Chapter 2

Review of Literature
2.1 Molecular farming and plants as a production system

Molecular farming or biofarming or transgenic non-food GM plant pharming is the production of pharmaceutically important and commercially valuable proteins in plants (Franken et al., 1997). It harnesses plants as heterologous protein expression systems for the large-scale production of recombinant proteins that are therapeutically valuable. The principal purpose of molecular farming is to evolve safe and inexpensive means for the mass production of recombinant pharmaceutical proteins using plants as heterologous protein expressing systems. There are two stages in molecular farming – the development of an optimized expression system and its scaling up to economic levels of production. However, principally, molecular farming implies the large scale and cost-effective production of recombinant pharmaceuticals outside their natural source i.e. by using heterologous expressing systems such as in bacteria, yeasts, viruses, cell culture, animals, and plants (Fischer and Emans, 2000). A classic example of molecular farming in microbes is the expression of recombinant insulin in bacteria which was the first recombinant protein approved for therapeutic use (Walsh, 1998).

Bacteria are often used for the protein expression system, while yeast cells or baculovirus infected insect cell systems are hardly used (Skerra, 1993; Taticek et al., 1994). Although bacteria are inexpensive and convenient production systems they are incapable of most of carrying out the post-translational modifications necessary for the activity of many proteins. This limitation and the cost of expression of proteins in mammalian cells prompted the exploration of plants as a cheap, safe, and efficient alternative.

The potential of using plants as a production system for recombinant pharmaceuticals was recognized between 1986 and 1990 with the successful expression of a human growth hormone fusion protein, an interferon and human serum albumin (De Zoeten et al., 1989; Sijmons et al., 1990). A crucial advancement came with the successful expression of functional antibodies in
plants in 1989 (Hiatt et al., 1989) and 1990 (Düring et al., 1990) which was a significant breakthrough showing that plants had the potential to produce complex mammalian proteins of medical importance. By analogy to the production of insulin in bacteria, the production of antibodies in plants had the potential to make large amounts of safe and inexpensive antibodies available. This was a notable achievement because plants could produce functional full-length antibodies, indicating that all the post-translational modifications necessary for antibody activity occurred, unlike that in E. coli. In the following 10 years, plants were shown to be able to produce a variety of antibody fragments, secretory IgA, blood substitutes and biological effectors including interleukins (Fischer and Emans, 2000).

2.2 Comparison of different expression systems used in Molecular farming

Plants represent an advantageous expression system for the production of complex heterologous proteins. Plants offer significant advantages over the classical expression systems based on bacterial, microbial and animal cells (Table 1). Plants provide a broad range of technical advantages compared to other expression systems e.g. product size, yield, propagation, protein assembly and folding accuracy, product quality, storage temperature and scale up capacity (Spök and Karner, 2008). Plants contain a higher eukaryote protein synthesis pathway, very similar to animal cells with only insignificant differences in protein glycosylation (Cabanes-Macheteau et al., 1999). Contrastingly, bacteria are unable to either produce full size antibodies or perform most of the important mammalian post-translational modifications. Proteins produced in plants accumulate to significantly high levels (Verwoerd et al., 1995; Ziegler et al., 2000) and plant derived antibodies are functionally equivalent to those produced by hybridomas (Hiatt et al., 1989; Voss et al., 1995). Moreover, concerns about contamination of expressed proteins with human or animal pathogens (HIV, hepatitis viruses) or the co-purification of blood-borne pathogens and oncogenic sequences, are entirely avoided by using plants.
Classical methods of protein expression often require a significant investment in recombinant protein purification (bacteria) or require expensive growth media (animal cells). Bacteria produce contaminating endotoxins that are difficult to remove and bacterially expressed recombinant proteins often from inclusion bodies, making labour- and cost-intensive in vitro refolding necessary. Mammalian cell cultivation can be difficult, requires sophisticated equipment and expensive media supplements, such as foetal calf serum. The use of transgenic animals as a source of recombinant antibodies is becoming limited by legal and ethical constraints (Echelard, 1996).

