Figure 3.11 which clearly suggests that Hc-Pro-amiR construct is more effectively transcribes the transgene and producing miRNA as compared to the CP-amiR construct. The amiRNA transgene expression level is not significant as compared to wild type control plants. To obtain the efficient expression level, current study was elaborated to develop the hairpin RNA transgenesagainst the *CP* and *Hc-Pro* targeted genes and analyzed their expression level.

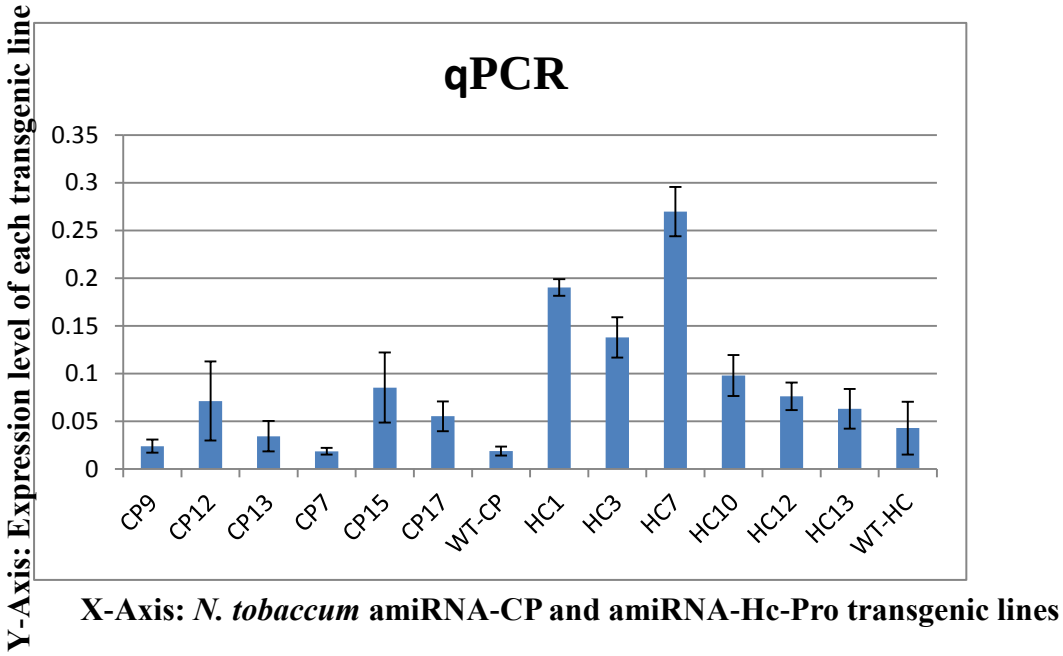

Figure 3.11: Expression analysis of amiRNA transgenes through qPCR. X-axis represents the independent transgenic lines transformed through CP-amiRNA and Hc-Pro-amiRNA constructs. However, WT-CP and WT-HC are the wild type plants transformed with RS-300 vector while Y-axis represents the expression value from each transgenic line.

3.4 Development of hairpin RNA vectors targeting *CP* and *Hc-Pro* (SCMV)

3.4.1 Selection of targeted gene

Selection of a suitable target sequence is important for successful knockdown by RNA silencing-based approaches. In this study, *CP* and *Hc-Pro* genes were selected as the target sequence because both of them are involved in virus spread and transmission. In addition, *Hc-Pro* functions as a suppressor of RNA silencing so knock-down of this gene could further enhance the efficiency of hpRNA transgene-mediated antiviral silencing. In particular, the selected 240 bp *CP* sequence covers the conserved DAG motif near the N-terminus, which is responsible for aphid mediated virus transmission (Dolja *et al*., 1994) while the 240 bp *Hc-Pro* sequence contains the N-terminus KITC motif that is required for viral interaction with aphid stylet (Dolja *et al*., 1993). To confer the resistance against multiple SCMV variants, *CP* and *Hc-Pro* multiple sequences were retrieved from NCBI database and 240 bp N-terminus conserved sequences of both genes were selected through multiple alignments subsequently. As shown in (Figure. 3.12), the *CP* and *Hc-Pro* sequences were joined together to form a chimera SCMV sequence.

Selected DNA bases were fused together with the addition of restriction enzymes sequences at the start and end of bases. About 480 bp fused gene sequence with specific restriction sites for cloning into monocot and dicot hairpin vector was synthesized from GeneArt™Gene Synethesis (ThermoFisher Scientific, Waltham, MA).

Synthesized sequence was cloned into monocot and dicot hairpin vectors (pStarling and pKannibal) according to procedure mentioned in section 2.5.2. To confirm the successful cloning of insert into hairpin cassette, restriction digestion was performed using *Not*I restriction enzyme. Positive clones containing hairpin insert produced two bands of 4 kb and 3 kb (Figure 3.13).

Figure 3.12: Gene sequence of *CP* (240 bp; in red) and *Hc-Pro* sequence (240 bp; in blue) fused together. Multiple restriction sites were also added in the start and end of fused sequence. *Kpn*I (ggtacc), *Xho*I (ctcgag) and *Bam*HI (ggatcc) are the restriction sites in the 5' end of sequence while *Sma*I (cccggg), *Hin*dIII (aagctt), *Spe*I (actagt) and *Kpn*I (ggtacc) are the restriction sites added in the 3' end of sequence.

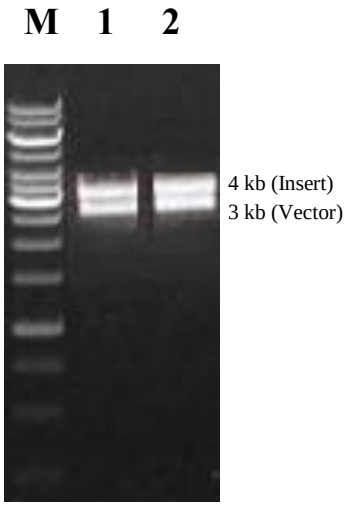

Figure 3.13: Restriction digestion of 35S-hpCP:Hc-Pro and Ubi-hpCP:Hc-Pro with *Not*I restriction enzyme. Lane M represents the 1 kb marker, Lane 1 is the digested products of 35S-hpCP:Hc-Pro and lane 2 is the digested products of Ubi-hpCP:Hc-Pro.

After the confirmation of successful cloning of synthesized sequence into the hairpin vectors, hairpin cassette was excised and ligated with *Not*I digested Vec8 and Vec2a binary vectors. Transformation was done into *DH5α* cells and positive clones were screened through restriction digestion. Transformed binary vectors plasmids were digested with *Bam*HI (for Ubi-hpCP:Hc-Pro) and *Kpn*I (for 35S-hpCP:Hc-Pro). Figure 3.14 represents the restriction pattern produced through BamHI restriciton enzyme. Vec8 containing hairpin insert produced three digested fragments; 10 kb vector backbone; 1.8 kb vector fragment and 4 kb hairpin cassette. Both orienations of Ubi-hpCP:Hc-Pro (a and b) produced same restriction digest pattern.

Restriciton digestion analysis of 35S-hpRNA showed two different digestion pattern (Figure 3.15). Orientation (a) of 35S-hpCP:Hc-Pro produced two bands; i.e. one of 10 kb while second of 2.1 kb. However, Orientation (b) of 35S-hpCP:Hc-Pro also produced two bands size of 10 kb and 1.8 kb.

3.4.2 Transient gene expression of dicot hairpin constructs in *Nicotiana benthamiana* through Agro-infiltration

Agro-infiltration of binary constructs in *N. benthamiana* provides the simple and efficient system to evaluate the transgene expression in relatively short period of time. 35S-hpCP:Hc-Pro construct were infiltrated in 4 weeks old *N. benthamiana* leaves along with the green florescent protein (GFP) which was used as visual marker to monitor and locate the infiltrated samples. After 4-5 DPA (days post Agro-infiltration) infiltrated leaves were

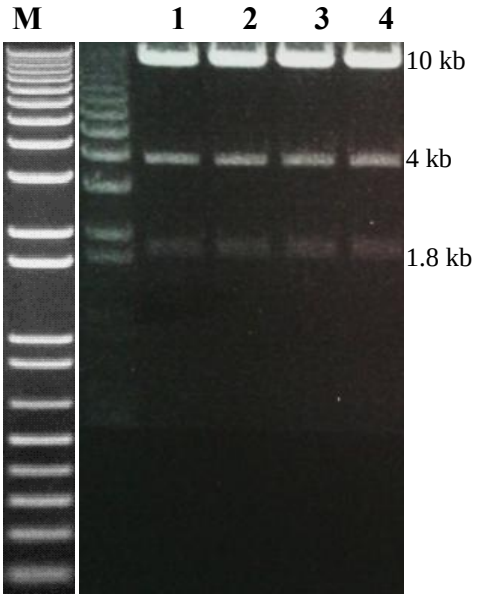

Figure 3.14: Restriction digestion of binary vector (Vec8) containing Ubi-hpCP:Hc-Pro cassette with *Bam*HI restriction enzyme. Lane M represents the 1 kb plus marker, Lane 1, 2, 3, 4 are the digested products of Ubi-hpCP:Hc-Pro (4 kb and 1.8 kb) and vector (10 kb).

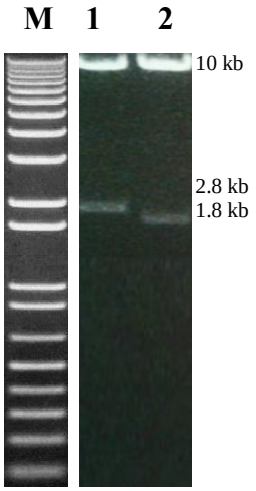

Figure 3.15: Restriction digestion of binary vector (Vec2a) containing 35S-hpCP:Hc-Pro cassette with *Kpn*I restriction enzyme. Lane M represents the 1 kb plus marker, lane 1 are the digested products of 35S-hpCP:Hc-Pro (a), lane 2 are the digested products of 35S-hpCP:Hc-Pro (b).

visualized under the blue light in dark. Agro-infiltrated leaf area emitted the green light in contrast with red background, was excised from the leaf and frozen in liquid nitrogen (Figure 3.16).

Different Agro combinations were prepared to inoculate the *N. benthamiana* leaves to evaluate the specificity and efficiency of 35S-hpCP:Hc-Pro construct. Agro-infiltration of constructs combinations were performed in triplicate. One replicate was used for histochemical staining by X-Gluc solution while rest was used for protein quantification.

3.4.3 Histochemical assay for GUS (β-glucuronidase) expression

Infiltrated *N. benthamiana* leaves samples were subjected to GUS (*β*-glucuronidase) staining to examine the expression of hpRNA construct driven by 35S constitutive promoter. GUS staining was performed on the selected leaves of combinations [(1) 35S-GUS:CP:Hc-Pro; (2) 35S-GUS:CP:Hc-Pro/35S-hpCP:Hc-Pro(a)/(b); (3) 35S-GUS:Y-sat/35S-hpCP:Hc-Pro(a)/(b)] on 2 DPA leaves of *N. benthamiana*. Expression pattern confirmed that constructs are functional. An identical staining pattern was noticed as observed from rice stained calli. Figure 3.17 explained the staining pattern of infiltration. **A** and **B** represent the infiltrated sample from the combination No. 1. As GUS:CP:Hc-Pro fused construct transcribed by 35S CaMV promoter, a constitutive dicot promoter which strongly expressed the GUS:CP:Hc-Pro gene and exhibits the blue color. **C** and **D** showed the infiltration of combination 2. As the previous staining pattern of rice calli exhibited the sequence specific degradation of GUS fused gene with Ubi-hpCP:Hc-Pro. Both sense and antisense 35S-hpCP:Hc-Pro staining pattern also displayed the low expression of GUS gene (consistent with the previous results) as dicot hairpin degrades its complementary sequences in 35S-GUS:CP:Hc-Pro through the production of siRNA. Additional staining pattern in **E** and **F** by infiltrating combination 3

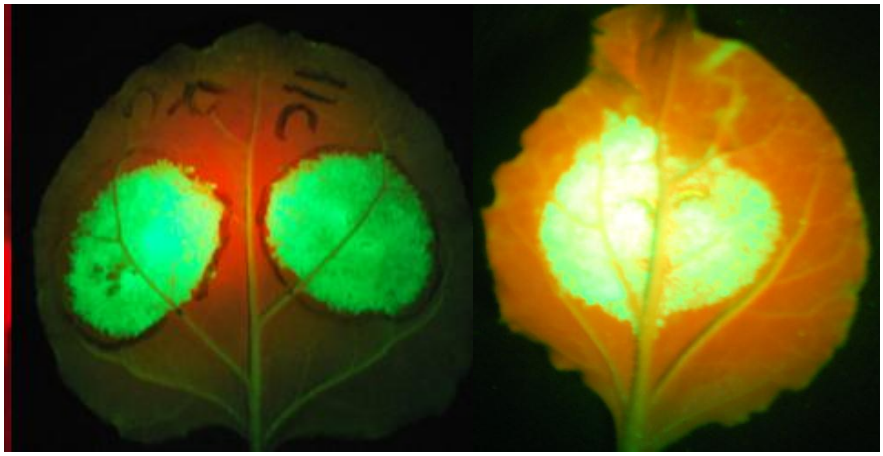

Figure 3.16: GFP imaging of 35S-hpCP:Hc-Pro binary construct in Agro infiltrated *N.benthamiana* leaves after 4 DPA.

