Chapter 6

How RNA Interference Combat Viruses in Plants

Bushra Tabassum, Idrees Ahmad Nasir, Usman Aslam and Tayyab Husnain

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772//51870

1. Introduction

RNA mediated silencing technology has now become the tool of choice for induction of virus resistance in plants. A significant feature of this technology is the presence of double-stranded RNA (dsRNA), which is not only the product of RNA silencing but also the potent triggers of RNA interference (RNAi). Upon RNAi induction, these dsRNAs are diced into short RNA fragments termed as small interfering RNAs (siRNAs), which are hallmarks of RNAi. Considerable resistance in transgenic plants against viruses can be created by exploiting the phenomenon of RNAi. In the current chapter, generation of potato virus Y (*PVY*) resistant potato and sugarcane mosaic virus (*SCMV)* resistant sugarcane by CEMB has been quoted as an example.

We are in the dawn of a new age in functional genomics driven by RNAi methods. RNA interference (RNAi) refers to a post-transcriptional process triggered by the introduction of double-stranded RNA (dsRNA) which leads to gene silencing in a sequence-specific manner. It is one of the most exciting discoveries of the past decade in functional genomics and is rapidly becoming an important method for analyzing gene functions in eukaryotes and holds promise for the development of therapeutic gene silencing and which is therefore currently the most widely used gene-silencing technique in functional genomics.

2. Need for resistance

Agriculture sector of any country strengthen the economy by contributing in its gross domestic product (GDP). In Pakistan, the major agricultural crops include cotton, wheat, rice, sugarcane, potato and tomato etc. All of the above mentioned crops has great potential for yield and contribute 24 % in gross domestic product (GDP) of Pakistan economy [1]. There is a major gap in the actual yield potential of each crop with respect to its harvested yield, possible reasons include disease attack, environmental damages and in some cases lack of quality seed. Disease attack as being the most common cause include infections by

pathogens like viruses, bacteria and insects etc. Viruses can cause most devastating effects due to their systemic infections and hence decrease crop productivity in primary infection and reduce seed quality for subsequent use through persistent infection. Viral epidemics are often associated with the emergence of a new form of the viral strain or a new form of vector.

Viruses are a major threat to agriculture all over the world. Up till now, more than 1200 plant viruses have been reported which include 250 of those viruses that cause significant losses in crop yield [2]. In nature, viral particles exist as obligate parasites which consist of hereditary material packed in a thick layered coat and completely depend on host cell throughout their life cycle. Viruses utilizes host resources like nucleic acid, amino acids and certain proteins for their replication and survival, thus disturbing host plant metabolism to a considerable extent. Most of the infecting plant viruses are ssRNA viruses like sugarcane mosaic virus, potato virus Y etc. In an infected plant, virus accumulation goes higher with increased progeny rate through its replication. The spread of the virus in a plant is achieved through its movement from infected cell to healthy one via plasmodesmata while long distance movement occurs through phloem. Entry of virus in plant usually occurs through physical injury like wound etc or via certain viral vectors like aphid, fly etc.

Cotton (Gossypium hirsutum) as being commonly known as 'white gold' is an important cash crop in many developing countries including Pakistan. It is a natural fibre and has many uses in industries. It accounts for 8.2 percent of the value added in agriculture and about 2 percent to GDP of Pakistan. The yield of the crop is severely affected by the viruses including geminiviruses (leaf crumple and leaf curl) and tobacco streak virus etc. These viruses can cause severe losses when infections occur on young plants; some infect cotton yield while others affect lint quality as well [3].

Potato (*Solanum tuberosum*) is the world's major food crop and is one of the leading vegetables. Viruses are a serious problem, not only because of effects caused by primary infection, but also because the crop is vegetatively propagated and the viruses are transmitted through the tubers to subsequent generations. Potato virus Y (PVY) is probably the most damaging and widespread virus of potato and is found wherever potato crops are grown, where losses are reported upto 10 to 90% [4]. PVY is transmitted through aphids.

Sugarcane (*Saccharum* spp. hybrid) is among the top 10 food crops of the world, and yearly provides 60% to 70% of the sugar produced around the world [5]. Yield harvested by the farmers of Pakistan is very low whose main cause is mosaic disease of sugarcane which continues to be a potential threat to the sugarcane production. It is a very common disease in all the major sugarcane growing regions, because of the perpetuation of the disease virus through vegetative propagules. Sugarcane mosaic virus (*SCMV*) is reported to infect sugarcane naturally and can cause severe losses to the farmers and lesser production to the industry [6,7]. Aphids are the vector for transmission of the disease. Seed produced by infected cane can also transmit the disease.

Tomato (*Lycopersicum esculentum*) ranks among the most widely grown vegetables all over the world. In general, viral diseases are not a routine problem in most tomato plantings but incidence of some viruses including tomato spotted wilt virus, tomato leaf rolling, tobacco mosaic virus and single and double virus streak viruses has devastating impact on crop yield with losses of upto 100% have also been reported [8]. Tobacco mosaic virus is one of the most stable viruses known because it is able to survive in dried plant debris as long as 100 years.

Various control measures have been taken to overcome losses caused by plant viruses which are expensive and also inadequate. Biotechnologists have developed and adopted several strategies for virus resistance in crop plants. These include cross protection, pathogen derived resistance and more recently RNA interference. Therefore, the development of virus resistant varieties seems the only economically feasible way to control viruses [9]. Today, use of resistant varieties has been advocated as the most promising and least expensive method of viral disease suppression. With the appealing results of RNAi in silencing target genes of attacking virus, RNAi seems to have potential for creating virus resistant crops.

A plant is said to be resistant if it has the ability to suppress viral disease symptoms by inhibiting its replication or by blocking the virus expression. Resistance mechanism in plants may be either protein mediated or RNA mediated, however final outcome of both is reduced accumulation of virus in the host plant. Acquired resistance could be either high almost reaching immunity with no disease symptoms or moderate to low where mild symptoms of particular viral disease can be seen. In contrast, when a viral infected plant shows normal growth rate with a good yield along with milder symptoms of disease, it is said to be a tolerant plants. In this case, the host plant supports multiplication of virus rather than blocking its replication [10,11].

3. History of virus resistance in transgenic plants

When a plant encounters virus, it reacts naturally through hypersensitive response (HR) and extreme resistance response (ER) which induces the production of secondary metabolites termed as response elements in plants. These response elements include elevated levels of ethylene, jasmonic acid, salicylic acid, nitric oxide and increased rate of ion flux, in combination these factors block the virus entry and /or helps eliminate the virus (figure 1).

The acquired virus resistance mechanisms in plants are of two types: a) gene silencing independent virus resistance and b) gene silencing dependable virus resistance via Post Transcriptional Gene Silencing (PTGS). The first includes coat protein-mediated, movement protein-mediated and replicase protein-mediated resistance, while second includes pathogen-derived resistance, antisense RNA mediated resistance and RNA-mediated resistance. PTGS is an evolutionary conserved mechanism in plants against potential harms by viruses and transposons. In this process, a plant defends itself by exploiting the requirement of plant RNA viruses to replicate using a double-stranded, replicative intermediate (dsRNA). The double-stranded RNA produced is cleaved into approximately

21 nucleotide fragments by the Dicer enzyme [12]. Evidence suggest that transgene loci and RNA viruses can generate double-stranded RNAs which are similar in sequence to the transcribed region of target genes, which further undergo endonucleolytic cleavage to generate small interfering RNAs (siRNA) that promote degradation of cognate RNAs.