Plants allow the cost-effective production of recombinant proteins on an agricultural scale (Dove, 2002; Giddings, 2001). According to these estimations the production of the biomass containing the target protein is likely to be much cheaper in case of open filed cultivation compared to microbes and mammalian cell. Recombinant proteins could be produced in plants at 2 to 10% of the cost of microbial fermentation system and 0.1% of the cost of mammalian cell culture (Giddings, 2001).

Another advantage of the use of plants in recombinant protein production is that the vaccine candidates can be expressed in edible plant organs, allowing these to be administered as unprocessed or partially processed material (Thanavala et al., 2006). From a commercial point of view, one of the major benefits of molecular farming is the reduced set-up and running costs. Once a transgenic plant line has been produced, it can be maintained, harvested and processed using existing agricultural infrastructure and conventional practice (Franconi et al., 2002). Various plant species have been used as bioreactors to produce heterologous proteins at large scale (Giddings et al., 2000; Ma et al., 2003; Hellwig et al., 2004).

Transgenic plants producing high levels of safe, functional recombinant proteins can be cultivated on an agricultural scale (Whitelam et al., 1993; Whitelam, 1996). Currently, pharmaceutical proteins like human serum proteins and growth regulators, antibodies, vaccines, industrial enzymes, biopolymers and molecular biology reagents are the major focus of this emerging technology (Ma
Various plant species have been used as bioreactors to produce heterologous proteins at large scale (Giddings et al. 2000; Ma et al., 2003; Hellwig et al., 2004).

Table 2.1 Comparison of features of recombinant protein production in plants, yeast and classical systems (Adapted and modified from Fischer and Emans, 2000)

<table>
<thead>
<tr>
<th>Features</th>
<th>Transgenic plants</th>
<th>Plant viruses</th>
<th>Yeast</th>
<th>Bacteria</th>
<th>Mammalian cell culture</th>
<th>Transgenic animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost/storage</td>
<td>Cheap/RT</td>
<td>Cheap/-20°C</td>
<td>Cheap/-20°C</td>
<td>Cheap/-20°C</td>
<td>Expensive/liquid N2</td>
<td>Expensive</td>
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<tr>
<td>Distribution</td>
<td>Easy</td>
<td>Easy</td>
<td>Feasible</td>
<td>Feasible</td>
<td>Difficult</td>
<td>Difficult</td>
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<tr>
<td>Gene size</td>
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<td>Unknown</td>
<td>Limited</td>
<td>Limited</td>
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<tr>
<td>Glycosylation</td>
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<td>Correct?</td>
<td>Incorrect</td>
<td>Absent</td>
<td>Correct</td>
<td>Correct</td>
</tr>
<tr>
<td>Multimeric protein assembly</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Production cost</td>
<td>Low</td>
<td>Low</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Production scale</td>
<td>Worldwide</td>
<td>Worldwide</td>
<td>Limited</td>
<td>Limited</td>
<td>Limited</td>
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<tr>
<td>Propagation</td>
<td>Easy</td>
<td>Feasible</td>
<td>Easy</td>
<td>Easy</td>
<td>Hard</td>
<td>Feasible</td>
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<tr>
<td>Protein folding accuracy</td>
<td>High?</td>
<td>Medium</td>
<td>Medium-high</td>
<td>Medium-high</td>
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<td>Protein yield</td>
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<td>High</td>
<td>Medium</td>
<td>Medium-high</td>
<td>High</td>
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<td>Protein homogeneity</td>
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<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
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<td>Medium</td>
<td>High</td>
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<td>Scale up cost</td>
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<td>High</td>
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<tr>
<td>Therapeutic risk</td>
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<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>High</td>
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</tbody>
</table>