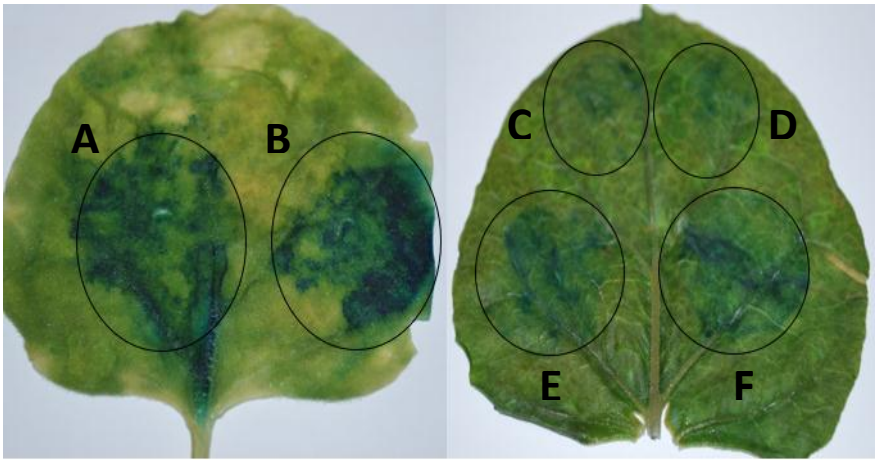

Figure 3.17: GUS (*β*-glucuronidase) staining pattern of Agro-infiltrated *N. benthamiana* leaves. **A, B** 35S-GUS:CP:Hc-Pro **C, D** 35S-GUS:CP:Hc-Pro/35S-hpCP:Hc-Pro(a) /(b)**E, F** 35S-GUS:Y-sat/35S-hpCP:Hc-Pro(a)/(b).

proved that the blue color appearance in the negative control is due to non-specificity of 35S-hpCP:Hc-Pro to 35S-GUS:Y-Sat sequence.

3.4.4 Fluorometric assay for GUS (β-glucuronidase) expression

Fluorescent *β*-Galactosidase Assay (MUG) is the very sensitive assay to detect the fluorescent produced by *β*-glucuronidase enzyme in the cell lysate of *N. benthamiana* Agro infiltrated samples. Beta galactosidase used 4-methylumbelliferyl-*β*-D-galactopyranoside (4-MUG) as a substrate and cleaved it to produce fluorophore 4-methylumbelliferone. Fluorescent produced was used to estimate the GUS protein expression level (Figure 3.18). *β*-galactosidase protein concentration was assessed through MUG assay which further confirmed the 35S-hpCP:Hc-Pro efficiency. A closer look into the fluorescence level, 35S-GUS:CP:Hc-Pro and 35S-GUS:Y-Sat construct has the highest fluorescent value. Dramatically, hairpin GUS repressed the GUS activity in both 35S-GUS:Y-Sat and 35S-GUS CP:Hc-Pro constructs and showed reduction in GUS fluorescent level when compared to their GUS peak without 35S-hpGUS. Consistently, 35S-hpCP:Hc-Pro constructs in both orientations (a and b) proficiently down regulated the GUS level about 50% compared to the actual 35S-GUS:CP:Hc-Pro expression. The reason for repression is due to 21-24 nts siRNA produced through sense and antisense hairpin RNA transgene which caused post transcriptional gene silencing corresponding to the GUS targeted reporter gene constructs.

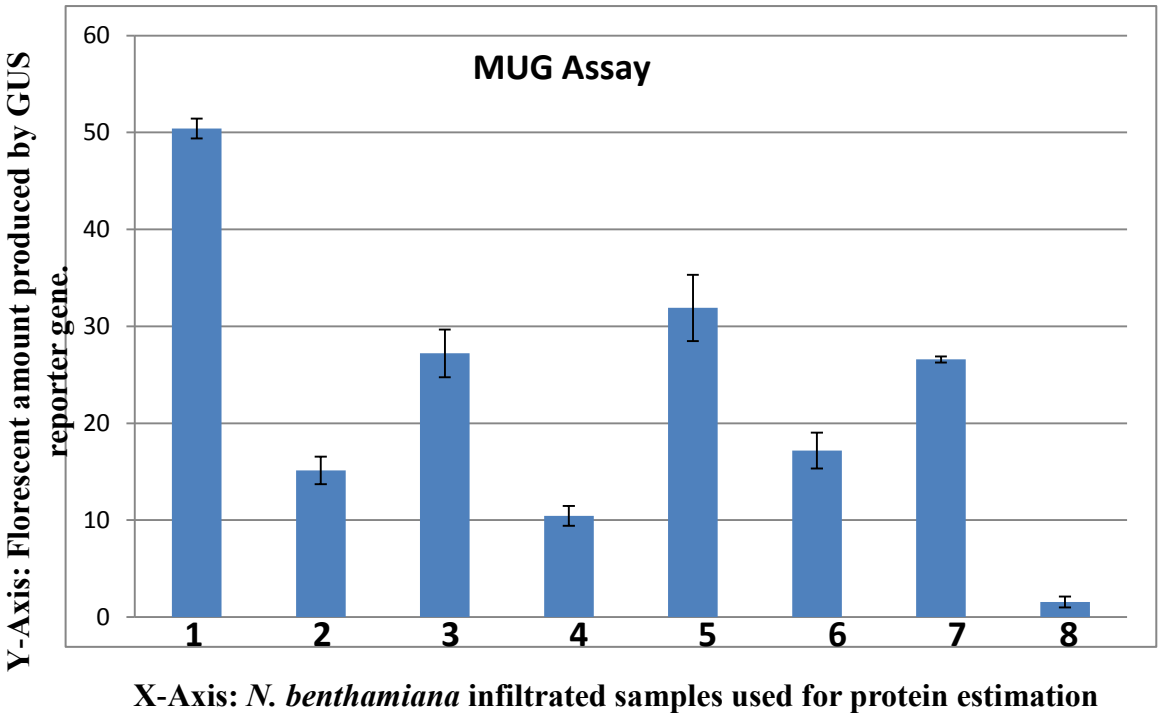

Figure 3.18: Fluorescent *β*-Galactosidase Assay (MUG) for the estimation of Beta Galactosidase activity from the Agro-infiltrated *N. benthamiana* leaves samples. Y-axis represents the fluorescent level produced by GUS reporter gene and X-axis represents the protein samples used for estimation. (1) 35S-GUS:CP:Hc-Pro (2) 35S-GUS:Y-Sat (3) 35S-GUS:CP:Hc-Pro+35S-hpGUS (4) 35S-GUS:Y-Sat+ 35S-hpGUS (5) 35S-GUS:CP:Hc-Pro+35S-hpCP:Hc-Pro(a) (6) 35S-GUS-Y-Sat+35S-hpCP:Hc-Pro(a) (7) 35S-GUS:CP:Hc-Pro+35S-hpCP:Hc-Pro(b) (8) 35S-GUS-Y-Sat+ 35S-hpCP:Hc-Pro(b).

3.4.5 Northern blot hybridization for detection of large RNA

To further confirmed the MUG results, extracted RNA from the Agro-infiltrated *N. benthamiana* leaves samples were run on 1.2% formaldehyde agarose gel and hybridized with the probe containing oligonucleotides specific for GUS gene to detect the GUS band. Probe hybridized to the GUS band transferred to the N+ Hybond membrane which was visible on X-ray film after overnight exposure to X-rays. Figure 3.19 displayed the detection of GUS band.

Data obtained was considerably co-related to the results obtained from the MUG analysis. Hypothetically, the Northern blot should expose the GUS band in all the lanes except where 35S-hpCP:Hc-Pro is efficient and degrade the GUS gene. The hybridization pattern followed the above hypothesis with the intensity of GUS band explained the functionality of hpRNA.

As shown in the figure 3.20. Lane (1) 35S-GUS:Y-Sat has very intense GUS band. However, lane (2 and 3) GUS band are very weak predicting the role of hairpin GUS in degrading the GUS gene in 35S-GUS:CP:Hc-Pro construct and 35S-GUS:Y-Sat construct. Interestingly, 35S-hpCP:Hc-Pro in both orientations (a) and (b) are equally effective to target its specific sequence in GUS gene which was judged by absence of GUS band in lane 4 and 6. The 35S-hpCP:Hc-Pro sequence specificity was approved when 35S-GUS:Y-Sat fused transgene showed inverse co-relationship to the GUS banding pattern.

3.4.6 Northern blot hybridization for siRNA detection

To investigate the production of siRNA from 35S-hpCP:Hc-Pro, RNA was extracted from the infiltrated *N. benthamiana* leaves. High quality RNA was extracted through TRIzol method (see section 2.1.1) was loaded on polyacrylamide gel and hybridized

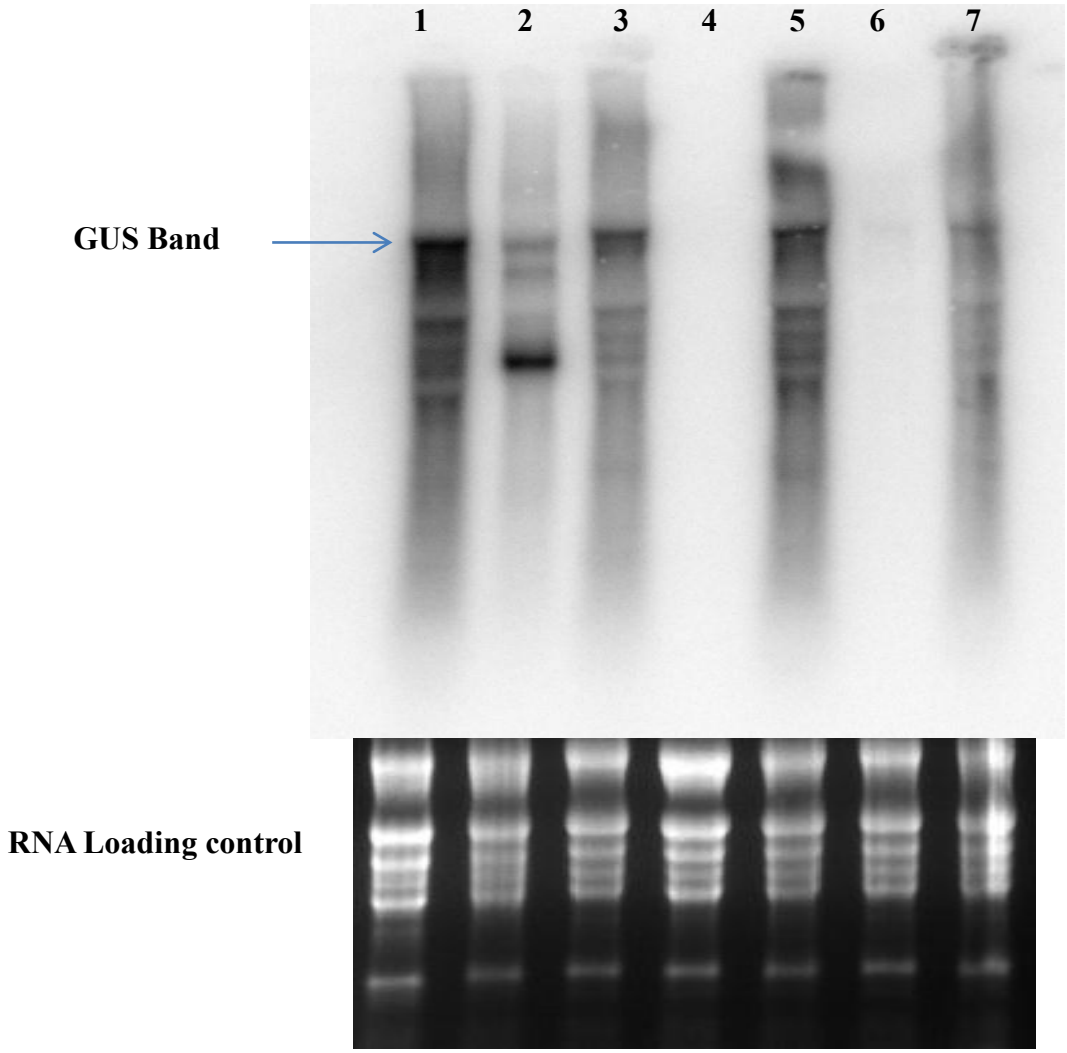

Figure 3.19: Northern blot of Agro-infiltrated *N. benthamiana* leaves for the detection of GUS band. Lane 1: 35S-GUS Y-Sat; lane 2: 35S-GUS CP:Hc-Pro+35S-hpGUS; lane 3: 35S-GUS-Y-Sat+35S- hpGUS; lane 4: 35S-GUS CP:Hc-Pro+35S-hpCP:Hc-Pro(a); lane 5: 35S-GUS-Y Sat+ 35S-hpCP:Hc-Pro(a); lane 6: 35S-GUS CP:Hc-Pro+ 35S-hpCP:Hc-Pro(b); lane 7: 35S-GUS-Y Sat+ 35S-hpCP:Hc-Pro(b). Bottom panel: RNA as a loading control.

with gene specific probe to confirm the production of siRNA through hairpin. As a positive control 35S-GUS:CP:Hc-Pro was also loaded on gel. Figure 3.20 illustrates the detection of 21-24nt siRNA bands in the lane of 35S-hpCP:Hc-Pro while the lane containing sample (35S-GUS CP:Hc-Pro) without hairpin had no siRNA band. U6 loading control showed the same amount of RNA samples were loaded on the gel.

3.4.5 Development of transgenic *N. tobaccum* using 35S-hpCP:Hc-Pro constructs through Agrobacterium

Nicotiana tobacum plants were transformed through Agrobacterium infection using leaf disc method (see section 2.8.1). Explants (leaf discs) were suspended in the Agro suspension of 35S-hpCP:Hc-Pro binary constructs which after co-cultivation with Agrobacterium started developing shoots on hygromycin selection media. Elongated shoots were further developed roots on the hygromycin selection rooting media. Plantlets were shifted into glass house for the elongation and growth of plants (Figure 3.21).Summary of different stages of *N. tobaccum* tissue culture is mentioned in Table 3.6.

3.4.6 Expression analysis in dicot stable expression system

To investigate the expression of 35S-hpCP:Hc-Pro in dicot stable expression system, *N. tobaccum* plants were screened through Northern blot analysis. About 15-20 independent stable lines were generated in the transformation event of *N. tobaccum* with 35S-hpCP:Hc-Pro. RNA was extracted from 4-5 transformed lines and run on polyacrylamide gel. Membrane was hybridized with the sequence specific probe to detect the production of small RNA. A representative blotting Figure 4.29 is shown below which clearly showed that 35S-hpCP:Hc-Pro produced typical pattern of 21-24 nt siRNA (Figure 3.22).