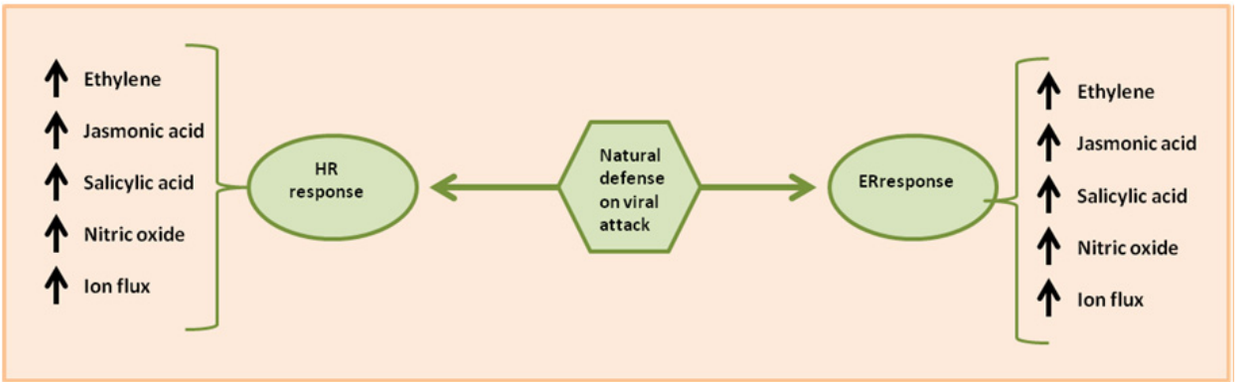

Figure 1. Natural response of plants against viral attack, where production of secondary metabolites cause extreme or moderate resistance.

The first approach made by plant agronomists was the inoculation of susceptible plant with a milder strain of the target virus. This technique was named as **cross protection** and was employed on crops like tomato, papaya and citrus [13-15]. Scientists were met with success as considerable resistance was achieved in transgenic plants through employment of this approach but the success was accompanied with a major drawback that the milder strain of the virus providing protection to one crop may cause serious diseases on varieties growing nearby.

To compensate the drawback of cross protection, **pathogen derived resistance** (PDR) based strategies were employed. These are based on the insertion of resistant genes that are derived from the pathogen (virus) into the host plant. Resistance was achieved by expressing viral genes in plants including coat protein, movement protein and replicase protein gene, each of them targets at a step crucial to virus replication. Coat protein gene is responsible for viral uncoating and is involved in virus replication [16], movement protein is crucial for cell to cell movement of the infecting virus [17] whereas the Rep protein is involved in virus replication and its genome integrity [18,19]. Resistance was either due to protein accumulation (coat protein mediated resistance, movement protein mediated resistance and replicase protein mediated resistance) or because of accumulation of small RNA sequences (replicase mediated resistance).

Uptill now, scientists have made considerable successful attempts to generate virus resistant transgenic plants by employing PDR concept [20, 21]. For example, virus-resistant potato varieties having *PVY* coat protein (CP) or P1 gene sequences has been reported in numerous studies [22-27]. Biotechnologists employed various genes of *PVY* and have met with mixed success in engineering *PVY* resistant transgenic potato plants [22,28-30,24,31,25,32]. In another study, the presence of the movement protein (pr17 protein) was reported to create resistance in transgenic plants against luteovirus Potato leaf roll virus [33].

Although pathogen derived resistance strategies hold promise for upto 90% resistance against target virus and are being employed still to date but some remarkable and potential threats are also associated with the use of this technology. The major one includes; the expression of a gene fragment derived from virus in transgenic plant confers resistance to particular virus but at the same time also raises environmental safety concerns regarding the constitutive expression of viral genes. It is supposed that infecting virus can interact with expression product in transgenic plants and can potentially modify the biological properties of the existing virus, ultimately leading to creation of new virus species which have novel pathogenic properties, host range and altered transmission specificity. In the initial experiments, the virus resistance was based on protein expression but resistance was neither so stable nor effective as compared to the resistance achieved through RNAi.

Among pathogen derived resistance strategies, **antisense RNA** complementary to part of the viral genome proves to have potential utility for protecting plants from systemic virus infection [34]. Antisense RNAs refer to small untranslatable RNA molecules that pair with a target RNA sequence on homology basis and thereby exert a negative control on interaction of target RNA with other nucleic acids or protein factors. Further, RNase H cause an increase in rate of degradation of double stranded RNA [35]. Antisense RNA technology was quickly adopted by plant researchers because other approaches like homologous recombination and gene-tagging mutagenesis used were based on reverse genetics and also these were not applicable in plants nor these were well developed. This background makes antisense RNA-mediated suppression more powerful tool for transgenic research and also for the development of commercial products [36].

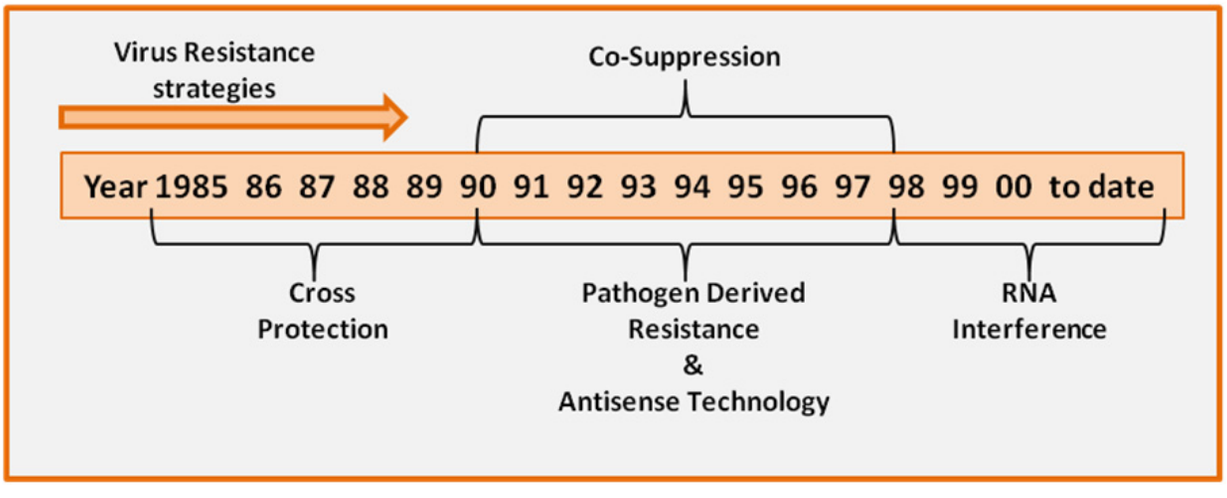

Figure 2. Major milestones in virus resistance strategies drawn to scale, starting form cross protection to RNA-mediated gene suppression.