2.3 Limitations of the plant expression system

Though plants offer significant advantages, however, there are some hurdles to use plants as protein expression vehicles. Among the barriers, stable plant transformation technology and efficient and stable transgene(s) expression in transgenics have become subjects of considerable importance in molecular farming technology (Scholthof, 2007; Fischer and Emans, 2000). In view of increasing popularity of molecular farming technology, establishments of these two important aspects need more attention. Plant transformation involves the chromosomal integration of a heterologous gene and this is becoming
straightforward. However, there are still technical and logistical hurdles to be overcome, such as developing efficient transformation techniques for all major crop species. However, after successful plant transformation events in most of the genetically modified plants, unfortunately, the introduced transgenes are either not expressed or the expression level is too low even under most favorable conditions. Transgene(s) expression varies considerably within populations of transgenic plants. Various factors including copy numbers, gene silencing, position effects, somaclonal variation and regulatory sequences represent potential impediments to achieve desirable and predictable transgene expression levels in plants (Butaye, 2005; Ma, et al., 2009). Among these, RNA silencing constitutes one of the foremost barriers for the expression of transgene(s).

2.4 Potential sources of variable transgene expression

Several factors that cause undesired and unpredictable levels of transgene expression in plants are briefly described below.

2.4.1 Copy numbers

The copy number of transgene has high impact on its expression profile. Indeed, multiple copies of the transgene DNA tend to integrate at one or a few insertion sites, probably as a result of extrachromosomal ligation of the transgenic DNA fragments before integration (Pawlowski and Somers, 1998; De Buck et al., 1999). Theoretically, an increase in transgene copy number is expected to increase the transgene expression level. However, multiple copy integration patterns often seem to be associated with low-level transgene expression, especially complex integration patterns such as tandem repeats (Sijen et al., 1996; Wang and Waterhouse, 2000) and inverted repeat structures (Hobbs et al., 1993; Depicker and VanMontagu, 1997; Stam et al., 1997; Muskens et al., 2000). For example, Xugang et al. (2001) had observed high GUS activity in transgenic tobacco with one or two copies, while plants with multiple copies of transgene exhibited no GUS activity. However, two analyses show that a single copy can also trigger silencing (gene silencing is an evolutionarily conserved mechanism in eukaryotes
protecting genomes from nucleic acid invaders) in cis whereas two other cases demonstrated the requirement of a particular inverted repeat to trigger silencing in trans (Fagard and Vaucheret, 2000).

**2.4.2 Position effects**

Another source of inter-individual variation of transgene expression is attributed to epigenetic position effects, i.e. the position within the genome into which the foreign DNA is integrated (Matzke and Matzke, 1998). In higher eukaryotic organisms DNA integrates mainly via nonhomologous end joining or illegitimate recombination (Gheysen et al., 1990; De Buck et al., 1999; Hohn and Puchta, 2003). As a consequence, the integration of foreign DNA in the plant genome can take place at virtually any site. Consequently, integration may occur in regions with higher or lower transcriptional activity, and the surrounding endogenous regulatory sequences, such as transcriptional enhancers and inhibitors, may influence transgene expression (Matzke and Matzke, 1998; Meyer, 2000; Francis and Spiker, 2005).

**2.4.3 Regulatory sequences**

Regulatory sequences, mainly promoters and terminators, employed to direct transgene expression also have an impact on transgene expression (Butaye et al., 2004). Promoters, for instance, affect not only the transgene expression levels but also the magnitude of expression variability among individual transformants (De Bolle et al., 2003). The widely used 35S promoter (p35S) of the *Cauliflower mosaic virus* (Odell et al., 1985) yields a bimodal expression pattern with high expression levels in a limited number of transgenic plants but very low expression levels in the majority of the transformants (De Bolle et al., 2003; Elmayan and Vaucheret, 1996). Other regularly used promoters, such as the derivatives of the promoter of the mannopine synthase gene (pMAS), never reach the high-level expression that is conferred by p35S in some transformants but result in normally distributed expression levels in populations of transformants (De Bolle et al., 2003).
2.4.4 Somaclonal variation

Another cause of transgene expression variation is the occurrence of somaclonal variation which is generally defined as genetic and phenotypic variation among clonally propagated plants of a single donor clone. This phenomenon has been yet attributed to the applied transformation methods or protocols (Phillips et al., 1994; Duncan, 1997; Kaeppler et al., 2000).