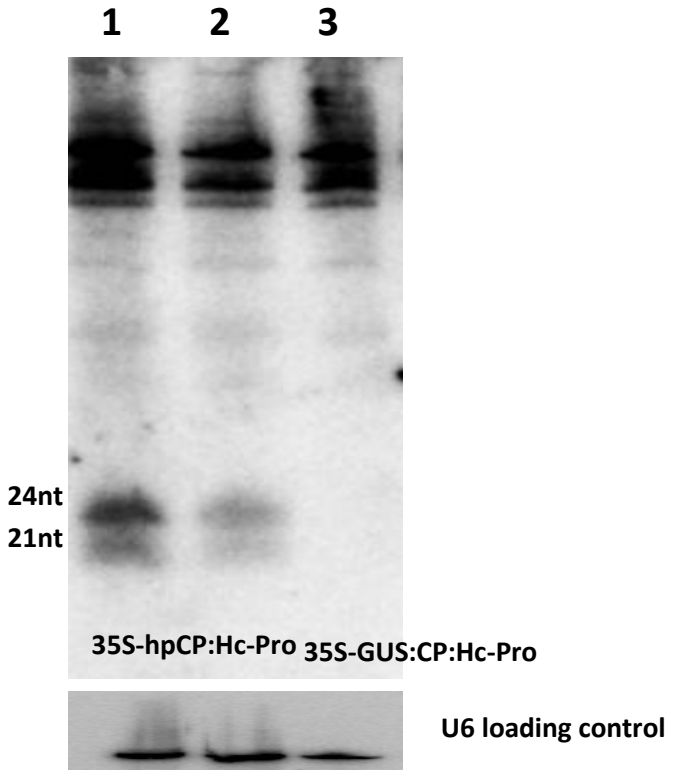

Figure 3.20: Northern blotting of Agro-infiltrated samples to detect the production of siRNA from 35S-hpCP:Hc-Pro. Lane 1 and 2 are the samples of 35S-hpCP:Hc-Pro, lane 3 represents the 35S-GUS:CP:Hc-Pro sample loaded as a positive control. U6 used as a sample loading control.

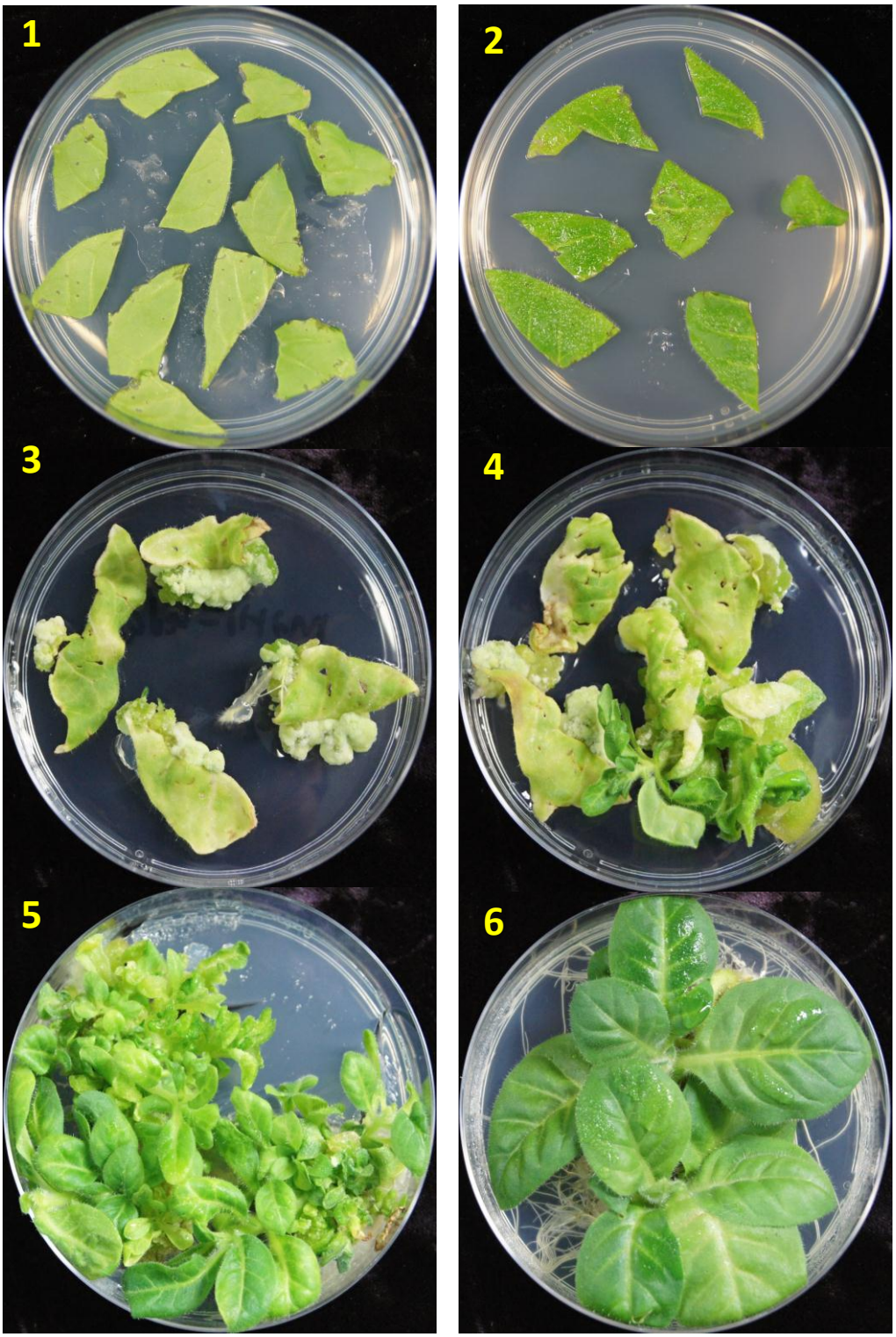

Figure 3.21: Schematic representation of Agrobacterium mediated *N. tobacum* tissue culture of 35S-hpCP:Hc-Pro through leaf disc method. **(A, B)** Co-cultivation of leaf discs with Agrobacterium harboring 35S-hpCP:Hc-Pro binary construct **(C, D)** Callus formation **(E)** Shoots regeneration on hygromycin selection shooting media **(F)** Roots development on rooting media containing hygromycin as plant selection marker.

Table 3.6: Summary of *N. tobacum* tissue culture mentioning hairpin constructs, components, duration on media.

Experiment Components	**Co-cultivation medium**	**Callus Induction medium**	**Shooting Medium**	**Rooting medium**
	48 hours (dark)	2-4 weeks (light)	2-4 weeks (light)	3 weeks (light)
35S-hpCP:Hc-Pro(a) + Hygro	Growth of Agrobacterium	Callus formation	Green shoots develop	Roots develop
35S-hpCP:Hc-Pro(b) + Hygro	-do-	-do-	-do-	-do-
Vec2a+Hygro (Empty vector)	-do-	-do-	-do-	-do-

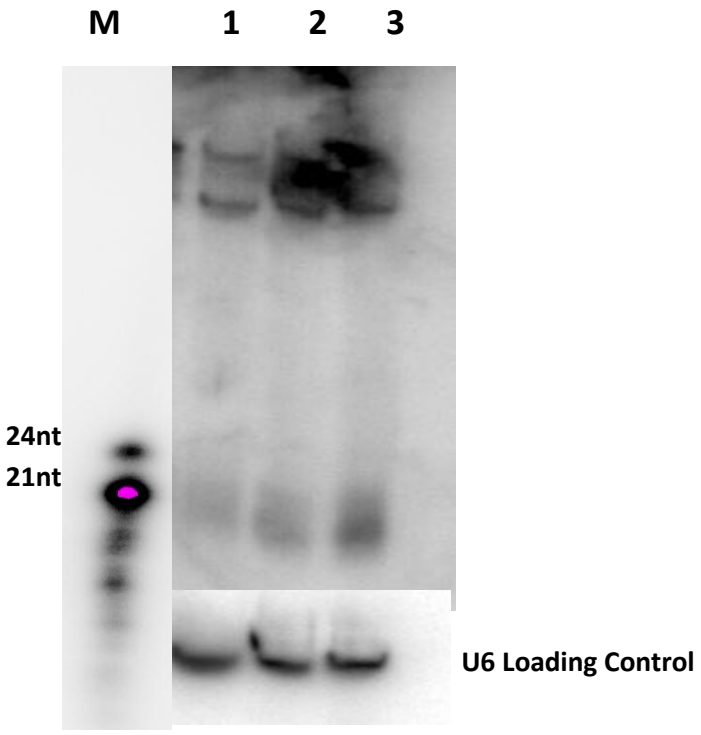

Figure 3.22: Northern blotting of RNA extracted from *N. tobacum* transgenic lines transformed with 35S-hpCP:Hc-Pro. Lane M: sRNA ladder; Lane 1, 2, 3: 35S-hpCP:Hc-Pro transgenic lines. Bottom panel: U6 loading control.

3.4.7 Analysis of transient gene expression induced by monocot hpRNA constructs in rice callus

To quickly estimate the efficiency of Ubi-hpCP:Hc-Pro vector, attempts were initially made by bombarding the callus of Nipponbare variety of rice seeds. Fresh and healthy looking rice callus were selected for the experiment as shown in Figure 3.23.

Rice calli were bombarded according to the standard protocol mentioned in the section 2.7.4. Different combinations of plasmids were used to confirm the Ubi-hpCP:Hc-Pro efficacies.

Bombarded rice calli were subjected to GUS (*β*-glucuronidase) staining for 3-4 hours as described in the methods section. Experimented calli were observed under florescent microscope and images were captured as presented below in Figure 3.24;

Multiple clear blue spots were observed in rice calli bombarded with 35S-GUS:CP:Hc-Pro and 35S-GUS:Y-Sat or co-bombarded with 35S-GUS:Y-Sat and Ubi-hpCP:Hc-Pro. In contrast, very few blue spots could be seen in rice calli co-bombarded with 35S-GUS:CP:Hc-Pro and Ubi-hpCP:Hc-Pro. This result indicated that the Ubi-hpCP:Hc-Pro, comprised of the *CP* and *Hc-Pro* sequence, induced effective sequence-specific silencing against the CP:Hc-Pro sequence in the 35S-GUS:CP:Hc-Pro fusion RNA, resulting in downregulation of GUS expression and hence diminished blue staining (Figure 3.24).

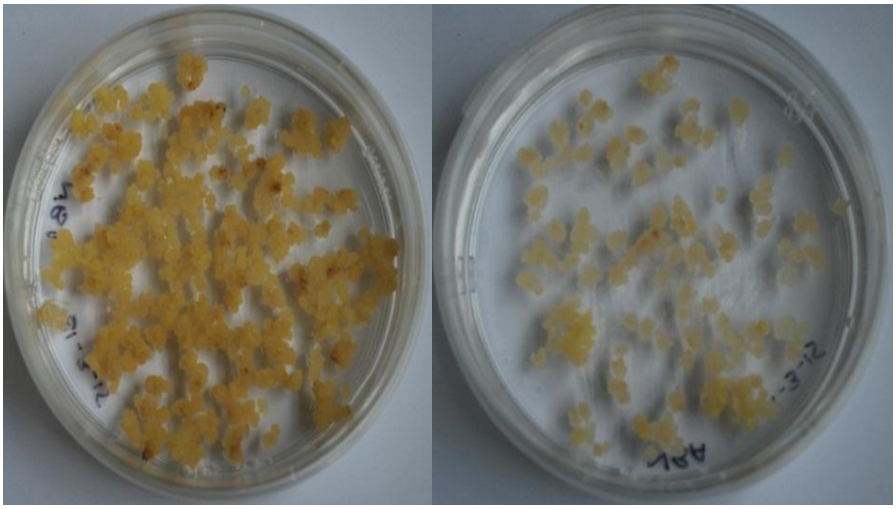

Figure 3.23: Nipponbare rice callus on NB (callus induction) media.

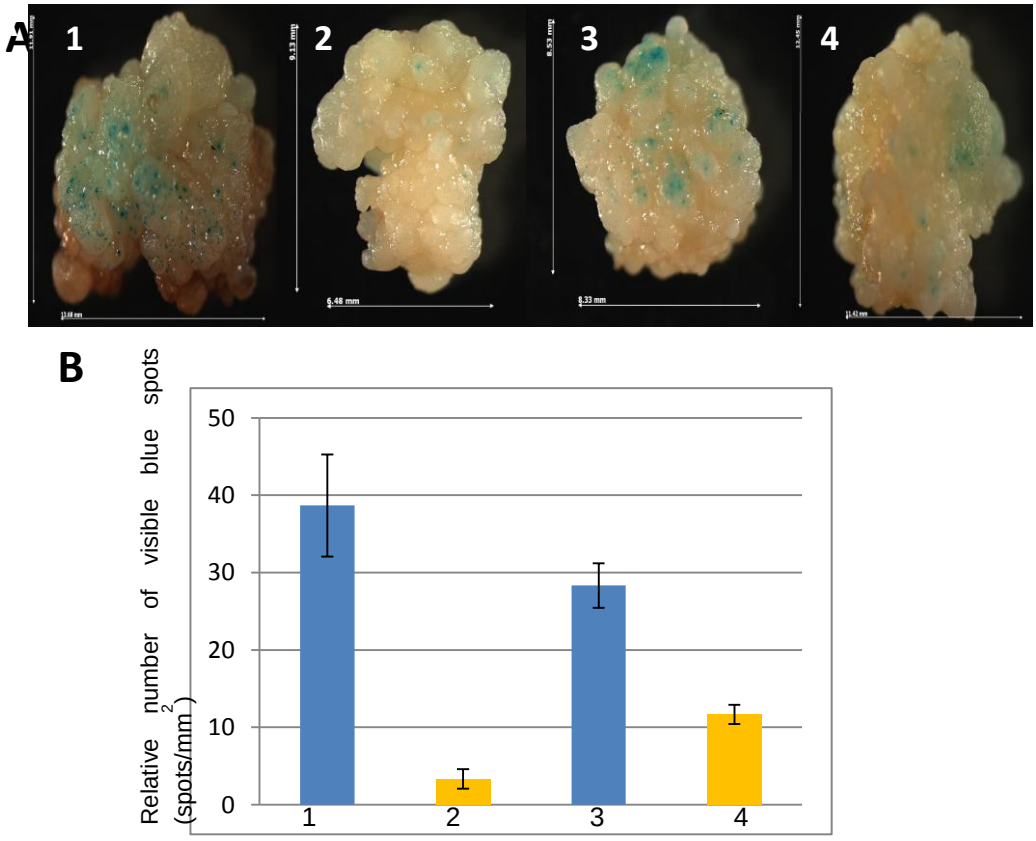

Figure 3.24: Transient expression assay to evaluate Ubi-hpCP:Hc-Pro construct in rice callus tissue. (A) X-Glucuronide staining of rice callus tissue bombarded with (1) 35S-GUS:CP:Hc-Pro, (2) 35S-GUS:CP:Hc-Pro + Ubi-hpCP:Hc-Pro, (3) 35S-GUS:Y-Sat and (4) 35S-GUS:Y-Sat + Ubi-hpCP:Hc-Pro constructs. Scale bar shows length and width of callus. **(B)** Histogram showing the average number of blue spots per square millimeter of bombarded rice callus tissue. Error bar represents standard deviation (n = 3).