4. RNA silencing

The development of the concept of pathogen derived resistance gave rise to strategies ranging from coat protein based interference of virus propagation to RNA mediated virus gene silencing. Virus resistance is achieved usually through the antiviral pathways of RNA

silencing, a natural defense mechanism of plants against viruses. The experimental approach consists of isolating a segment of the viral genome itself and transferring it into the genome of a susceptible plant. Integrating a viral gene fragment into a host genome does not cause disease (the entire viral genome is needed to cause disease). Instead, the plant's natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner via a cascade of events involving numerous proteins, including ribonucleases (enzymes that cleave RNA) is activated. This targeted degradation of the genome of an invader virus protects plants from virus infection.

a. Transcriptional Gene Silencing (TGS)

In plants, silencing is of two types: transcriptional and post transcriptional gene silencing. In both types, the inactivated genes are in trans position as homologous genes upon interaction reside on opposite chromosomes. TGS and PTGS differ from each other with respect to the underlying mechanism they exhibit. TGS requires sequence homology between promoters as compared to PTGS which require homology between coding region of the interacting genes. In TGS, an inactive allele residing on one chromosome can render another allele silenced. The mechanism behind transcriptional gene silencing is suggested to be DNA-DNA interaction which is thought to play an important role [37,38]. In other studies, it was proposed that RNA molecules interact with DNA and subsequently induce DNA methylation which then leads to gene silening [39-42], however it is not clear whether methylation of DNA alone is sufficient for silencing or not. It is proposed that DNA methylation in promoter region has a strong negative effect on interaction of certain transcription factors with promoter. Possible mechanism of TGS is depicted in figure 3.

b. Post Transcriptional Gene Silencing (PTGS)

'RNA interference' is a conserved mechanism of post transcriptional gene silencing (PTGS). It has rapidly gained favor as a "reverse genetics" tool to knock down the expression of targeted genes in plants. The term RNAi was coined in 1998 by Fire and Mello to describe a gene-silencing phenomenon based on double-stranded RNA [43]. PTGS mechanism controls processes including development, the maintenance of genome stability and defense against molecular parasites (transposons and viruses). Several reports pointed out that PTGS in plants is strictly linked to RNA virus resistance mechanism [44-46].

5. Mechanism of RNAi/PTGS

RNAi (RNA interference) is a natural defense pathway evolved in plants against viruses and potential transposons. It is a cellular pathway in which target sequences are degraded on homology basis at mRNA level by small RNAs, thereby preventing the translation of target RNAs. In plants, two functionally different RNAs; microRNA (miRNA) and small interfering RNA (siRNA), have been characterized. The miRNAs are small 21-26nt long dsRNAs that are genome coded and are endogenous to every cell. Structurally, they comprised of a stem region which is double stranded and a loop region which is single stranded. The miRNAs generated from endogenous hpRNA precursors and are basically

involved in the regulation of development [47]. On the other hand, siRNAs are generated from long dsRNA and are involved in defense through RNA interference [48, 49].

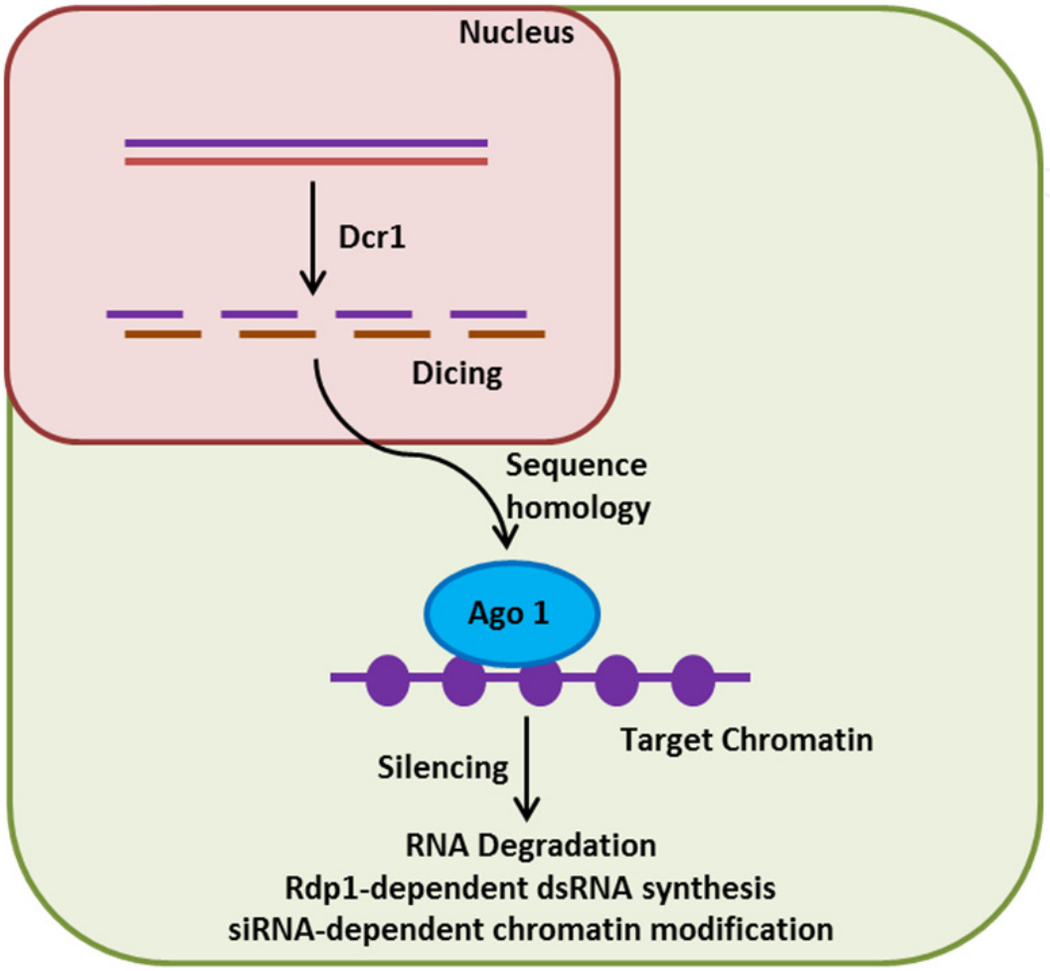

Figure 3. Mechanism of transcriptional gene silencing, active in chromatin modification.

RNAi is an immune system in plants which is directed against viruses [50]. Upon viral attack, long dsRNAs are produced from the replication intermediates of viral RNAs that act as substrate for an endonuclease termed Dicer which is located in the cytosol [51]. Dicer recognizes these dsRNAs and cleave them into duplex siRNA (21-25 nt) [52]. The siRNA duplex comprised of two strands; strand complementary to target mRNA is guide strand and other is passenger strand. The guide strand of short siRNA duplex is incorporated into the RNA-induced silencing complex (RISC) and then siRNA programmed RISC degrade viral RNA. As the RISC complex encounters a foreign mRNA which could be of virus origin, it has two consequences. 1) If the homology of guide strand and target mRNA is 100%, then perfect complement form between them resulting in mRNA cleavage and subsequent degradation or 2) in case of imperfect complement, where few mismatches exist between guide strand of RISC and target mRNA, translation of target mRNA is inhibited (figure 4).