2.4.5 Gene silencing

Gene silencing is an evolutionarily conserved surveillance mechanism in eukaryotes protecting genomes from nucleic acid invaders. This phenomenon probably results from the activation of defense host mechanisms (Fagard and Vaucheret, 2000). Gene silencing, which plays dominant roles in the establishment of transgene expression variation, has been considered as major obstacles for effective transgene(s) expression. Transgene expression can be inhibited due to gene silencing at the transcriptional level (transcriptional gene silencing; TGS) or at the post-transcriptional level (post-transcriptional gene silencing; PTGS). Over the years, PTGS and TGS have become major determinants believed to influence transgene expression in plants (Butaye, 2005). Therefore, gene silencing has become a major impediment for production of commercial GM crops with predictable and stable transgene performance.

2.4.5.1 Transcriptional gene silencing (TGS)

Transcriptional gene silencing (TGS) corresponds to a block of transcription and is associated with methylation of homologous promoter sequences of the transgene (Mette et al., 2000). TGS has been shown to affect sequences that are integrated in the genome and not extrachromosomal DNA. TGS is both mitotically and meiotically heritable. Earlier, TGS and PTGS were believed to involve two distinct mechanisms. However, recently, the difference between TGS and PTGS has blurred. TGS is viewed as a nuclear side of RNA silencing while PTGS is considered as a cytoplasmic RNA silencing. Small interfering RNAs, which are key actors of RNA silencing, may also operate as regulators of gene expression at
a transcriptional level in the nucleus, via chromatin remodeling or RNA-directed DNA methylation (RdDM) (Aufsatz et al., 2002; Pickford and Cogoni, 2003; Matzke et al., 2004). In RdDM, specific RNA transcripts are produced from a genomic DNA template, and this RNA forms double-stranded RNA molecules. The double-stranded RNAs (through small RNAs) direct de novo DNA methylation of the original genomic location that produced the RNAs (Aufsatz et al., 2002). TGS in plants has been shown to be carried out by a generally larger size class of siRNAs (24-26nt) (Hamilton et al., 2002; Zilberman et al., 2003).

2.4.5.2 Post-transcriptional gene silencing (PTGS)

PTGS, generally referred to as RNA silencing, is a conserved eukaryotic defense mechanism thought to defend the plant against viruses and transgenes, protect the genome from transposons and regulate gene expression (Baulcombe, 2004). This pathway, which exists in various organisms including fungi (quelling), animals (RNA interference, RNAi), and plants (PTGS), have similar genetic requirements and biochemical features although there are some significant differences in the silencing pathways engaged by these various organisms (Lindbo et al., 1993, Fire et al., 1998; Zamore et al., 2000; Denli and Hannon, 2003; Waterhouse and Helliwell, 2003; Baulcombe, 2004; Susi et al., 2004; Ding and Voinnet, 2007; Molnar et al., 2007). The phenomena of RNA silencing are described in greater details in the following sections.

2.5 RNA silencing or RNAi

RNA silencing is a phenomenon which refers to the regulation of gene expression through nucleotide sequence specific interaction mediated by RNA. Over the last few years RNA silencing and/or RNA interference have become widely studied biological systems. Initially being discovered as a side effect of transgene expression in plants and a process by which transgenic virus resistance could be obtained, it has since been implicated in natural virus resistance and basic biological processes such as development, gene regulation and chromatin condensation. Due to the biochemical dissection of components of the silencing
pathway in several model organisms, such as *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster*, the general understanding about RNA silencing mechanisms have greatly advanced in recent years.

### 2.5.1 Discovery of RNA silencing

RNA silencing phenomena were first described in plants where overexpression of *chalcone synthase* (*CHS*) in pigmented petunia petals by introducing a chimeric petunia *CHS* gene unexpectedly created a block in anthocyanin biosynthesis (Napoli et al., 1990). It was later confirmed that the introduction of the *CHS* transgene led to the inhibition of endogenous gene expression. Further studies have established that such inhibition is not due to the reduced transcription but due to the degradation of the transcript through a partial mRNA duplex formation. This phenomenon was termed as RNA silencing. In another case, transgenic tobacco plants expressing untranslatable parts of the plant viruses proved to be highly resistant to the homologous virus, but not to related viruses (de Haan et al., 1992; Lindbo and Dougherty, 1992). Moreover, even nontransgenic plants that recovered from a viral infection showed resistance against an unrelated virus which carried a sequence insert from the first inoculated virus (Ratcliff et al., 1999). These observations were subsequently described as ‘co-suppression’, ‘post-transcriptional gene silencing’ or ‘virus induced gene silencing’.