3.4.8 Development of transgenic *Oryza sativa* through Ubi-hpCP:Hc-Pro binary construct

Rice, a model monocotyledonous species, as a surrogate of sugarcane was used because its diploid small size genome (~430 Mb) is easy to transform as compared to the other polyploid monocot crops such as wheat, maize and barley. Besides transformation efficiency, comparison of genetic maps within family *Poaceae* indicates the similarity in the genes function due to conservation of gene sequences. Thus, among other cereal crops, rice model plant was approachable to quickly test the efficacy of the Ubi-hpCP:Hc-Pro construct at inducing resistance to SCMV. Agrobacterium containing binary construct of monocot hairpin (Ubi-hpCP:Hc-Pro) was transformed into rice callus. pWBVec8 was also transformed as a control experiment. To initiate the monocot transformation sterilized Nipponbare rice seeds were grown on the N6D media (see section 2.7.2) (figure 3.25; 1). Rice calli were propagated on the callus induction medium and were ready to transform after 4-6 weeks (Figure 3.25; 2). Healthy calli were co-cultivated on the media plates (NBA plates; no antibiotic) with Agrobacterium (Figure 3.25; 3). After 48 hours of incubation period, calli were started growing under light on the NBHT50 media which contained hygromycin as plant selectable marker, followed by shoots development under dim light on PRHT50 media also containing hygromycin (Figure 3.25; 4,5). Once shoots were developed, roots started appearing on the resistant plantlets on RHT50 media which were ready to transfer to the soil in glass house (Figure 3.25; 6). As soon as plants were grown enough size, RNA was extracted from leaves of transformants for screening. Different stages of *O. sativa* tissue culture are mentioned in Table 3.7.

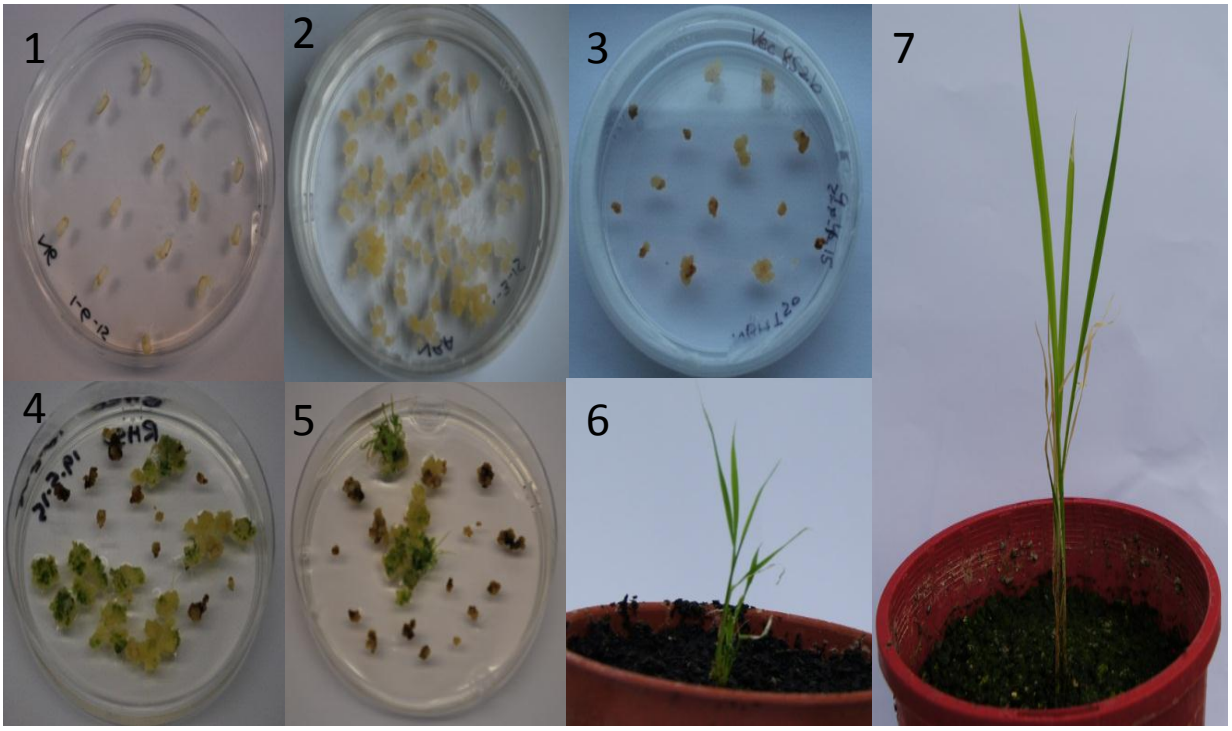

Figure 3.25: Different stages of *Oryza sativa* transformation with Ubi-hpCP:Hc-Pro through Agrobacterium. (1) Callus development on N6D media from Nipponbare variety of rice seeds (2) Propagation of callus on callus induction media (3) Co-cultivation of rice calli with Agrobacterium containing hairpin construct (4) Shoots development on the shooting media (NBHT50) containing hygromycin (5) Shoots propagation and roots development PRHT50 and RHT50 media (6) Regenerated plantlets shifted into glass house (7) Transformed lines are ready to screen

Table 3.7: Summary of *Oryza sativa* tissue culture mentioning hairpin constructs, components, duration on media.

Experiment Components	**Co-cultivation medium (NBA media)**	**Callus Induction medium (NBHT50)**	**Shooting Medium (PRHT50)**	**Rooting medium (RHT50)**
	48 hours (dark)	2-4 weeks (light)	2-4 weeks (Dim light)	3 weeks (light)
Ubi-hpCP:Hc-Pro(a) + Hygro	Growth of Agrobacterium	Callus formation	Green shoots develop	Roots develop
Ubi-hpCP:Hc-Pro(b) + Hygro	-do-	-do-	-do-	-do-
pWBVec8+Hygro (Empty vector)	-do-	-do-	-do-	-do-

3.4.9 Analysis of rice T_0 transgenic plants-Southern hybridization

Five independent Ubi-hpCP:Hc-Pro transgenic lines and one control transgenic line was selected to determine the transgene copy number. *Spe*I digested DNA was hybridized with the probe targeting the specific sequence of hairpin. Hybridization signals appeared suggest that rice genome integrated different copies of transgene while absence of hybridizing signals in control sample proved it was the successful transformation event (Figure 3.26). Five copies of transgene were existed in rice transgenic line# 1, as one band hybridized more strongly indicate that two copies are located at the same position in genome. However, lines # 2, line # 3 and line# 4 integrated single copy of hairpin RNA in the genome while, in lane # 5 multiple DNA banding pattern (upto ten) was detected from the rice genome. The size of the hybridizing band for the lines # 2, # 3, # 4 appears to be the same, which seems to indicate that they came from a single transgenic event. However, this is unlikely because the three plants were derived from different callus lines. The influence of copy number was noticeable on the seed production of transgenic. Single copy number lines produced more seeds as compared to multiple copy number lines.

3.4.10 Analysis of rice T_0 transgenic plants-Northern blotting for siRNA detection

Transformed rice plants were analyzed through Northern blot hybridization to confirm the production of 24-21nts siRNA. RNA extracted from young leaves of rice plants which were transformed with Ubi-hpCP:Hc-Pro and empty hairpin (Vec8) vector. Small interfering RNA (siRNAs) of 21-24 nts in size, corresponding to the CP:Hc-Pro sequence of the hpCP:Hc-Pro, were readily detected by Northern hybridization in all the five independent lines (Figure 3.27), indicating that the Ubi-hpCP:Hc-Pro is expressed and efficiently processed by DCLs into siRNAs. The size distribution of the siRNA is consistent with that of hpRNA-derived siRNAs in Arabidopsis, consisting of 21, 22 and 24 nt size classes (Fusaro *et*

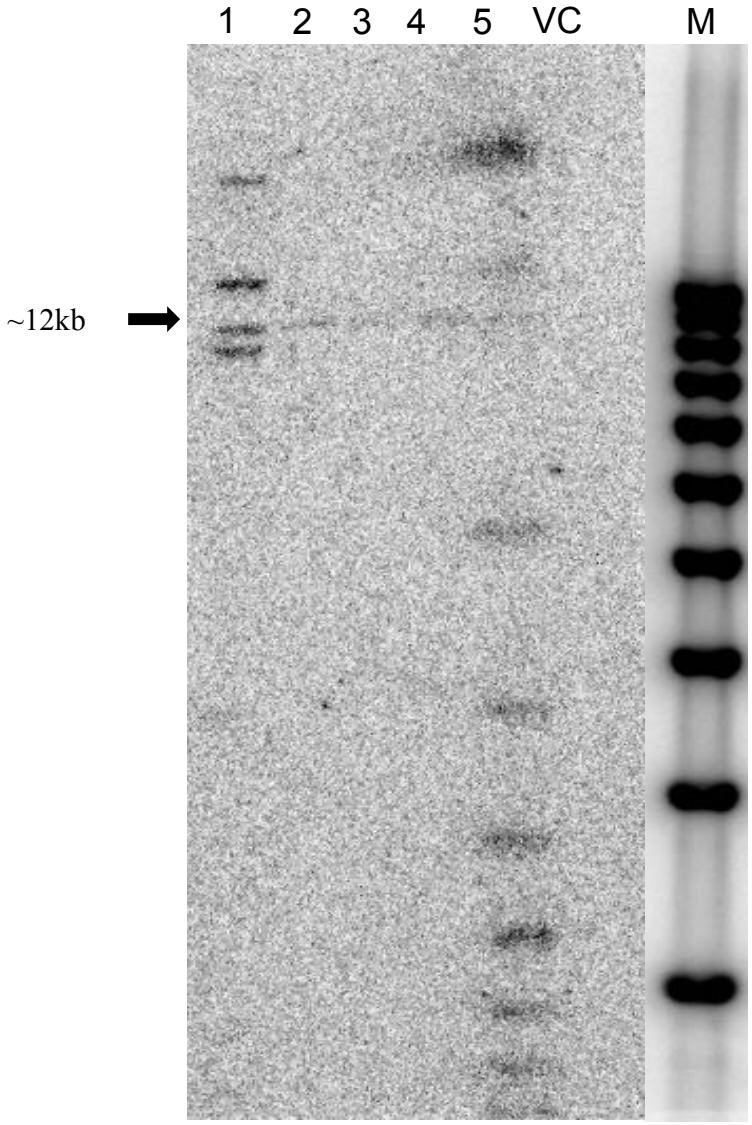

Figure 3.26: Southern blot analysis of T_0 transgenic rice plants. DNA extracted from transgenic rice leaves was digested with *Spe*I restriction enzyme, separated in 0.8% agarose gel, transferred to HyBond-N^+ Nylon membrane and hybridized with radioactive labeled CP:Hc-Pro DNA probe. The numbers 1-5 indicate five Ubi-hpCP:Hc-Pro transgenic lines. VC, transgenic rice plants containing the empty vector control transgene. M, DNA marker (1 kb plus Gene Ruler). The arrow indicates the single transgene band in lines 2, 3 and 4.

al., 2006), although the 21 and 22 nt species were not clearly separated (Figure 3.27). This indicates that the hpRNA is processed in transgenic rice by DCL4, DCL2 and DCL3, the same pattern as in Arabidopsis. In the previous reports, DCL4 dependent 21nt species abundantly produced in the positive sense RNA viruses (Garcia *et al*., 2010). Moreover, DCL2 and DCL4 worked redundantly in maize plants and Arabidopsis to accumulate higher level of 21nt and 22nt siRNAs (Garcia *et al*., 2010; Xia *et al*., 2014). It is noteworthy that in the different transgenic lines the siRNA hybridization signals showed different intensity, indicating a variable level of siRNA in these lines. Interestingly, the high-copy-number transgenic lines #1 and #5 did not accumulate more siRNAs, instead, they appear to generate slightly less siRNAs, suggesting that the multiple copy transgene insertion may have resulted in transcription repression of the hpRNA transgene (Wang and Waterhouse, 2000).

3.4.11 Assessment of hairpin transgene efficiency using qPCR

Functionality and expression of Ubi-hairpin transgene was validated by qPCR. Primers sequences used for the amplification of target gene along with the house keeping gene (Actin) sequence are mentioned in Appendix A.