Same mechanism operates in microRNA triggered gene silencing. miRNAs processed from stem loop precursors (shRNA and/or hpRNA) and requires Dicer activity [53] followed by

RISC assembly and subsequent degradation of homologous RNA in a sequence specific manner.

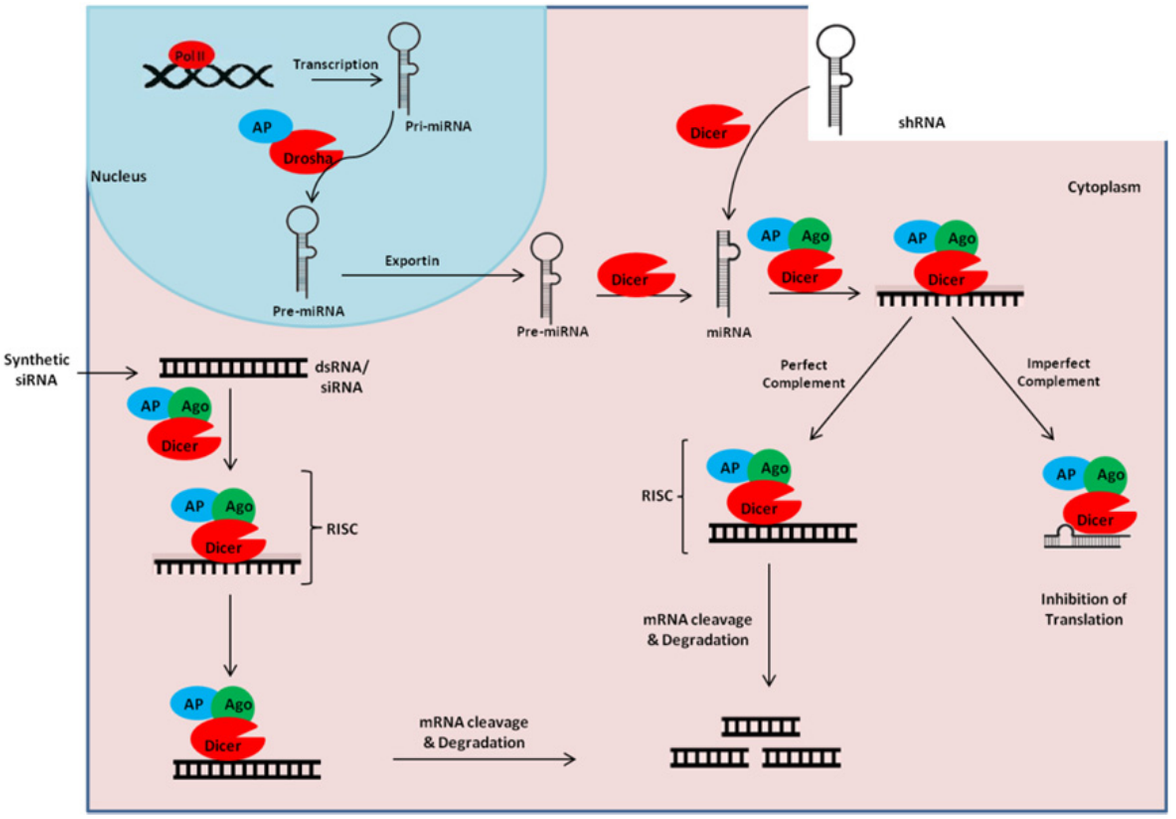

Figure 4. Model for RNA silencing, an ordered biochemical pathway which is triggered by dsRNA of viral origin. The source of dsRNA is either the synthetic siRNA or pre-microRNA. Genome encoded pri-miRNAs are processed by Drosha (an RnaseIII enzyme) into pre-miRNAs which are exported in the cytosol. dsRNA (siRNA or miRNA) subsequently joins Dicer, Ago and some other accessory proteins located in the cytosol forming RISC (RNA induced Silencing Complex). The degree of complementarity between the RNA silencing molecule and its cognate target determines the fate of the mRNA: blocked translation or mRNA cleavage/ degradation.

RISC is a combination of Dicer (an endonuclease enzyme), some accessory proteins namely argonaute (ago1, 4, 6, 9; catalytic endonucleases) and RNA binding proteins (RBP), and some trans-acting RNA-binding proteins (TRBP) [54,55].

Stability of RNAi induced silencing is based on enzymatic methylation of siRNA. This reaction is catalyzed by the enzyme methyltransferase (HEN1) which methylates the siRNA at 3′ end, hereby preventing it from oligouridylation and subsequent degradation [56].

6. Systemic spread of RNAi

When RNAi is induced at one site in an organism including plant, a mobile signal is generated which spread cell to cell and systemically throughout the organism [57-59,43,60,61] and make RNAi response obvious in distant tissues of the plant. This silencing signal moves inside plant either through the intercellular spaces called plasmodesmata or

through the phloem as shown in figure 5 [59,60]. Presence of a mobile signal has been proposed to be an integral part in systemic spread of silencing. The first evidence of the presence of a mobile silencing signal came from the study of Agro infiltration assay or particle bombardment in development of transgenic tobacco plants [59,60,62]. Subsequently, in silenced tissues of Agro-infiltrated plants, T-DNA or Agrobacterium was detected which suggests that mobile signal is responsible for propagation of silencing from one tissue to another [59] and this signal can also cross graft junction [59,60,62]. Candidates proposed to be responsible for mobile silencing signal involve siRNAs, Aberrant RNAs and dsRNA [63].

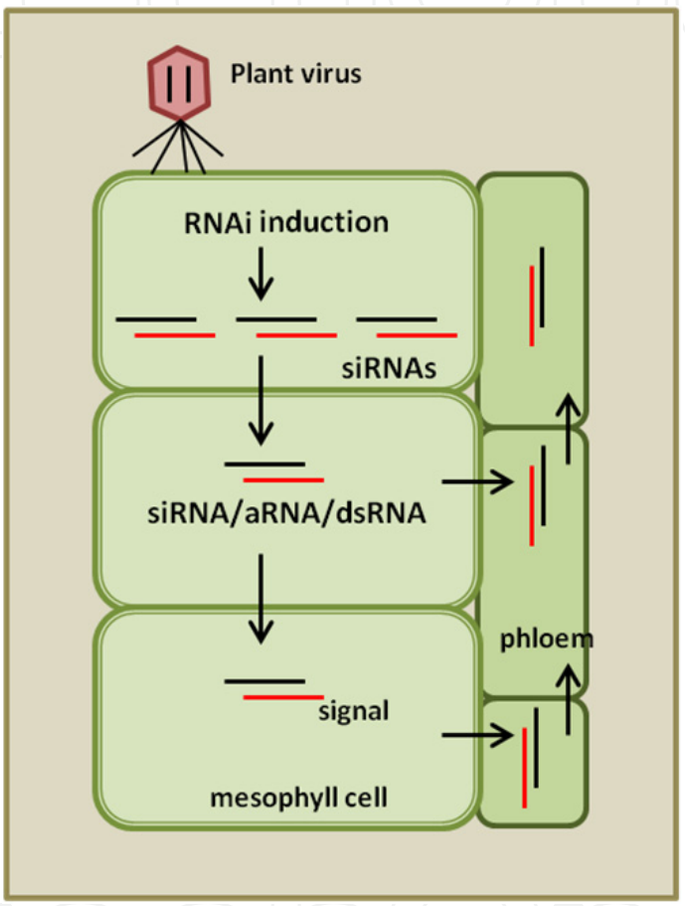

Figure 5. Mobile silencing signal passes from infected cell to healthy cell upon RNAi induction. Candidate of RNA silencing could siRNA, aRNA or dsRNA and travel through plasmodesmata and/or phloem.