Similar phenomenon had been described in *Neurospora crassa*, where introduction of homologous RNA sequences caused sequence-specific RNA degradation or ‘quelling’ of the endogenous gene (Romano and Macino, 1992). In animals, introduction of sense or antisense RNA to endogenous mRNA also resulted in the degradation of the endogenous messenger in *Caenorhabditis elegans* (Guo and Kemphues, 1995). A breakthrough in the animal world came with the observation that injection of dsRNA in *C. elegans* resulted in the degradation of endogenous mRNA and this was introduced as ‘RNA interference’ (RNAi) (Fire et al., 1998). When dsRNA was injected into one region of *C. elegans* it caused
systemic silencing, a phenomenon that is also observed in plants. This led to the hypothesis that RNA silencing was mediated by a stable silencing intermediate.

Other than siRNA mediated gene silencing, recently, the identification of endogenous small RNA molecules, now known as micro (mi)RNAs, are also involved in RNA silencing/repression phenomenon as well as play a vital role in development. *lin-4* and *let-7* miRNAs were shown to be required for proper larval developments in *C. elegans* unraveling the important role played (Lee et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Wightman et al., 1993). Subsequently, cloning and computational approaches have identified hundreds of animal and plant miRNAs and potential target sites in mRNAs, which suggest that a large proportion of plant and animal transcripts are miRNA regulated (Brennecke et al., 2005; Doench and Sharp, 2004; Lewis et al., 2003; Llave et al., 2002; Reinhart et al., 2002).

**2.5.2 Biology of RNA silencing**

Recent years have seen an exponential expansion of research in RNA silencing or interference (RNAi) involving the sequence-specific cellular degradation of target RNAs. The RNAi pathway is complex and although operates through diverse pathways, they follow few common steps as depicted in Figure 2.1. Basically, RNAi is triggered off by dsRNA precursors. The first step of RNAi involves the recognition of double-stranded RNA (dsRNA) regions. The bimolecular or folded-monomolecular dsRNA is recognized by an effector referred to as DICER, a dsRNA specific RNase III-type endonuclease. DICER processes dsRNAs into small interfering RNAs (siRNAs) that vary in length from 20-26nt (Fire et al., 1998; Hamilton and Baulcombe, 1999; Elbashir et al., 2001; Wesley et al., 2001, Agarwal et al., 2003). The siRNAs are double stranded RNAs with characteristic 3' two-nucleotide overhang and a 5' phosphate. These siRNAs make complex with a large repertoire of proteins, known as RNA-induced silencing complex (RISC). RISC most likely involves the participation of one or more Argonaute-like (AGO) proteins (Baumberger and Baulcombe, 2005), which form the catalytic component of the RISC. The incorporated siRNA functions as a search-and-strike module to
specifically position RISC onto RNAs that are complementary to the RISC-bound siRNA. Once such a target RNA is identified and the complementarity is perfect, the catalytic activity of RISC causes endonucleolytic cleavage of the target RNA molecule (Figure 2.1); in case of minor mismatches, translational repression might occur instead of RNA degradation (Voinnet, 2005). The key initiator molecule (dsRNA) can be delivered exogenously or produced in vivo by RNA dependent RNA polymerases (RdRP) or by transcription either through inverted repeats or from converging promoters or short hairpin RNA (shRNA).