Reference gene was optimized according to the pre-mentioned normalization strategy used for primers. Standard curve was generated by amplifying the actin gene in triplicate using serial dilution of RNA samples. Same conditions were used for qPCR as described in material and methods. Raw data was processed and analyzed to validate the

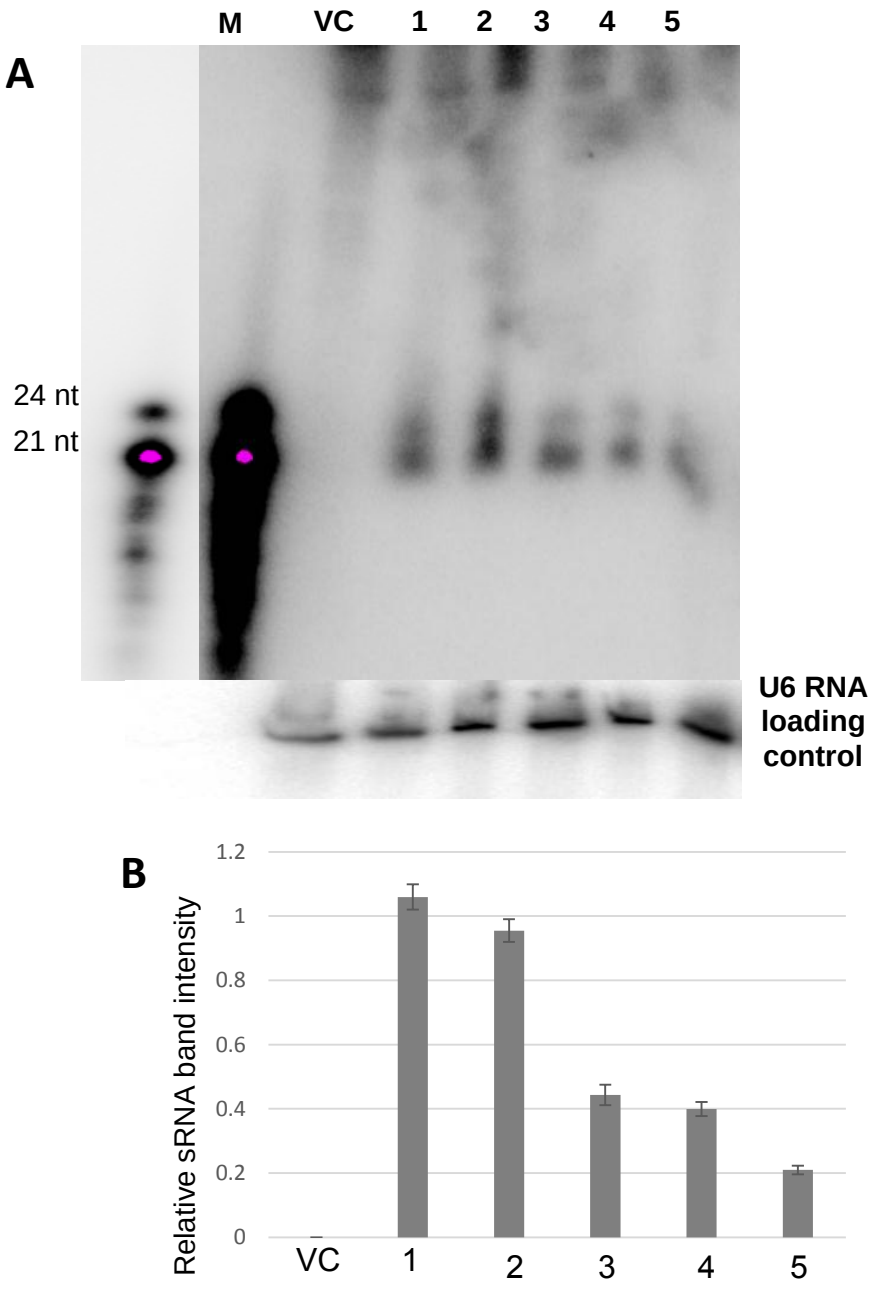

Figure 3.27: T_0 transgenic rice plants express 21-24 nt siRNAs. (A) Northern blot hybridization for the detection of siRNA from transgenic rice lines. Twenty five µg of total RNA extracted from leaves of transgenic plants was hybridized with antisense CP:Hc-Pro RNA probe (the upper panel). The U6 small nuclear RNA was hybridized as a loading control (the lower panel). Lane M represents the 21-24 nt radiolabeled RNA size marker, with a less-exposed picture given on the left to clearly identify the 21 and 24 nt bands. VC is transgenic rice plants containing the empty vector control transgene while, Lane 1-5 are the same five Ubi-hpCP:Hc-Pro transgenic rice lines shown in Figure 3. **(B)** Histogram representing the relative intensity of the sRNA band on the northern blot above.

quantitative difference between the expression level of Ubi-hpCP:Hc-Pro transgenic plants and control plants.

Expression pattern of the rice transgenic lines are depicted in the Figure 3.28A. As compared to control, all the transgenic lines were expressing higher level of Ubi-hairpin transgene. Gene expression percentage was validated in Figure 3.28B, in which transgenic line 3 had maximum expression among all other transgenic lines. These results demonstrate that rice transformed plant with empty hairpin vector did not display any expression while line# 3 showed percentage expression upto 700 folds or 7%.

3.4.12 Analysis of Ubi-hpCP:Hc-Pro transgene in T_1 rice generation

3.4. 12.1 Analysis of Ubi-hpCP:Hc-Pro transgene –Particle bombardment

To confirm that the Ubi-hpCP:Hc-Pro transgene in the transgenic plants could induce effective silencing against the target viral sequences, transient expression assay was first employed in transgenic rice lines. Seeds were harvested from T_0 transgenic lines which were grown in soil for further screening of T_1 progeny. T_1 leaves were collected and bombarded by 35S-GUS:CP:Hc-Pro target construct into transgenic rice leaves using gene gun followed by GUS expression assay. Strong GUS staining was observed in bombarded leaves of plants transformed with the empty vector but not in the leaves of the Ubi-hpCP:Hc-Pro transgenic plants, indicating that siRNAs derived from the Ubi-hpCP:Hc-Pro induced effective silencing against the 35S-GUS:CP:Hc-Pro transgene (Figure 3.29). These results proved that hairpin transgene was inherited in all progenies as a dominant trait and all lines showed high level of silencing against the targeted reported gene in transient assay.

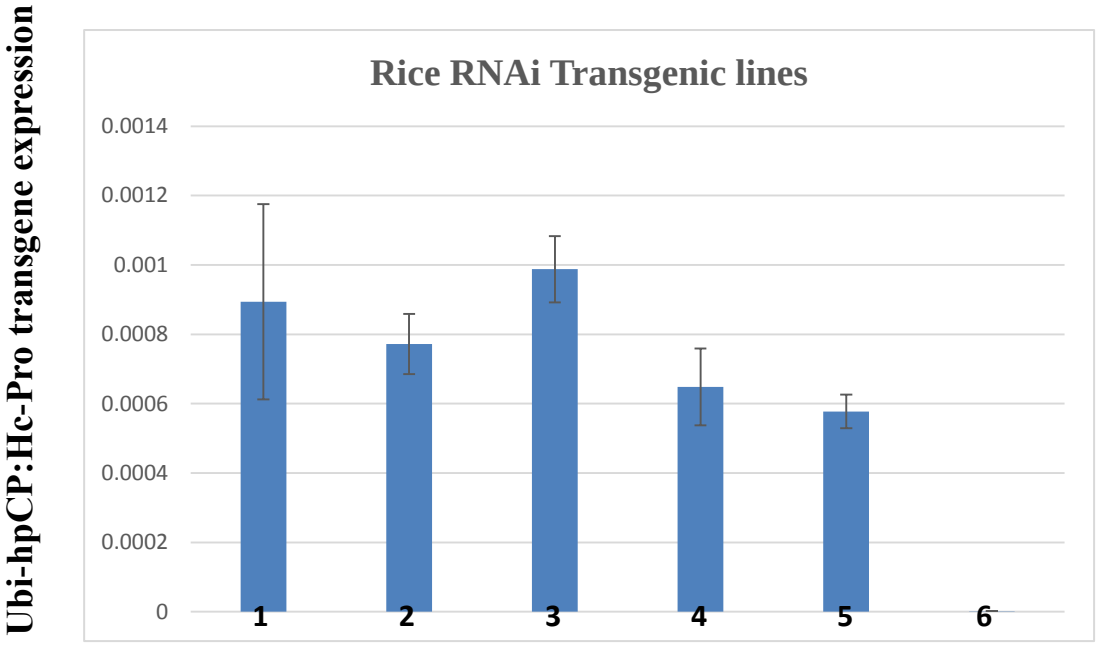

Figure 3.28A: Gene expression profile of Ubi-hpCP:Hc-Pro rice transformed lines (1-5) and control plant (6). Analysis of siRNA accumulated in transgenic lines by qPCR. Error bars indicate the standard deviation. Data presented in the graph are the combined results of three replicates for all samples.

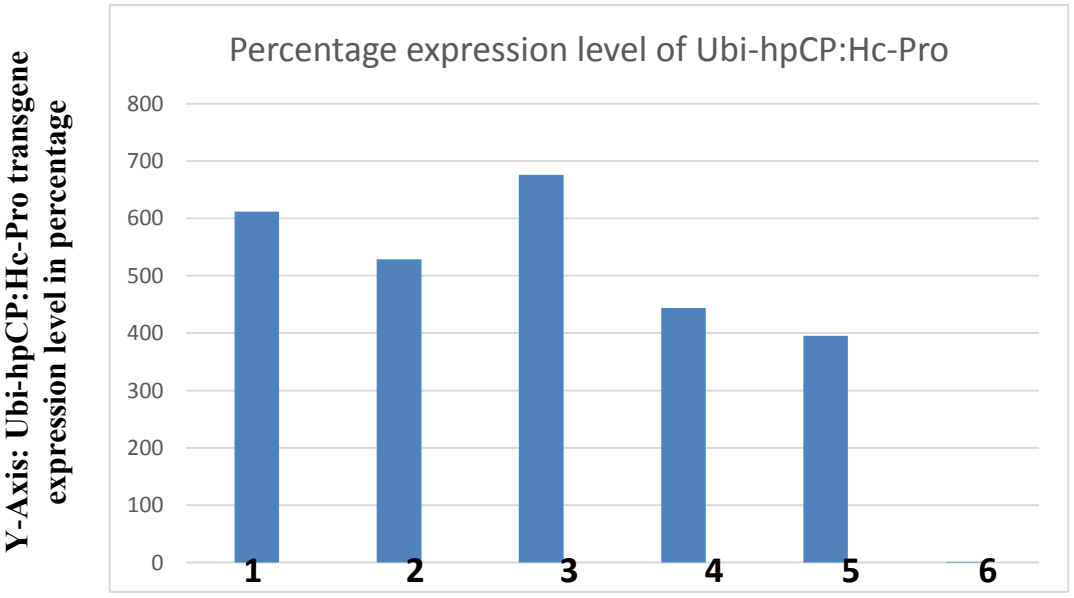

Figure 3.28B: Percentage data of siRNA produced from the rice transgenic lines (1-5) in comparison with control sample (6).

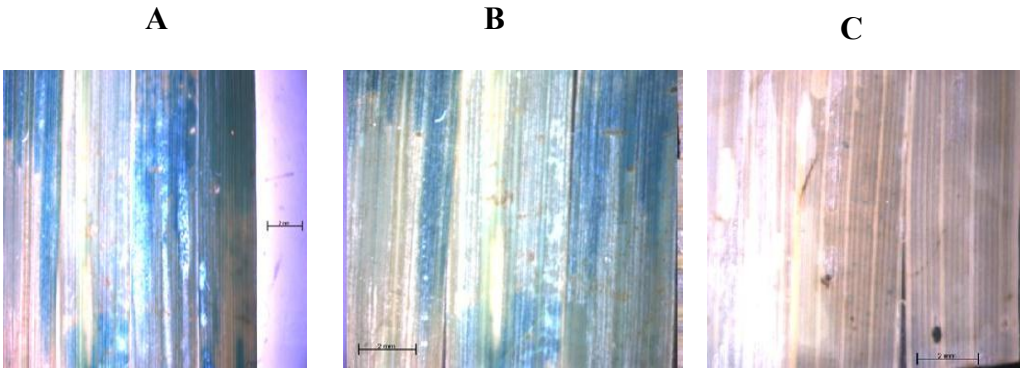

Figure 3.29: GUS expression assay in the Ubi-hpCP:Hc-Pro transgenic rice leaves. In the representative images, leaves of the rice plants transformed with the empty vector control **(A, B)** and those transformed with Ubi-hpCP:Hc-Pro (line #3) **(C)** were bombarded with 35S-GUS:CP: Hc-Pro fusion construct, and stained with X-Gluc. Prominent GUS expression (blue spots) was detected in the empty vector control leaves but GUS expression was hardly discernable in the leaves derived from rice plants transformed with Ubi-hpCP:Hc-Pro, indicating that GUS silencing was induced by the Ubi-hpCP:Hc-Pro transgene. Scale bar = 2 mm. The experiment was repeated for three times.

3.4.12.2 Analysis of resistance in mechanically inoculated transgenic leaves - reverse transcriptase PCR (RT –PCR)

To investigate either Ubi-hpCP:Hc-Pro transgenic plants were resistant to SCMV infection, seed was collected from lines #2, #3, #4 and #5 and plants of T_1 generation were grown and assayed for transgene expression and SCMV resistance. 4-6 weeks old transgenic leaves were mechanically inoculated with the SCMV (Brisbane, isolate A) according to the procedure mentioned in the material and methods. 25 days of post infection, RNA was extracted from the inoculated leaves. RNA from SCMV infected sugarcane leaves was used as a positive control in the experiment. *CP* detection primers were used for the RT-PCR amplification sequence mentioned in Appendix A. Viral infection in rice plants was proved through amplified *CP* band of about 180bp from the leaves of control sample. All of the infected Ubi-hpCP:Hc-Pro transgenic rice plants showed resistance against SCMV infection with no amplification of SCMV *CP* gene, except the transgenic line#1 and line #5 (which repressed the transgene expression due to multiple copy numbers) (Figure 3.30).

3.4.12.3 Analysis of resistance in mechanically inoculated transgenic leaves - real time PCR (qPCR)

To investigate if the Ubi-hpCP:Hc-Pro transgenic plants were resistant to SCMV infection, seed was collected from lines #2, #3, #4 and #5 primary transgenic plants (while line #1 did not produce any fertile seed) from which T_1 plants were established and assayed for transgene expression and SCMV resistance. The Ubi-hpCP:Hc-Pro transgene expression, as indicated by the hybridizing signals of the unprocessed Ubi-hpCP:Hc-Pro on the Northern blot, was clearly detected in the T_1 progeny of the single-copy lines #2, #3 and #4, indicating stable inheritance from the primary transgenic plants. However, for line #5, the

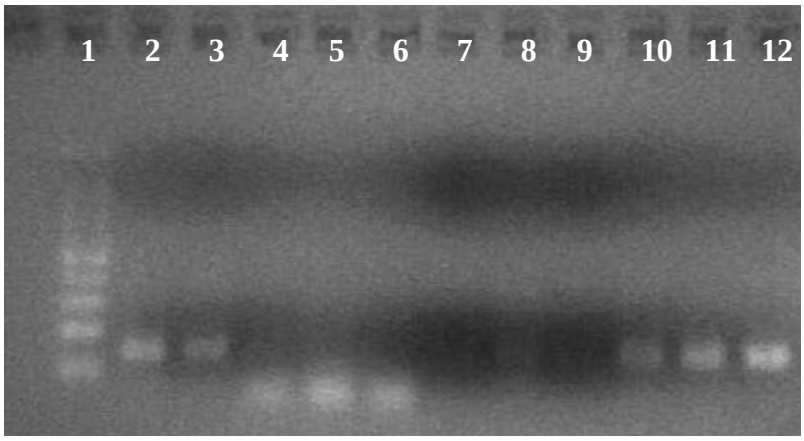

Figure 3.30: Agarose gel electrophoresis of RT-PCR from the mechanically inoculated rice transgenic leaves. Lane 1 is the 1 kb marker; Lane 2, 3 are the transgenic line #1, Lane 4, 5 are the rice transgenic line #2, Lane 6, 7 are the samples from line #3, Lane 8, 9 are the transgenic line #4, Lane 10, 11 are the line #5 while Lane 12 is the SCMV infected sugarcane sample (positive control).

highest-copy-number transgenic line, three of the four T_1 plants analyzed showed no Ubi-hpCP:Hc-Pro signal, which may indicate the transcriptional suppression of the Ubi-hpRNA transgene in these individuals. It is also noticeable that plant #5 from line #3, also showed no Ubi-hpCP:Hc-Pro hybridization signals (Figure 3.32A). This lack of Ubi-hpCP:Hc-Pro expression in plant #5 is likely due to segregation of the Ubi-hpCP:Hc-Pro transgene in T_1 progeny, which followed the Mendelian's pattern (3:1) of inheritance.