Conclusively, transgenic approach mediated by RNAi pre-programmed an existing antiviral defense in plants [21,64-66]. Plant viruses are the strong inducers of RNAi as well as a target. The simplicity and specificity of RNAi has made RNAi a routine tool for the generation of virus resistant crops.

7. Effective RNAi inducers

In general, gene silencing has proven fruitful with both sense- and antisense transgenes in plant cells [67,68]. An RNA molecule that contains a fragment of a sense strand, an antisense strand and a short loop sequence between the fragment making a tight hairpin turn is

termed as short hairpin RNA (shRNA) which has the ability to suppress the expression of desired genes via RNA interference [69]. Silencing can be more efficiently achieved by utilizing shRNA cassettes [70-72] which usually include a specific plant promoter and terminator sequences to control the expression of inversely repeated sequences of the dsRNA. Upon subsequent delivery of shRNA cassette in the plant cells, dsRNA molecules comprised of a loop (single-stranded) and a stem region (double-stranded) are formed. Further, stem region is used by Dicer as a substrate and trigger RNAi mechanism [72-74]. RNA silencing mediated by the use of shRNA cassette enforces stable and heritable gene silencing [67] as it utilizes the specific promoter to ensure that the shRNA is always expressed. Another reason which justify that the silencing efficiency can be more powerful when using shRNA cassette is due to the fact that dsRNA are being fed into a later step in the silencing pathway where they act as a substrate for Dicer (RNaseIII like enzyme) and therefore bypasses the step in which dsRNAs need plant encoded RdRps for their production [75].

Practically in development of virus resistant transgenic plants, specific hairpinRNA expression constructs have been designed for transformation. In this strategy, small dsRNAs which are hallmark of PTGS, are produced from the transformed construct and ultimately induce silencing. Scientists have used hpRNA construct for silencing of viral gene in potato and obtained efficient silencing results accompanied with production of siRNA [76]. Similarly, some others have compared various constructs in terms of their silencing potential and confirmed that most efficient and strong silencing in tobacco can be achieved through the expression of an intron containing construct, which trigger PTGS [77]. However, in another study where *PVY* resistant potato plants were obtained through CP gene expression, evidence for existence of both protein and RNA mediated mechanisms was verified [27].

While considering the appealing outcome of RNAi in development of virus resistant transgenic plants as reviewed in this article and the use of hairpin RNA for strong silencing, production of transgenic potato resistant against potato virus Y and sugarcane plants resistant against sugarcane mosaic virus developed by [69] at Centre of Excellence in Molecular Biology (CEMB), University of the Punjab has been quoted as an example in following chapter.

8. Development of *PVY* and *SCMV* resistant transgenic plants

Tabassum *et al*. [79] have developed *PVY* and *SCMV* resistant potato and sugarcane plants respectively through siRNA technology by targeting capsid protein gene of respective virus. In the study, the respective plant was equipped with shRNA cassette that reacts continuously against invading virus specifically, thus resulting in degradation of viral mRNA in a sequence-specific manner. Specialty of this shRNA cassette is that it contained screened siRNA (the one most efficient in *in-vitro* experiments) out of bulk. The 22nt long siRNA was used as core sequence in shRNA cassette while loop sequence and flanking sequences were taken from highly active regulatory microRNA of respective host plant.

Initially, for screening of siRNA out of bulk, a strategy based on transient transfection assay was optimized in mammalian cell line (CHO). mRNA knockdown efficiency of capsid

protein gene of target virus was analyzed by real-time PCR. In case of *PVY* capsid gene, one specific siRNA out of a total six was found to be the most effective for knockdown of respective mRNA in transfected CHO cells by up to 80-90%. Data obtained showed that all six siRNAs used reduced the mRNA expression of target gene to some extent but only siRNA1 significantly reduced CP-*PVY* mRNA expression by up to 12.25 fold and, as is clearly shown in figure 6, expression was almost diminished or very faint in cells transfected with siRNA1 as compared to the control where scrambled siRNA was transfected. The remaining siRNA knockdown values were: siRNA 2 - 7x decrease ; siRNA 3 - 8x decrease; siRNA 4 - 10.8 x decrease; siRNA 5 - 9x decrease and siRNA 6 - 10x decrease. These values were based on Ct values obtained from real-time PCR studies [78].

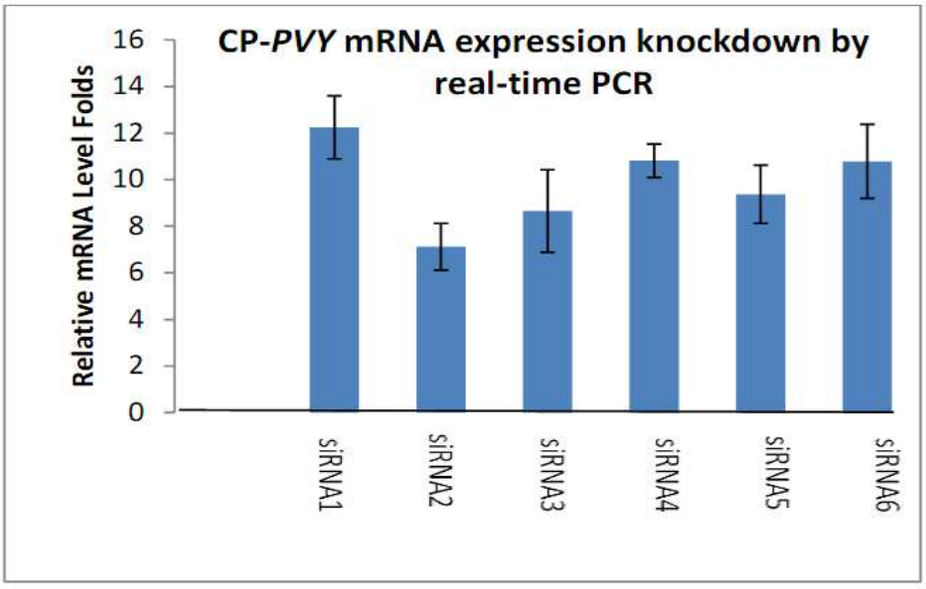

Figure 6. Relative measure of the knockdown of mRNA expression of CP-*PVY* gene in transient transfection assays. Knockdown values are based on relative Ct values obtained in realtime PCR assay; GAPDH was used as internal control to normalize the results.