![Figure 2.1. Schematic representation of RNA silencing pathway showing the sequence-specific cellular degradation of target mRNA.](image)

**2.6 Gene silencing acts as a plant defense mechanism**

Gene silencing is an adaptive defense mechanism of the plants against foreign nucleic acids invaders such as viruses, bacteria, etc. (Baulcombe, 2004). Gene silencing is one of the major principles behind ‘Pathogen-Derived Resistance (PDR)’ towards the plant viruses and thus it acts as a major adaptive immune
system (Voinnet, 2005; Baulcombe, 2004). Upon viral infections long dsRNAs are generated as replicative intermediates or due to secondary structure or convergent transcription of viral RNAs that serve as a potent trigger of RNA silencing. The long dsRNA is converted into functionally different short (21-22 nt) and long (24-26 nt) siRNA duplexes (Hamilton et al., 2002) by different DICER-like enzymes (DCLs) (Tang et al., 2003). The elevated siRNA levels of viral transcripts are correlated with the reduction in viral titer (Szittya et al., 2002; Ratcliff et al., 1997), as the viral siRNAs guide RISC to sequence specific cleavage of the viral RNA (Hammond et al., 2000; Tang et al., 2003). Thus, the host RNA silencing mechanism serves as antiviral defense to protect the plants from viral infection (Voinnet, 2001).

In plants, the RNA silencing machinery not only elicits antiviral activity at a localized site but the silencing signal also spreads systemically, from cell to cell, and triggers RNA silencing in the distant tissues of the infected plants. Short siRNAs play a role in the cell-to-cell movement for short distance movement of the silencing signals (Himber et al., 2003) while the long siRNAs are involved in DNA methylation and systemic spread of silencing (Himber et al., 2003; Hamilton et al., 2002). If these signals spread and the silencing condition is established ahead of a viral infection, viral RNAs are degraded before viral replication at the viral infection front (Voinnet et al., 2000). This phenomenon has also been described as host 'recovery' phenotype, in which the newly emerging leaves lack viral symptoms and remain substantially free of virus.

RNA silencing phenomenon, set off in response to one mild virus, may also cross-protect the infected plant against virulent one carrying sequences homologous to the pre-infecting one (Ratcliff et al., 1997). The phenomena are now known as Virus-induced Gene Silencing (VIGS). In VIGS, the effector siRNAs serve as molecular memory which counters the virus on re-infection. Hence, the RNA silencing activated against the infecting virus not only helps the host from the initially virulent infection so that the new growth is both symptom and virus free but also bestows resistance to a secondary challenge by the same or homologous viruses.
2.7 RNA silencing affects transgene expression in plants

RNA silencing is a blessing phenomenon as it is a defence mechanism of plants, however, the same can be an undesired event in some plant molecular research and applications. RNA silencing approaches have been proven to be effective for investigating plant gene function in a high throughput, genome-wide manner (Wang and Waterhouse, 2002; Waterhouse and Helliwell, 2003). Mostly the development of hairpin-constructs (Helliwell and Waterhouse, 2003) and virus induced gene silencing (VIGS)-vectors (Johansen and Carrington, 2001; Lu et al., 2003; Burch-Smith et al., 2004) to down-regulate gene expression has greatly improved the potential of functional genomics research. On the other hand, RNA silencing strongly complicates phenotype analyses and the production of commercial crops with predictable and stable transgene performance.

RNA silencing plays a dominant role in the establishment of transgene expression variation. Many laboratories have reported the occurrence of transgene silencing either during the developmental stage or in subsequent generations (Matzke et al., 1989; Napoli et al., 1990; Van der Krol et al., 1990; Vaucheret et al., 1998; Alvarez et al., 2000). Many selected transgenic lines (based on Southern blots) produced little or no foreign protein, presumably due to RNA silencing (Scholthof, 2007). Besides complex arrangements of multiple transgenic DNA insertions such as IRs, other triggers of gene silencing in plants include dsRNA, concurrent expression of sense and antisense genes, homology between transgenes and endogenous genes, aberrant RNA production (premature transcripts, breakdown products or antisense RNA) and high levels of transgene expression i.e. exceeding a certain threshold level (Matzke et al., 2002). After incorporation of transgene(s) into plant chromosome these transgene(s) transcribe into ssRNAs which are converted into dsRNAs by RdRP (Figure 2.2) (Dalmay et al., 2000). RdRP converts single stranded aberrant, immature or truncated transcripts into double stranded RNAs which trigger the gene silencing machinery in plants. DICER cleaves these dsRNA to yield small (~21 to 26nt) short interfering RNAs (siRNAs). These siRNAs could trigger two kinds of gene
silencing viz. RITS (RNAi-induced transcriptional gene silencing) mediated transcriptional gene silencing (TGS) and RISC (RNA-induced silencing complex) mediated post-transcriptional gene silencing (PTGS) (Figure 2.2). TGS inhibits transcription initiation, while in PTGS, siRNAs serve as guides for cleavage or degradation of specific homologous RNA molecules. PTGS can be triggered locally and then spread throughout the organism via a mobile signal (Mallory, 2001). Therefore, even after successful transformation event stable and predictable transgene expression is not achieved due to gene silencing. Moreover, the plant mutants, defective in one or several of the RNA silencing pathways, are found to show enhanced and stable transgene expression in plants (Butaye et al., 2004).