To examine if the Ubi-hpCP:Hc-Pro transgenic could confer effective resistance to SCMV infection, a number of T_1 plants of line #3, shown to express high levels of Ubi-hpCP:Hc-Pro in the Northern blot (Figure 3.31A), together with T_1 plants of an empty vector control line, were inoculated with SCMV. The 12 T_1 Ubi-hpCP:Hc-Pro plants showed visibly higher growth vigor than the 12 empty vector control plants at 6 weeks post inoculation, implying that the Ubi-hpCP:Hc-Pro line# 3 of T_1 generation proved to be resistant to SCMV infection (Figure 3.31B). No clear mosaic symptoms were observed in the SCMV-inoculated plants. Possible explanation for the lack of mosaic phenotypes is due to the SCMV-strain A used in this experiment, which is a relatively mild strain compared to the other strains of SCMV. As SCMV infection studies verified that plants infected with strain A typically showed stunted growth with mild mosaic pattern without necrosis (Summers *et al*., 1948; Abbott and Tippett, 1964). To confirm the SCMV resistance, RNA was extracted from a subset of the inoculated plants and analyzed for the presence of SCMV using RT-qPCR with *CP* and *Hc*-Pro specific primers. The empty vector control plants showed high levels of SCMV *CP* and *Hc-Pro* RNA (Figure 3.31C). In contrast, no significant amount of SCMV RNA was detectable in all five T_1 transgenic plants analyzed, indicating that the Ubi-hpRNA transgenic plants (from line #3) were highly resistant to SCMV infection.

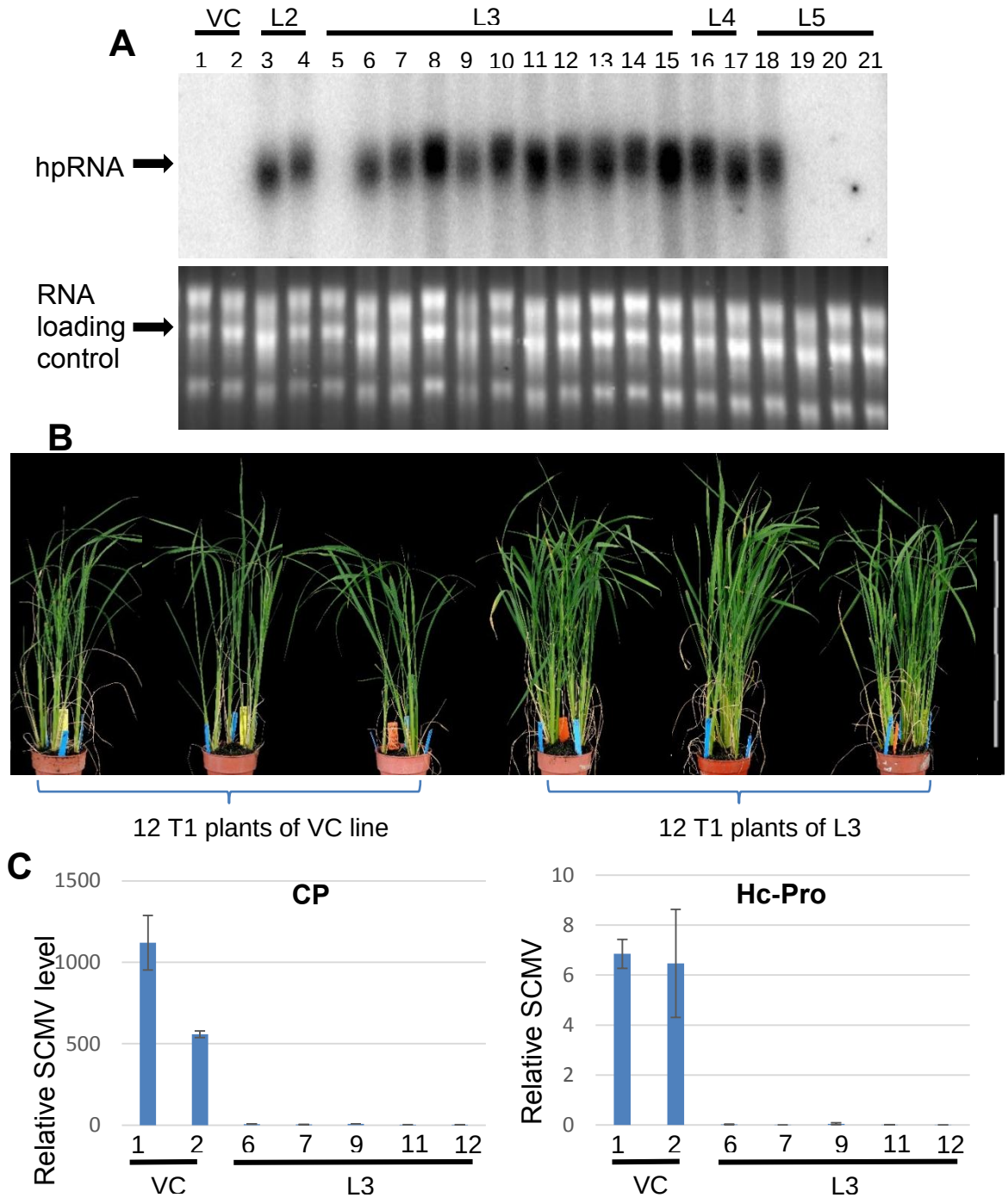

Figure 3.31: Ubi-hpCP:Hc-Pro transgene expression and SCMV resistance analysis in T_1 generation. (A) Northern blot hybridization of T_1 progeny of four independent Ubi-hpCP:Hc-Pro lines (#2, #3, #4 and #5) and an empty vector control line (VC) using ^{32}P-labelled antisense CP:Hc-Pro RNA as probe, which shows Ubi-hpCP:Hc-Pro expression from #2, #3 (except for one plant), #4 and one of #5 plants, but not from the vector control and the other three #5 plants. Lower panel shows RNA loading control. Asterisks indicate plants analysed in (C). **(B)** Vector control and Ubi-hpCP:Hc-Pro transgene T_1 transgenic rice plants inoculated six weeks prior with SCMV. **(C)** Quantitative real-time RT-PCR analysis of SCMV accumulation in infected VC and hpRNA transgenic T1 plants using *CP* (left) or *Hc-Pro* (right) primers.

3.5 Comparison of amiRNA and hpRNA strategy

In this study, processing and efficiency of amiRNA and hairpin transgenes were compared. Expression analysis of both transgenes was estimated through real time PCR data using comparative Ct method. Ct values obtained showed the very low expression in amiRNA transgene, with the maximium value of about 0.08% and 0.27% in CP-amiR-15 and Hc-Pro-amiR-7 transgene, respectively. However, hpRNA transgenic line # 3 showed maximum expression upto 7% as compared to the control plants. This reduced expression of amiRNA collectively correlated to the series of factors. It might be due to position effect of amiRNA and complimentarity of target gene. Further, 5' region and central region of amiRNA is critical for binding with RISC (Duan *et al*., 2012). Another limitation is the durability of amiRNA because miRNA silencing signals are cell autonomous and do not spread through out plant and 21-22nt amiRNA causes only tissue specific silencing (Kamthan *et al*., 2015).

Though, amiRNA and hpRNA techniques both are effectively used to silence the targeted genes as they together shared the same biochemical processing pathways. Despite of the similarities, slight differences among them contribute hpRNA technique remarkably approachable as compared to the amiRNA. Naturally occurring miRNAs are the key regulators for the expression of endogenous genes. MicroRNA (miRNAs) constitute a group of endogenous single stranded small RNAs (21-22 nucleotides) having negative regulatory function on gene expression (Mishra and Mukherjee, 2007), whereas siRNAs, act as a defenders to maintain the integrity of genome when foreign particles such as viruses, transgenes or transposons enters the cell (Meister *et al*., 2004). AmiRNA are 21 nucleotide sequences derived from guide strand of pre-amiRNA strand which preferentially avoid perfect complementarities to minimize transitivity problems and easy to predict the off-

targets (Schwab *et al*., 2006; Ossowski *et al*., 2008). This offers the advantage to target the different members of same family and even to provide capacity to target alleles of a single gene (Schwarz *et al*., 2006). In contrast, hpRNA generates multiple 21-24 nts siRNA from long dsRNA structure caused sequence specific degradation. Previously, it is also reported that amiRNAs are mostly caused local silencing (Alvarez *et al*., 2006) and inactivation of gene only at specific time and stage. While, 21-24 mer siRNAs can facilitate systemic silencing as well as modification in chromosome structure (Hamilton *et al*., 2002; Zilberman *et al*., 2003). Advantage of silencing caused by hpRNA are not only production of sequence specific siRNA but also amplification of silencing signals and their spread to the neighboring cells which results in the strong silencing of targeted transcript (Dunoyer *et al*., 2005; Deleris *et al*., 2006). Expression analysis data of amiRNA proved the less production and accumulation of amiRNAs. More understanding of utilization of effective pre-miRNA backbone, optimization of amiRNA processing and the complementarity features of amiRNA with targeted genes are required for maximum efficiency and silencing. Conversly, abaundant production of 21-24nt siRNA from long hpRNA provides strong SCMV silencing.

Chapter 4

DISCUSSION

Sugarcane mosaic virus (SCMV) is considered to be one of the most economically significant virus which causes severe reduction of sugarcane production all over the world. SCMV is transmitted through six different aphid species i.e. *Myzus persicae, Schizaphis graminum*, *Aphis gossypii*, *Rhopalosiphum maidis*, *Rhopalosiphum padi* and *Sitobion avenae* (Hasan *et al*., 2003). Besides that, Noone *et al*. (1994) identified that *R. maidis, R. padi, A gossypii* and *M. persicae* efficiently transmit the *Sugarcane mosaic potyvirus* (SCMV-SC) non-persistently to the different susceptible crops.

Virus naturally caused infection in numerous *Poaceae* species which mainly includes sugarcane, maize, sorghum, rye, rice, barley, cereals and various wild grasses (Teakle *et al*., 1989). However, Yahaya *et al*. (2014) first time reported the infection of SCMV in five different weeds species *Commelina benghalensis* (Mayere), *Cymbogon giganteus* (Dan Kwaire), *Sorghum arundinaceum* and *Arundo donox.* Incidence of infection in *Commelina benghalensis* weed species which belongs to the family *Commelinaceae,* first time reported the occurrence of SCMV in the other genera as well as along with the family *Poaceae*. However, Mollov *et al*. (2016) also first time reported the occurrence of SCMV in Columbus Grass (*Sorghum almum*) in the USA. They said that it may be an alternate host for the epidemiology of SCMV.

Primarily, to determine the sequence diversity of *CP* of SCMV from Pakistan, sugarcane leaf samples with mosaic symptoms were collected from sugarcane fields from different areas of Punjab and KPK Provinces. High infection rate of SCMV was observed in most of sugarcane growing regions of the Pakistan. The possibility for high incidence of infection may be due to more susceptibility of sugarcane varieties to *Sugarcane mosaic virus*

and another possible reason is the presence of more insect vector aphids or their reservoir near sugarcane fields which is responsible for transmission of disease (Luo *et al*., 2003; Zhou *et al*., 2007). Infection of SCMV in the cultivated crops such as capsicum also suggests that they could be the alternate source of virus spread to the nearby sugarcane fields. Similarly, dual infection of Sorghum crop with SCMV and SrMV, increases the risk of potential of infection in intercropped sugarcane fields. Another source of virus epidemics are the SCMV infected broad leaf crop such as *Commelina benghalensis* whose stands can even survive in the dry seasons after harvesting which rapidly developed new leaves along with the virus as soon as the rainy season starts (Yahaya *et al*., 2014). Infectivity level is also high due to frequent rate of mutations and recombination between the related groups of RNA viruses and particularly due to lack of RNA dependent RNA polymerase proof reading activity (Domingo and Holland, 1997, Sztuba-Solinska *et al*., 2011).

4.1 *CP* Gene based *Sugarcane mosaic virus* diversity in Pakistan

The present study was conducted to determine the disease incidence on the basis of coat protein gene of SCMV and their phylogenetic relationships was estimated with the rest of the world. Total twenty sugarcane leaf samples, showing mosaic and streak symptoms, collected from different sugarcane growing region of the country were positive for *CP* of SCMV. The available 20 *CP* DNA sequences shared highest (95-100%) nucleotide sequence identities among them and (85-100%) with the rest of the world. One sugarcane leaf sample showing streak symptoms was also positive for *CP* gene of SCMV, the possible explanation for which is dual or mixed infection of SCMV and *Sugarcane streak virus* (SSV). It is well known that dual infection is beneficial for viruses for causing local as well as systemic infection (Fraile *et al*., 2008). Since all the twenty *CP* sequences of present study shared a maximum nucleotide sequence identity between 95-100% hence these are the isolates of same species i.e SCMV. According to species demarcation criteria of *Potyviridae* published

in 9Th report of International Committee on Taxonomy of Virus (ICTV), nucleotide sequence identity either in coat protein or with in entire genome should be less than 76% and CP amino-acid sequence identity must be less than 80% (Adam *et al*., 2011).