Similar findings were met when knockdown in mRNA expression of CP-*SCMV* was studied *in-vitro* through transient transfection assays. As clear from figure 7, siRNA1 reduced the mRNA expression of target gene by upto 96%, while inhibition by siRNA2 was 46%, siRNA3 and siRNA4 inhibited target gene mRNA expression upto 50% and 77% respectively (figure 7).

Subsequently, the screened siRNA for both viruses was used in shRNA cassette which is thought to synthesize target specific siRNAs that continuously guard the plant against respective viral attack. shRNA cassette cloned in pCAMBIA1301 vector and transformed in potato and sugarcane through Agrobacterium- and particle bombardment method respectively. Results were compared with control non-transgenic plants. Figure 8 and 9 depicts the results, clearly indicating that in transgenic potato having shRNA1 cassette integrated in them, mRNA knockdown was upto 96% whereas in transgenic potato plants

having shRNA4 cassette in them, *PVY* knockdown was upto 57% as compared to control where *PVY* infection was maximum.

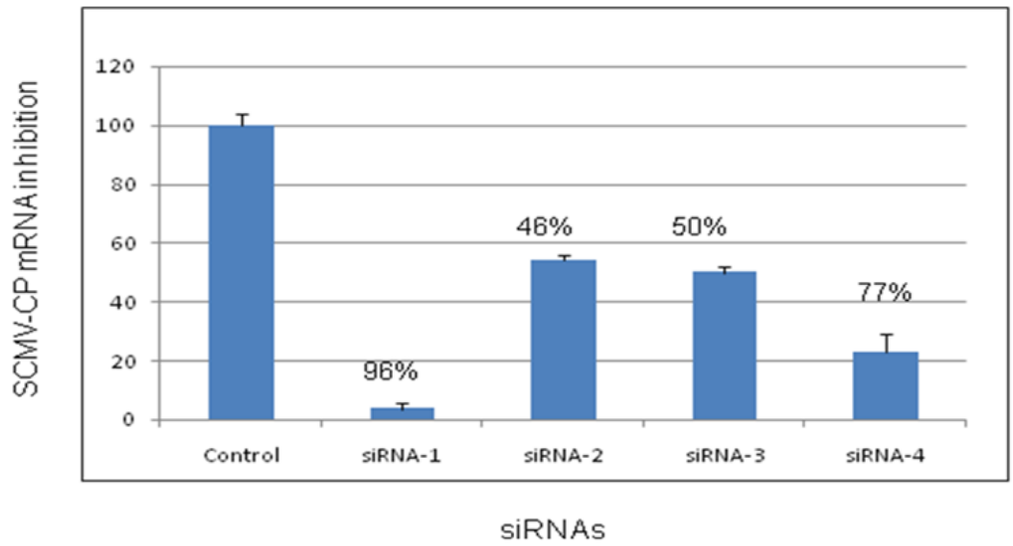

Figure 7. Relative measure of the knockdown of mRNA expression of CP-*SCMV* gene in transient transfection assays.

Similarly, in transgenic sugarcane plants, shRNA1 reduced the mRNA expression of *SCMV* to lesser extent with 30% reduction only while shRNA4 caused maximum knockdown of 95% as compared to the control non-transgenic sugarcane plant.

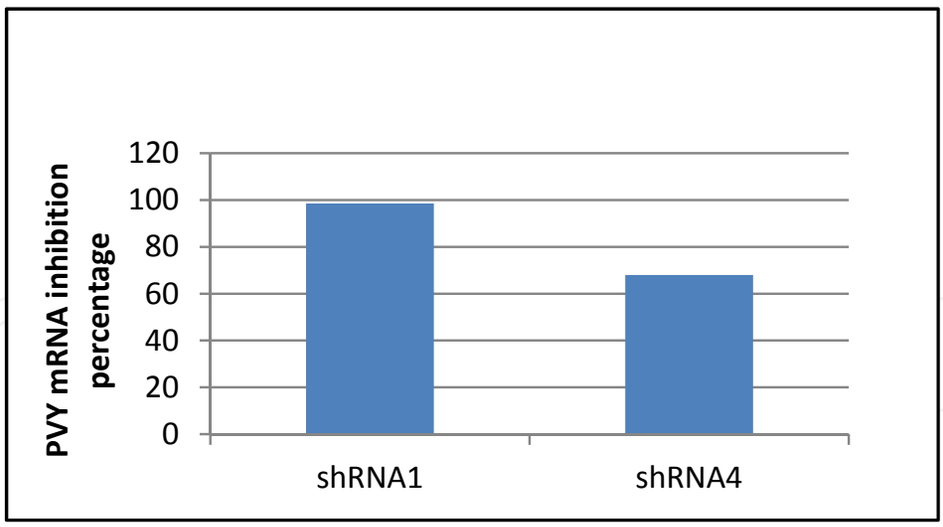

Figure 8. Percentage inhibition in mRNA expression of *PVY* rendered by integrated shRNA1 and shRNA4 cassette in potato plants. Transgenic plants were subjected to bioassay by *PVY* inoculation and RT-PCR was performed 30 days post *PVY* inoculation.

In conclusion, we have developed transgenic potato and sugarcane plants that were highly resistant against *PVY* and *SCMV* infection respectively. This resistance was because of the shRNA cassette integrated in them that is targeted against capsid protein gene of each virus.

These shRNAs are supposed to create long-term targeted gene inhibition in cells and whole plant. Our shRNA construct designing was based on the hypothesis that if we express potentially effective screened siRNA in hairpin form which is further combined with the power of most active regulatory microRNA in respective plant, the level of resistance will be far more effective. Applying this theme, we were able to obtain transgenic potato and sugarcane plants where resistance level against targeted virus was upto immunity.