2.8 Remedy of transgene silencing

In plants, RNA silencing serves as an adaptive immune response that restricts accumulation or spread of inducing viruses (Ye et al., 2003; Voinnet, 2001; Waterhouse et al., 2001). As a counter defensive mechanism, members of different viruses encode proteins known as RNA silencing suppressors (RSS) that suppress RNA silencing at different stages of the pathway (Vaucheret et al., 2001; Tijsterman et al., 2002; Moissiard and Voinnet, 2004; Roth et al., 2004; Li and Ding, 2006). Plants perceive transgenes like virus molecules and endeavour to build a defence mechanism against invaded transgene(s) like virus-induced gene silencing (VIGS). However, viral suppressors can overcome this RNA silencing barrier to replicate and establish viruses in the host plants. Recently, these suppressors have been explored to ameliorate low transgene expression in plant transformation technology. The use of such viral suppressors has proven to be an efficient strategy to inhibit the negative influence of RNA silencing on transgene expression (Mallory et al., 2002; Voinnet et al., 2003; Alvarez et al., 2008). Hence, an attractive prospective application of viral-encoded suppressors is for use in transgenic plants to obtain and maintain substantially elevated levels of the foreign protein.
Figure 2.2. Transgene(s) silencing in plants. Gene silencing is an evolutionarily conserved mechanism in plants protecting its genome from foreign nucleic acid invaders. After incorporation of foreign gene(s) into plant chromosome these gene(s) transcribe into ssRNAs which are converted into dsRNAs by plant endogenous RdRP. These dsRNAs, potential targets of the gene silencing mechanism, are recognized by a dsRNA specific nuclease (DICER) to yield small (~21 to 26 nt) short interfering RNAs (siRNAs). These siRNAs can trigger two kinds of gene silencing viz. RITS (RNAi-induced transcriptional gene silencing) mediated transcriptional gene silencing (TGS) and RISC (RNA-induced silencing complex) mediated post-transcriptional gene silencing (PTGS). TGS inhibits transcription initiation. On the other hand, in PTGS, siRNAs serve as guides for cleavage or degradation of specific homologous RNA molecules.
2.9 Discovery of RNA silencing suppressors (RSS)

The viruses have been found to counter the host RNA silencing defense mechanism by encoding protein(s) known as RNA silencing suppressor (RSS). The viral suppressor is a virus encoded protein or an RNA element that blocks silencing of viral nucleic acid sequences guided by siRNAs or miRNAs resulting in more severe pathogenic symptoms and diseases. The discovery of RSS itself played an important role in establishing RNA silencing as a natural antiviral response.

The preliminary indication that viruses encode RSS came from seminal experiments aimed at understanding the phenomenon of synergism during co-infection. Following synergism, the weak viral symptoms are aggravated by co-infection of a second, unrelated virus (Vance et al., 1995). The study of specific viruses like the potexvirus and potyvirus has led to the identification of the potyviral Helper component proteinase (HC-Pro) as the synergism determinant (Pruss et al., 1997). This determinant later turned out to be a strong suppressor of RNAi.