Phylogenetic analysis depicts the SCMV distribution occurs throughout the world with very minor sequences variations. Phylogenetic analysis of SCMV *CP* sequences from the present study and sequences collected from databases divide it into three clusters I, II, III. SCMV *CP* sequences from various regions of Pakistan clustered in a single clade, representing very minor sequence variations between them. Previously reported two SCMV *CP* sequences (AM040436, DQ648195) from Pakistan were also grouped with present study isolates together with the sequences obtained from Australia, China, France, Argentina, South Africa and Iran. Like China, India is a neighboring country but the SCMV *CP* sequences obtained from India occupied a distinct position in the phylogenetic tree with high bootstrap value (98). The two SCMV *CP* sequences obtained from USA occupied independent positions in the tree and do not fit in any cluster. The most probable reason may be their collection from geographically diverge regions of USA. Correspondingly, Chinese isolates also distribute themselves into different clusters; two sequences grouped in cluster I while rest of the four sequences segregates with cluster III, so these are geographically distinct isolates of SCMV.

This wide spread of virus transmission is possibly through diseased seeds as SCMV can be transmitted through seeds (Haider *et al*., 2011). In Pakistan, sugarcane varieties probably got infection through import of SCMV from Australia. As Perera *et al*. (2008) reported the similar results of co-relationship of Pakistan and Australian isolates. Xie *et al*. (2016) also studied the genetic diversity and numerous strains of SCMV in different regions

of China. They reported that negative selection and recombination is the major factor for the wide distribution of SCMV isolates.

4.2 Analysis of conserved region of Coat protein

CP gene sequences have been extensively analyzed to assess the classification of species and strains (Adam *et al*., 2011) and to study the differences within and between the *Potyvirus* species (Urcuqui-Inchima *et al*., 2001). CP has highly variable N-terminus region which is exposed to the surface of virus (Shukla *et al*., 1988) while core region and C-terminus regions are conserved. However, C terminus exposed only few amino acids to the surface of virus (Shukla *et al*., 1988).

Alignment of amino-acids of all studied isolates highlighted the conserved DAG motif. Importance of conserved DAG motif (Asp-Ala-Gly) was previously proven in a study for its responsibility for transmission of *Potyvirus* through its insect vector aphids (Harrison and Robinson, 1988). Despite of highly variable N terminus region, its DAG motif conservation is important for the virus transmission while decline in virus spread also co-relates with the alteration of DAG region (Atreya *et al*., 1991). However, the variations in the conserved region were also reported such as isolates of *Pea seed-borne mosaic virus* comprises of DAS conserved aminoacids (Johansen *et al*., 1996) while *Peanut mottle virus* contains DAA motif (Flasinski and Cassidy, 1998) and *Plum pox virus* contains DAL sequences (Lopez-Moya *et al*., 1995). Experiments performed to analyze the mutational effect in DAG motif to the binding efficiency of *CP* with *Hc-Pro*. Evidences reported indicate the transmission of virus still occur but reduction in transmission efficiency (Blanc *et al*., 1997).

4.3 RNAi based resistance against SCMV

RNA silencing is a natural defense mechanism and it also regulates the endogenous gene expression. Improvement of plant resistance against virus infection through gene

silencing mechanism has verified to be effective in various plant biosystems. According to the previous data, transgenic plants resistant against *Tobacco mosaic virus* was first time developed when *CP* genes of *Tobacco mosaic virus* was transformed into tobaccum (Abel *et al*., 1986). Joyce *et al*., (1998) transformed the sugarcane with sense *CP* gene of SCMV which showed different phenotypes when challenged with SCMV. Furthermore, Ingelbrecht *et al*. (1999) also generated the multiple sugarcane resistance lines through the transformation of the coding sequence of *CP* gene of *Sorghum mosaic virus* (SrMV) strain H into sugarcane. Subsequently, some of these sugarcane transgenic lines displayed the mosaic symptoms after challenged with SCMV-H in field trials (Yao *et al*., 2004). Similarly, multiple genes of *Potyvirus* were separately introduced into the different plants to obtain the resistant lines (Gammelgard *et al*., 2007). Further, Leibman *et al*. (2011) studied the plant virus resistance development due to accumulation of transgenic small interfering RNA. Hairpin mediated gene silencing was developed against the *Hc-Pro* gene of *Zucchini yellow mosaic virus* (*Potyvirus*). Systemic silencing was observed in cucumber and melon transgenic lines due to accumulation of transgenic siRNA (t-siRNA). Moreover, Guo *et al*. (2015) developed the transgenic sugarcane plants resistant against the CP gene of *Sugarcane mosaic virus* through RNAi technology. Resistance development was screened through artificial inoculation of *Sorghum mosaic virus* (strain of SCMV) to the transgenic plants and then through RT-PCR to analyze the anti-*Sorghum mosaic virus* resistant plants. SCMV resistant transgenic lines were generated showing different level of virus resistance. Wang *et al*. (2016) used the long hpRNA strategy to develop the resistant rice plants against *Rice black-streaked dwarf virus* (RBSDV). Their results proved that long hpRNA targeting multiple genes of virus was useful for the development of stable virus resistance in rice and other plants as well. However, in this study, it was focused to develop the resistance against SCMV in model crops through silencing of targeted viral genes through amiRNA and hpRNA strategies.

4.4 Development of Artificial microRNA based binary constructs

4.4.1 Selection of amiRNA targets and transgene processing

Single stranded RNA genome of *Sugarcane mosaic virus* processed into ten mature proteins (Riechmann *et al*., 1992). Among ten functional proteins of SCMV, coat protein and helper component proteinase genes were targeted for amiRNA construction. These constructs targeted the conserved region of SCMV Coat Protein and Helper-component proteinase (RNA silencing suppressor) genes. In the present study two artificial microRNAs and two hairpin RNA gene silencing constructs were developed. Conserved regions of both genes were preferably targeted as Jiang *et al*. (2011) determined the efficacy of microRNAs generated against the different regions of coat protein of *Potato virus* Y (PVY). They constructed eight amiRNAs which cover the whole *CP* of PVY. Northern blotting and various other assays proved that out of 8 amiRNAs, only amiRCP-8 which target the 3' end of *CP* exhibited the highest virus resistance, while amiRCP-6 targeting the 3 proximal region of *CP* (highly variable region) exhibits the lowest resistance against virus. Their results confirmed that the appropriate selection of target region is crucial for derivation of pathogen derive resistance through amiRNA.

Careful selection of 21 base pair regions was based on the multiple functions of both proteins. As it was studied earlier, for the transmission of *Potyvirus*, DAG conserved region of *CP* and KITC motif of *Hc-Pro* interacts with each other (Atreya *et al*., 1995; Atreya and Pirone, 1993). For the effectiveness of amiRNA against diverse range of virus strains, *CP* and *Hc-Pro* isolates sequences were aligned and 21 nt conserved region including DAG and KITC motifs were selected. Arabidopsis (*Arabidopsis thaliana*) miR319a backbone was selected for the development of CP and Hc-Pro artificial microRNA constructs. Previously, thirteen miRNA families from Arabidopsis have been identified which includes miR156,

miR157, miR158, miR159, miR161, miR163, miR166, miR168, miR169, miR171, miR172, miR173, and miR319/miRJAW (Llave *et al*., 2002b; Palatnik *et al*., 2003). Among these thirteen miRNA families, miR319a produced more robust phenotypic changes as compared to the other derivatives (Schwaab *et al*., 2006). However, to develop the amiRNA constructs multiple key points were considered to be important. To increase the 5' instability, AU base pairs should be added in the start of transcript (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003) while 3' end must be stable as compared to 5' end. Insertion of C base at the position 19 is crucial [considered as last paired nucleotide miRNA/miRNA* duplex (Reynolds *et al.*, 2004)], which is supposed to be necessary for efficient processing of amiRNA constructs. Furthermore, in the amiRNA guide strand position 11 should be unpaired with the base at 9 position of amiRNA* strand to maintain the original amiR319a structure (Carbonell *et al*., 2014). Argonuate proteins further detect the mismatch position at 9^{th} position and cleaved the target transcript at the 10^{th} or 11^{th} position of targeted transcript (Lingel *et al*., 2003; Song *et al*., 2004). In the current study, real time PCR was used to verify the production and accumulation of *CP* and *Hc-Pro* amiRNA derived from amiR-319a backbone in the *N. tobaccum*. In this study, expression level of CP-amiRNA was not high as expected. However, the expression of Hc-Pro-amiRNA was significantly much more as compared to CP-amiRNA constructs. Possibly this may be due to reduced processing of amiRNA/amiRNA* duplex through RISC complex or may be due to non-cleavage of target region. Previous data also suggests that translational repression and methylation of RNA (which are involved in the regulation of gene expression) also cause the low expression or accumulation of amiRNA (Li *et al*., 2013; Ma *et al*., 2013; Fu *et al*., 2014). Thus, the amiRNA expression data encouraged us to generate the new hairpin constructs against the same regions of both *CP* and *Hc-Pro* genes.

4.5 Development of hairpin RNA mediated constructs

Hairpin RNA strategy was designed to silence the *CP* and *Hc-Pro* genes of SCMV that contain duplexed RNA sequences whose homology are similar to targeted gene. Previous research demonstrates that coat protein derived resistance was one of the most effective approaches to mediate antiviral resistance but many predict its limitation to the closely related virus species (Hamilton and Baulcombe, 1999). Here, in this study viral resistance was broaden by expressing the viral *CP* and *Hc-Pro* sequences including its conserved motifs. Thus, these RNAi constructs will be effective against broad range of SCMV strains. Constructs used contained the complementary RNA sequences whose homology is identical to targeted gene.

The main goal of the study was to target the conserved regions of multiple genes in a single construct. Addition of multiple transgenes on a single vector gives various benefits which includes successful co-transformation of transgene with equal dosage of gene and confirms that transcription units are equally influenced by the same position of chromosome. Hairpin RNA technology has also been used from many years to down-regulate the multiple genes (Halpin *et al*., 2001). It was also proved that long hairpin RNA construct targeting multiple genes of *Rice black streaked dwarf virus* was successfully used to regenerate stable and resistance lines against virus in rice (Wang *et al*., 2016).

For increasing the stability of hpRNA, maize ubiquitin promoter (Ubi) known to give strong constitutive expression in monocotyledonous plants and successfully used to drive the expression of hpRNA against *Barley yellow dwarf virus* in barley (Wang *et al*., 2000), was chosen to drive the expression of the SCMV hpRNA. However, to test the efficiency of hpRNA in dicot system, strong 35S CaMV promoter was chosen to direct the inverted repeat transgene. To further increase the strength of hpRNA optimal fragment length of insert was

selected. Optimum fragment size was selected in the current study to achieve the 100% efficiency of hpRNA construct as fragment size less than 50 bp results in low frequency of silencing and very larger hpRNA size results in recombination of bacterial host species (Wesley *et al*., 2001). Length of intron in hpRNA is also important factor which increased vector stability. Previously, it was proved that spacer region between complementary regions have no role in PTGS but specifically it increases the efficiency of transformants up to 100% (Waterhouse, *et al*., 1998; Smith, *et al*., 2000). For proper termination of transgene, Agrobacterium Octopine synthase gene terminator sequence (OCS terminator) was used which contains the signals for polyadenylation and cleavage (Depicker *et al*., 1982).

4.6 Transient expression analysis of hpRNA

Preliminary in the transient expression studies, microprojectile bombardment was carried out on *Oryza sativa* callus, for rapid detection of correlation between GUS and Ubi-hpCP:Hc-Pro construct. PDS-1000/Helium gene gun was used to rapidly co-integrate the multiple genes in a single bombardment. However, Tang *et al*., (1999) reported that 100% co-integration of multiple genes occurred in rice calli using gene gun. Standardization of protocol for bombardment and condition of calli are the main factors affecting the transformation efficiency. Ramesh and Gupta, (2005) mentioned that 6-7 weeks old calli are suitable for maximum GUS expression in rice calli. Selection of Nipponbare rice variety for plasmid bombardment was based on the observation that increased in GUS expression was noticed when Nipponbare variety of rice calli was used as compared to other varieties (Parveez *et al*., 1998).

Aforementioned studies validated that GUS reporter gene fused with gene of interest was successfully used to analyze chimera gene expression in transient system. This system is so sensitive that it detects the expression even from relatively few numbers of cells (Jefferson

et al., 1986). Noticeable GUS expression in the targeted reporter genes 35S-GUS:Y-Sat and 35S-GUS:CP:Hc-Pro fused constructs further confirmed the designed hypothesis. It was believed that in co-bombardment of Ubi-hpCP:Hc-Pro and 35S-GUS:CP:Hc-Pro fused plasmid, Ubi-hpCP:Hc-Pro is 100% specific to 35S-GUS:CP:Hc-Pro fused plasmid which downregulates the GUS expression by producing siRNA.

4.7 Analysis of stable transformed rice transgenic lines

Moreover, rice, a model monocotyledonous species, was used as a surrogate of sugarcane to test the efficacy of the Ubi-hpCP:Hc-Pro construct at inducing SCMV resistance. Rice is economically important cereal crop all over the world. Nipponbare rice variety was selected for transformation of hairpin constructs due to natural infection of *Sugarcane mosaic virus* on rice plant (Teakle *et al*., 1989). Hairpin RNA generated transgenic lines were able to further screen the level of resistance acquired against SCMV infection.

Multiple rice transgenic lines were screened for the production of 21-24nts siRNAs which provides insights into the processing of Ubi-hpCP:Hc-Pro. In this study, detection of large number of siRNAs was performed through Northern blot analysis. Successful identification of siRNA represents that primary transcripts undergo processing in the nucleus by Dicer enzymes. According to the literature, four distinct classes of Dicer enzymes are generated in *Arabidopsis thaliana*, DCL1 and DCL4 produced siRNA of about 21 nucleotides. However, DCL2 generates 22 nucleotide siRNAs while DCL3 are specific for the production of 24-26 nts siRNAs (Xie *et al.*, 2004). Small RNA produced is primarily associated with posttranscriptional modes of gene regulation (Lippman and Martienssen, 2004). In this study, it was identified that the size distribution of the siRNAs is consistent

with the typical pattern of hpRNA-derived siRNAs (Fusaro *et al*., 2006), with the 21-22 nt siRNAs being more abundant than the 24-nt species.