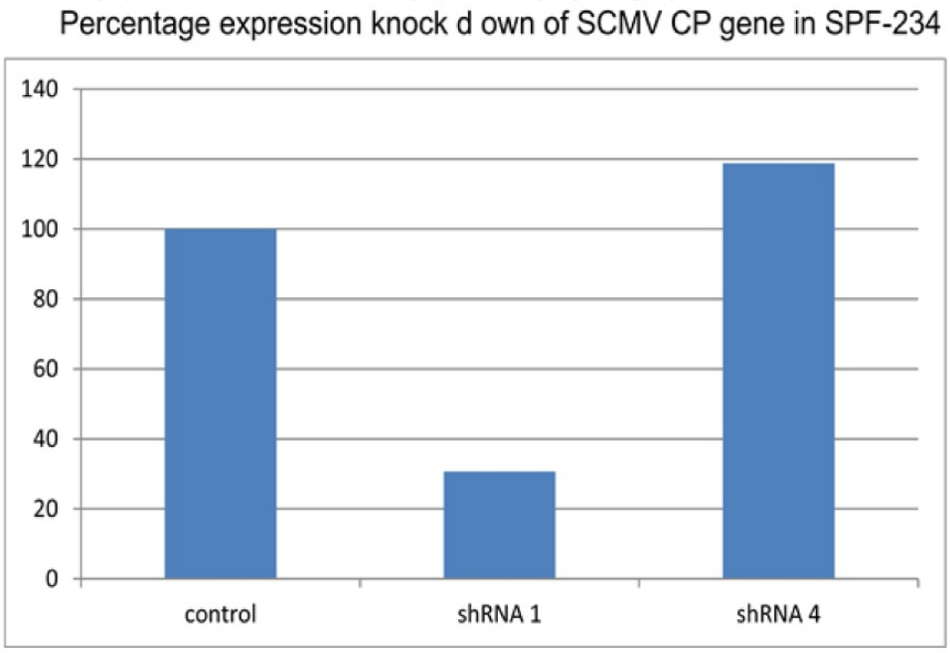

Figure 9. Percentage inhibition in mRNA expression of *SCMV* rendered by integrated shRNA1 and shRNA4 cassette in sugarcane plants. Transgenic plants were subjected to bioassay by *SCMV* infection and RT-PCR was performed 30 days post inoculation.

One important aspect of this strategy in engineering *PVY*-resistant plants is the fact that the integrated shRNA sequence is not of viral origin nor it is translated into a protein. Moreover, the actual RNA transcript is almost undetectable because it gets cleaved quickly in small fragments through RNAi pathway. These two features limit the environmental risks of this strategy, such as trans-encapsidation or recombination of the transgene with an incoming virus.

Author details

Bushra Tabassum, Idrees Ahmad Nasir, Usman Aslam and Tayyab Husnain
National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan

Abbreviations

RNAi (RNA interference); *PVY* (Potato virus Y); *SCMV* (Sugarcane mosaic virus); PTGS (Post Transcriptional Gene Silencing); siRNA (small interfering RNA); shRNA (short hairpin

RNA); ssRNA (single stranded RNA); dsRNA (double stranded RNA); RdRp (RNA dependent RNA polymerase); hpRNA (hairpin RNA).

9. References

[1] Pakistan economic Survey (2010-2011) Government of Pakistan; Ministry of finance. http://www.finance.gov.pk/survey_0910.html.

[1] Beachy RN (1997) Mechanisms and applications of pathogen-derived resistance in transgenic plants. Curr. Opin. Biotechnol. 8:215–220.

[2] Briddon RW, Markham PJ (2000) Cotton leaf curl virus disease. Virus Res. 71:151-159.

[3] Novy RG, Nasruddin A, Ragsdale DW, Radcliffe EB (2002) Genetic Resistances to Potato Leafroll Virus, Potato Virus Y, and Green Peach Aphid in Progeny of *Solanum etuberosum.* American Journal of Potato Research 79(1): 9-18.

[4] Anonymous (1997) Sugar and sweetener situation and outlook yearbook. US department of Agriculture Washington DC.

[5] Hema M, Joseph J, Gopinath K, Sreenivasulu P, Savithri HS (1999) Molecular characterization and interviral relationships of a flexuous filamentous virus causing mosaic disease of sugarcane (*Saccharum officinarum* L.) in India. Arch. Virol. 144: 479–490.

[6] Koike T, Martin DP, Johnson EM Jr (1989) Role of calcium channels in the ability of membrane depolarization to prevent neuronal drath induced by trophic factor deprivation: evidence that levels of internal calcium determine NGF dependence of sympathetic ganglion cells. Proc. Natl. Acad. Sci. 86:6421-25.

[7] Akhtar KP, Ryu KH, Saleem MY, Asghar M, Jamil FF, Haq MA, Khan IA (2008) Occurrence of Cucumber mosaic virus Subgroup IA in tomato in Pakistan. J. Plant Dis. Protect. 115: 2-3.

[8] Solomon-Blackburn RM, Barker H (2001) Breeding virus resistant potatoes (Solanum tuberosum): a review of traditional and molecular approaches. Heredity 86:17-35.

[9] Cooper JI, Jones AT (1983) Responses of plants to viruses, proposals for the use of terms. Phytopathology 73:127-128.

[10] Walkey DGA (1991) Applied plant virology. 2nd edition. Chapman and Hall India.

[11] Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, et al (2001) Construct design for efficient, effective and high throughput gene silencing in plants. The Plant Journal 27(6): 581-590.

[12] Beachy RN, Loesch-Fries S, Tumer NE (1990) Coat protein mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-74.

[13] Gadani F, Mansky LM, Medici R, Miller WA, Hill JH (1990) Genetic engineering of plants for virus resistance. Arch. Virol. 115:1-21.

[14] Hull R, Davies JW (1992) Approaches to nonconventional control of plant virus diseases. Crit. Rev. Plant Sci. 11:17-33.

[15] Yusibov V, Loesch-Fries LS (1995) High-affinity RNA-binding domains of alfalfa mosaic virus coat protein are not required for coat protein-mediated resistance. Proc. Natl. Acad. Sci. USA 92:1–5.

[16] Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8:1669-l681.

[17] Carr JP, Marsh LE, Lomonossoff GP, Sekiya ME, Zaitlin M (1992) Resistance to tobacco mosaic virus induced by the 54-kDa gene sequence requires expression of the 54-kDa protein. Mol. Plant–Microbe Interact. 5: 397–404.

[18] Golemboski DB, Lomonossoff GP, Zaitlin M (1990) Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proc. Natl. Acad. Sci. USA 87:6311–6315.

[19] Lomonossoff GP. Pathogen-derived resistance to plant viruses. Annu. Rev. Phytopathol. 33:323-343.

[20] Baulcombe DC. RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Molecular Biology 32(1-2): 79-88.

[21] Farinelli L, Malnoe P, Collet GF (1992) Heterologous encapsidation of potato virus Y strain^{O} (PVY-^{O}) with the transgene protein of PVY strain (PVY-^{N}) in *Solanum tuberosum* cv. Bintje. Biotechnology 10:1020–1025.

[22] Kollar A, Thole V, Dalmay T, Salamon P, Balazs E (1993) Efficient pathogen-derived resistance induced by integrated potato virus Y coat protein gene in tobacco. Biochemie 75:623–629.

[23] Malnoe P, Farinelli L, Collet G, Reust W (1994) Small-scale field tests with transgenic potato, cv. Bintje, to test the resistance to primary and secondary infections with potato virus Y. Plant Mol. Biol. 25: 963–975.

[24] Smith HA, Powers H, Swaney S, Brown C, Dougherty WG (1995) Transgenic potato virus Y resistance in potato: evidence for anRNA-mediated cellular response. Phytopathology 85:864–870

[25] Maki-Valkama T, Pehu T, Santala A, Valkonen JP, Koivu K, Lehto K, Pehu E (2000) High level of resistance to potato virus Y expressing P1 sequence in antisense orientation in transgenic potato. Molecular Breeding 6: 95–104.