In the present study, copy number of transgenic plants was detected through Southern blot hybridization. Copy number is defined as number of transgenes insert per haploid genome. However, it is reported that single copy of transgene is preferable because it is stable over subsequent generations (Meyer, 1998). Current study determined that majority of transgenic event contain single copy number. The influence of copy number was noted on the seed production of transgenic. Single copy number lines produced more viable seeds as compared to multiple copy number lines. It is known that transgenic copy number are inversely proportional to level of expression, as multiple copy number may cause silencing or co-suppression of transgene (Vaucheret *et al*., 1998; Fagard and Vaucheret 2000). Here, it was reported that the abundance of siRNAs showed no positive correlation with the copy number of hpRNA transgene insertion, and it appeared that the higher the copy number, the less the siRNA accumulation.

It is illustrated that transformation of RNAi constructs targeting the *CP* and *Hc-Pro* gene of SCMV was lead to production of siRNA in majority of transformants. Further these transformants were analyzed to check the resistant against virus infection. It is already reported that resistance against viruses via RNA silencing mostly based on the expression of dsRNA which are processed into siRNA and leads to the degradation of viral RNA. Several reports proved that there is a co-relationship between viral specific siRNA production and virus resistance (Chen et al., 2004; Negri *et al*., 2005; Kalantidis *et al*., 2002; Missiou *et al*., 2004; Smith *et al*., 2000; Wang *et al*., 2000). Target reporter gene was silenced in the transgenic lines expressing the siRNA. This *in-vitro* analysis was proved further when SCMV strain A was artificially inoculated on the T_1 transgenic leaves. As among the different strains

of SCMV, strain A, B, D and E are more prominent in natural infection (Rishi and Rishi, 1985; Rao *et al*., 1998). Bain's and Matz's optimized protocol was adopted for maximum infection (i.e. 60%) of SCMV through mechanical inoculation of rice leaves.

Analysis of hpRNA accumulation in the subsequent T_1 population indicated that the hpRNA transgene is subject to transcriptional silencing when the number of transgene insertions is very high (Fagard and Vaucheret 2000; Wang and Waterhouse 2000). Ubi-hpCP:Hc-Pro transgene expression in the high-copy line #5 was totally lost in some of the T_1 progeny, confirming the detrimental effect of high copy number transgene insertion on transgene transcriptional stability. This suggests that for achieving stable virus resistance transgenic plants with a simple transgene insertion pattern should be selected.

Virus infection assays of the single-copy transgenic line expressing high levels of the Ubi-hpCP:Hc-Pro showed that the transgenic plants are highly resistant to SCMV, which displayed a more vigorous growth than vector control transgenic plants, with almost no detectable SCMV RNA in contrast to abundant SCMV RNA in the vector control. SCMV-inoculated plants were failed to developed typical mosaic symptoms. This asymptomatic infection in rice is due to the inoculation of SCMV-strain A in the infectivity experiment. This is because strain A show mild molting and green mosaic pattern along with little growth retardation in the later stages of infection as compared to the other strains of SCMV (Abbott and Tippett 1966). Current study generated transgenic *O. sativa* plants that were resistant to SCMV infection. Infectivity data showed that high-level SCMV resistance was achieved using long hpRNA transgene targeting two essential viral genes at the same time.

CONCLUSION & FUTURE PROSPECTS

Sugarcane mosaic virus is a significant disease of sugarcane which causes extensive yield loses of host plant all over the world. To control this disease, it is important to understand the virus genetic diversity which provides complete understanding of the mechanism of virus-host interaction. Sugarcane infected SCMV samples from different regions of Pakistan were collected which reported that there is no significant diversity among the SCMV populations. High infection rate was also noted which could be possibly due to the import of diseased seeds. Data obtained also identify the conserved motifs (DAG) in the coat protein gene which was further selected in this study as a target region to develop the resistant model plants.

RNA silencing is a natural defense mechanism and it also regulates the endogenous gene expression. In the present study two artificial microRNAs and two hairpin RNA gene silencing constructs were developed. These constructs targeted the SCMV Coat Protein and Helper-component proteinase (RNA silencing suppressor) genes conserved regions to compare the silencing efficiency of both techniques.

To analyze the resistance against SCMV, developed amiRNA constructs were first transformed into model dicot plant (*Nicotiana tobacum*). Expression analysis of both constructs through the real time PCR showed very low production of 21nt amiRNA which might be due to low processing efficicency of amiRNA construct in RISC complex.

Hairpin RNA constructs were designed in such a way that instead of developing two constructs for two different genes of SCMV, a novel strategy was adopted in which long hpRNA approach was used which simultaneously targeted the two different genes in a single transgene. *CP* and *Hc-Pro* genes were fused together and cloned in two hairpin vectors,

specifically contained maize Ubiquitin promoter to drive the transgene expression in monocots and 35S CaMV constitutive promoter which transcribed the hpRNA insert in dicot host system.

Firstly, *N. benthamiana* leaves and *O. sativa* calli were used to rapidly assess the Ubi-hpRNA functionality and efficiency. Agroinfiltration and bolistics data from *N. benthamiana* and *O. sativa*, respectively confirmed the efficient processing of hpRNA. Evalution of specificity of Ubi-hpRNA was proved through the 35S-GUS:CP:Hc-Pro and 35S-GUS:Y-Sat which used as a positive and negative control, respectively.

Transient data expanded through stable transformation of hpRNA in *N. tobaccum* and *O. sativa* to study the mechanism of PTGS and the suppression of virus infection after virus inoculation. The available evidences obtained through the Northern blotting and qRT-PCR indicated that efficient processing and expression of hpRNA in both dicot and monocot model plants. Further, virus infection data showed that PTGS derivatives moved from infected parts to the non-infected parts of plant and prevented the spread of virus infection in the transgenic infected SCMV rice plants. —Transgenic *O. sativa* plants were generated that were resistant to SCMV infection. Our infectivity data showed that high-level SCMV resistance was achieved using long hpRNA transgene targeting two essential viral genes at the same time. While this strategy has not been tested in sugarcane due to the complexity of transformation of polyploidy genome, we it is expected that in future this hpRNA construct will confer strong SCMV resistance when transformed in to sugarcane. Forthcoming, it is important to study diversity of SCMV from diverse areas of Pakistan based on the complete genome rather than on the only one gene.

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APPENDIX

Appendix A: List of primers used for amplification of transgene, RT-PCR, qRT-PCR and amiRNA constructs.

Primer Name		Primer Sequences (5'-3')
CP-RT-PCR	CP-F	TTACAACGAAGATGTTTTCC
	CP-R	CTGAAATAGTAAATACGAGG
amiRNA CP Primers	Oligo-I	GATATGATGCCATAAAGAAGGATCTCT CTTTTGTATTCC
	Oligo-II	GATCCTTCTTTATGGCATCATACTCAA AGAGAATCAATGA
	Oligo-III	GATCATTCTTTATGGGATCATACTCAC AGGTCGTGATATG
	Oligo-IV	GAGTATGATCCCATAAAGAATGATCTA CATATATATTCCT
amiRNA Hc-Pro primers	Oligo-I	GATTTAAAATCACATGCAAAACGTTCT CTCTTTTGTATTCC
	Oligo-II	GAACGTTTTGCATGTGATTTTAAATCA AAGAGAATCAATGA
	Oligo-III	GAACATTTTGCATGTCATTTTATATCAC AGGTCGTGATATG
	Oligo-IV	GATATAAAATGACATGCAAAATGTTCT ACATATATATTCCT
amiRNA vector primer	Primer A	CTGCAAGGCGATTAAGTTGGGTAAC
	Primer B	GCGGATAACAATTTCACACAGGAAAC AG
amiRNA detection primer	CP-amiRNA	GATCCTTCTTTATGGCATCATAC
	CP amiRNA*	GTGAGTATGATCCCATAAAGAATGA
	Hc-Pro- amiRNA	GAACGTTTTGCATGTGATTTTAAA
	Hc-Pro amiRNA*	GATATAAAATGACATGCAAAATGTTC
Real Time PCR Primers for amiRNA	CP-amiRT-F	TCCTTCTTTATGGCATCATAC
	CPamiRT-R	CATTCTTTATGGGATCATACTC
	Hc-amiRT-F	CGTTTTGCATGTGATTTTAAATC
	Hc-amiRT-R	ACATTTTGCATGTCATTTTATATC
	Actin-amiRNA-F	GGCTCCTCTTAACCCAAAGG
	Actin-amiRNA-R	ACCCTCGTAGATTGGCACAG
Real Time Primers for hpRNA expression	EF-1α-hpRNA-F	CACTCTTGGTGTGAAGCAG
	EF-1α -hpRNA-R	GACTTCCTTCACGATTTCATC
	Ubi-hpRNA-F	CTCACCGACTACAGCTTAGC
	Ubi-hpRNA-R	CAGACCGAACAGTCGTGTT
Real Time Primer for SCMV expression	UBQ-10-F	GGTCAGTAATCAGCCAGTTTG
	UBQ-10-R	CCACAAATACTTGACGAACAG
	CP-RT-F	ATGTAGATGCTGGTACGACAG
	CP-RT-R	TCTTGTTGTTGCGGTTTGTATG

Appendix B

MEDIA COMPOSITION FOR *N. TOBACCUM* TISSUE CULTURE

MSO-No antibiotics	**Amount per liter**
Macronutrients 20X	50 mL
Micronutrient 1000X	1 mL
MS vitamins 100X	10 mL
MS Iron/EDTA 200X	5 mL
Sucrose	30 g
Myoinositol	100mg

MS9-SHOOT REGENERATION MEDIA	**Amount per liter**
Macronutrients 20X	50 mL
Micronutrient 1000X	1 mL
MS vitamins 100X	10 mL
MS Iron/EDTA 200X	5 mL
Sucrose	30 g
Myoinositol	100mg
BAP (0.2mg/mL stock)	5 mL
IAA (1mg/mL stock)	500 µL

MS4-ROOT REGENERATION MEDIA	**Amount per liter**
Macronutrients (20X)	50 mL

Micronutrient (1000X)	1 mL
MS vitamins (100X)	10 mL
MS Iron/EDTA (200X)	5 mL
Sucrose	30 g
IAA (1 mg/mL stock)	50 µL

MS MACRONUTRIENT (20X)	**PER LITER**
NH_4NO_3	33g
$CaCl_2.2H_2O$	8.8g
KNO_3	38g
$MgSO_4.7H_2O$	7.4g
KH_2PO_4	3.4g
MS MICRONUTRIENTS (1000X)	**PER 500 mL**
H_3BO_3	3.1g
$MnSO_4.4H_2O$	11.2 g
$ZnSO_4.7H_2O$	4.3g
KI	415mg
$Na_2MoO_4.2H_2O$	125mg
$CuSO_4.5H_2O$	12.5mg
$CoCl.6H_2O$	12.5mg
MS VITAMINS 1000X (Store at 4 ^oC)	**PER 100 mL**
Nicotinic Acid	50 mg
Pyridoxine HCl	50mg
Thiamine HCl	10 mg

Glycine	200 mg
MS Iron/EDTA (200X)	**Per 500 mL**
Di-Na-EDTA	3.35g
$FeCl_3.6H_2O$	2.70g

Appendix C

Media composition for Rice Tissue Culture

N6 Macro element (20X)	**g/L**
$(NH_4)SO_4$	9.3
KNO_3	56.6
KH_2PO_4	8
$MgSO_4.7H_2O$	3.7
$CaCl_2.2H_2O$	3.3
B5 Micro-element (100X)	**mg/1000mL**
$MnSO_4.4H_2O$	1000
$Na_2MoO_4.2H_2O$	25
H_3BO_3	300
$ZnSO_4.7H_2O$	200
$CuSO_4.5H_2O$	3.87
$CoCl_2.6H_2O$	2.5
KI	75
B5 Vitamins (100X)	**g/250mL**
Gamborgs Vitamin powder (Sigma Cell Culture)	2.8
FeEDTA (200X)	**g/200mL**
Ferric-sodium Salt (Sigma Cell culture)	1.47
NB Media	**Amount/Litre**
N6 macro element (20X)	50 mL
B5 micro-element (1000X)	1 mL
B5 vitamins (100X)	10 mL

FeEDTA (200X)	5 mL
2,4 D (1mg/mL)	2 mL
Sucrose	30 g
Proline	500mg
Glutamine	500 mg
CEH (Casein Enzymatic Hydrolysate)	300mg
NBHT50	**Amount/Liter**
NB media	
Hygromycin (50mg/L)	
Timentin (150 mg/mL)	
PRHT50	
NB media (with no 2,4 D)	
BAP	3 mg/L
NAA	0.5mg/L
Hygromycin	50mg/L
Timentin	150mg/mL
½ MS	**Amount/L**
MS salts and vitamin mixture	2.21 g
Sucrose	10 g
Add 2.5 g phytogel/L	
NAA (0.05 mg/L)	
Timentin (150mg/mL)	
N6 Vitamins (1000X)	**mg/100mL**
Glycine	200

Thiamine-HCl	100
Pyridoxine-HCl	50
Nicotinic acid	50
N6D media	**Amount/Liter**
N6 macro (20X)	50mL
N6 micro (1000X)	1 mL
N6 vitamins (1000X)	1mL
MS Iron/EDTA	5 mL
Myoinositol	100 mg
Casamino acid	300 mg
Proline	2.9 g
2,4 D (1mg/mL)	2 mL

- **2,4 D (1mg/mL) 2,4- dichlorophenoxyacetic acid**

  Dissolve 100mg of 2,4 in 1 mL absolute ethanol, add 3 ml of 1 N KOH, adjust to pH 6 with 1 N HCL, make to 100 mL with distill water.

- **BAP (2mg/mL) 6-Benzylaminopurine**

  Weigh 100 mg BAP, add 1N KOH dropwise until powder is dissolved, make to 50 mL with distill water.

- **NAA Naphthaleneacetic acid (1 mg/mL)**

  Dissolve 100mg of NAA in 1mL of absolute ethanol, add 3 ml of 1N KOH, adjust volume to 80 mL with distill water, adjust pH to 6 with 1N HCl, make to 100 mL with water.

- **ABA Abscisic Acid (2.5 mg/mL)**

  Dissolve 250mg of ABA in a minimum volume of DMSO, make to 100 mL with distill water.

- **Acetosyringone 100mM**

  Dissolve 196 mg of 3-5 dimethoxy-4-hydroacetophenone in small amount of DMSO. Bring volume to 10 mL with distill water. Syringe filters the solution.