[26] Gargouri-Bouzid R, Jaoua L, Mansour R B, Hathat Y, Ayadi M, Ellouz R (2005) PVY resistant transgenic potato plants (cv. Claustar) expressing the viral coat protein. J. Plant Biotechnol. 3:1–5

[27] Hassairi A, Masmoudi, K, Albouy J, Robaglia C, Jjullien M, Ellouz R (1998) Transformation of two potato cultivars `Spunta' and `Claustar' (Solanum tuberosum) with lettuce mosaic virus coat protein gene and heterologous immunity to potato virus Y. Plant Sci. Limerick. 136: 31-42.

[28] Kaniewski W, Lawson G, Sammons B, Haley L, Hart J, Delan-nay X, Tumer NE (1990) Field resistance of transgenic Russet Burbank potato to effects of infection by potato virus X and potato virus Y. Biotechnology 8: 750–754.

[29] Lawson G, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer NE (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Biotechnology 8: 1277–134.

[30] Okamoto D, Nielsen SVS, Albrechtsen M, Borkhardt B (1996) General resistance against potato virus Y introduced into a commercial potato cultivar by genetic transformation with PVYN coat protein gene. Potato Research 39:271-82.
[31] Pehu TM, Maki-Valkama TK, Valkonen JPT, Koivu KT, Lehto KM, Pehu EP (1995) Potato plants transformed with a potato virus Y P1 gene sequence are resistant to PVY^{o}. American Potato Journal 72: 523-532.
[32] Tacke E, Salamini F, Rohde W (1996) Genetic engineering of potato for board-spectrum protection against virus infection. Nature Biotechnology 19:1597-1601.
[33] Bejarano ER, Lichtenstein CP (1992) Prospects for engineering virus resistance in plants with antisense RNA. Trends Biotechnol. 10:383–388.
[34] Culver JN (1995) Molecular strategies to develop virus-resistant plants. CRC Press, Inc.
[35] Chi-Ham CL, Clark KL, Bennett AB (2010) The intellectual property landscape for gene suppression technologies in plants. Nat. Biotechnol. 28(1):32-36.
[36] Stam M, de Bruin R, Kenter S, van der Hoorn RAL, van Blokland R, Mol JNM, et al (1997) Post-transcriptional silencing of chalcone synthase in petunia by inverted transgene repeats. Plant J.12:63–82.
[37] Luff B, Pawlowski L, Bender J (1999) An inverted repeat triggers cytosine methylation of identical sequences in Arabidopsis. Mol. Cell. 3:505-511.
[38] Mette MF, van der Winden J, Matzke MA, Matzke AJ (1999) Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. EMBO J. 18: 241–248.
[39] Pelissier T, Thalmeir S, Kempe D, Sanger HL, Wassenegger M (1999) Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. Nucleic Acids Res. 27:1625-1634
[40] Wassenegger M (2000) RNA-directed DNA methylation. Plant Mol. Biol. 43: 203–220.
[41] Jones AL, Thomas CL, Maule AJ (1998) De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. EMBO J. 17: 6385–6393
[42] Fire A, Xu S, Montgomery M, Kostas S, Driver S, Mello C (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391(6669):806–811.
[43] Kasschau KD, Carrington JC (1998) A counter defensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95:461-470.
[44] Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, et al (1998) A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA 95:13079-84.
[45] Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. 17:6739–6746.
[46] Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116:281–97.
[47] Lecellier CH, Voinnet O (2004) RNA silencing: no mercy for viruses? Immunol. Rev. 198:285–303.
[48] Vastenhouw NL, Plasterk RH (2004) RNAi protects the *Caenorhabditis elegans* germline against transposition. Trends Genet. 20:314–319.

[49] Baulcombe DC (2004) RNA silencing in plants. Nature. 431(7006):356-63.
[50] Tang G, Reinhart BJ, Bartel DP, Zamore PD (2003) A biochemical framework for RNA silencing in plants. Genes Dev. 17:49–63.
[51] Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. EMBO J. 21:4671–4679.
[52] Tijsterman M, Plasterk RH (2004) Dicers at RISC; the mechanism of RNAi. Cell. 117:1–3.
[53] Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing. Cell. 123:631–640.
[54] Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell. 115:199–208.
[55] Li J, Yang Z, Yu B, Liu J, Chen X (2005) Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis. Curr. Biol.15:1501-1507.
[56] Fagard M, Vaucheret H (2000) Systemic silencing signal(s). Plant Mol. Biol. 43(2-3):285-93.
[57] Palauqui JC, Elmayan T, Pollien JM, Vaucheret H (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 15: 4738–4745.
[58] Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. Nature. 389(6651):553.
[59] Voinnet O, Vain P, Angell S, Baulcombe DC (1998) Systemic spread of sequence-specific transgene RNA degradation is initiated by localised introduction of ectopic promoterless DNA. Cell. 95:177–187.
[60] Winston WM, Molodowitch C, Hunter CP (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science. 295: 2456–2459.
[61] Palauqui JC, Balzergue S (1999) Activation of systemic acquired silencing by localised introduction of DNA. Curr. Biol. 9: 59–66.
[62] Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB (2002) RNA silencing and the mobile silencing signal. Plant Cell 14:289–301.
[63] Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. Science 286:950-952.
[64] Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. EMBO J. 21:4671–4679.
[65] Plasterk RHA (2002) RNA silencing: the genomes immune system. Science 296:1263-1265.
[66] Bruening G (1998) Plant gene silencing regularized. Proc. Nat. Acad. Sci. 95:13349-13351.
[67] Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. 95:13959–13964.
[68] Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. 16: 948–58.

[69] Horiguchi G (2004) RNA silencing in plants: a shortcut to functional analysis. Differentiation. 72:65-73.
[70] Watson JM, Fusaro AF, Wang M, Waterhouse PM (2005) RNA silencing platforms in plants. FEBS Lett. 579:5982–5987.
[71] Hirai S, Oka SI, Adachi E, Kodama H (2007) The effects of spacer sequences on silencing efficiency of plant RNAi vectors. Plant Cell Reports. 26(5):651-659.
[72] Meyer S, Nowak K, Sharma VK, Schulzer J, Mendel RR, Hansch R (2004) vector for RNAi technology in Poplar. Plant Biol. (Stuttg) 6. 100-103.
[73] Miki D, Shimamoto K (2004) Simple RNAi Vectors for St in Rice. Plant Cell Physiol. 45(4):490–495.
[74] Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M (2008) Strategies for antiviral resistance in transgenic plants. Molecular plant pathology. 9(1):73–83.
[75] Missiou A, Kalantidis K, Boutla A, Tzortzakaki S, Tabler M, Tsagris M (2004) Generation of transgenic potato plants highly resistant to potato virus Y (PVY) through RNA silencing. Molecular Breeding 14: 185–197.
[76] Smith HA, Powers H, Swaney S, Brown C, Dougherty WG (1995) Transgenic potato virus Y resistance in potato: evidence for anRNA-mediated cellular response. Phytopathology 85:864–870.
[77] Tabassum B, Nasir IA, Husnain T (2011) Potato virus Y mRNA expression knockdown mediated by siRNAs in cultured mammalian cell line. Virol Sin. 26(2):105-13.
[78] Tabassum B, Nasir IA, Aslam U, Husnain T (2012) PhD dissertation. CEMB, University of the Punjab Lahore, Pakistan.