Synergistic viral diseases of higher plants are caused by the interaction of two independent viruses in the same host and are characterized by dramatic increases in symptoms and in accumulation of one of the co-infecting viruses. One of the classical examples is potato virus X (PVX)/potyviral synergism. PVX, by itself, causes mild symptoms but multiplies vigorously during co-infection and it has been shown that increased pathogenicity and accumulation of PVX are mediated by the expression of potyviral RSS, HC-Pro (Pruss et al., 1997). It was proposed that HC-Pro suppresses a host defense response similar to RNA silencing that normally restricts accumulation of these viruses (Ruiz et al., 1998). This hypothesis was subsequently verified independently by three research groups. In the course of these experiments, the 2b protein of cucumber mosaic virus (CMV) was also identified as a RSS (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Similar synergism has been evidenced in other viruses as well, including the geminiviruses where the differential role of...
AC2 and AC4 governs the synergism between two different cassava geminivirus strains (Vanitharani et al., 2004).

Hence RNA induced silencing was probably a general constraint to virus accumulation in plants and suppression of silencing was the likely shared property of many plant viruses. These findings were also coupled to the earlier investigations that characterized HC-Pro and CMV2b as pathogenicity determinants, i.e. factors that were not strictly required for viral replication but needed for efficient accumulation at the cellular and/or whole plant level. A reinvestigation of pathogenicity factors from diverse viruses interestingly identified many of them as potent silencing suppressors (Voinnet et al., 1999). Since then several additional proteins have now been shown to act as RSSs (Table 2). The ubiquitous nature of silencing suppression indicates that it is probably an absolute requirement for plant viruses if they are to accumulate and spread in plants.

Even though majority of the RSS have been identified from plant viruses, there are a few reported from the insect and animal viruses as well. It is now well established that the RNA silencing machinery is present in animal systems and it likely functions as an important defense against transposons and viruses. It is therefore not unreasonable to expect that animal viruses would have been exposed to similar selection pressure as plant viruses during the course of evolution and would be expected to encode proteins with silencing suppressor activity. It is somewhat surprising, therefore, that the definitive reports of animal viral proteins with such suppressor activity are meager compared with reports on plant viruses. Interestingly, the first insect virus suppressor of RNA silencing described is the B2 protein encoded by Flock house virus (FHV) (Li et al., 2002). Using plant, insect or mammalian cell-based assays, a number of mammalian viruses encoded double stranded RNA binding proteins, majority of which serves as interferon or protein kinase R antagonists, have been demonstrated to have RNAi suppressor activity.
Table 2. List of RSS identified from plant, animal and insect viruses

<table>
<thead>
<tr>
<th>Genus/Family</th>
<th>Virus</th>
<th>Suppressor</th>
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<tbody>
<tr>
<td><strong>Plant viruses</strong></td>
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<tr>
<td>Tombusvirus</td>
<td><em>Tomato Bushy stunt virus</em></td>
<td>P19</td>
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<td>Potyvirus</td>
<td><em>Potato virus-Y</em></td>
<td>Hc-Pro</td>
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<td>Luteovirus</td>
<td><em>Beet western yellow virus</em></td>
<td>P0</td>
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<td>Cucumovirus</td>
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<td><em>Rice yellow mottle virus</em></td>
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<td><em>African cassava mosaic virus</em></td>
<td>AC2, AC4, AV2</td>
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<td><em>Turnip crinkle virus</em></td>
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<td>Closterovirus</td>
<td><em>Citrus tristeza virus</em></td>
<td>P20, P23, CP</td>
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<td>Nodavirus</td>
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<td><strong>Animal viruses</strong></td>
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<td><em>Influenza A virus</em></td>
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<td>Flaviviridae</td>
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2.10 Characteristics of RNA silencing suppressors

2.10.1 Cross kingdom activity of RSS

Cross kingdom activity is an interesting feature of viral RSS. RSS has the ability to suppress RNA silencing in both animal and plant cells, regardless of their host preference (Voinnet et al., 1999). The known RSS of insect and animal virus origin, including B2 of FHV, NS1 of influenza virus, E3L of vaccinia virus, and Tas of PFV-1 can efficiently suppress RNA silencing in plant systems (Li et al., 2002 and 2004). Similarly, plant virus suppressors such as P19 of TBSV, CP of TCV, P15 of *Peanut clump virus* etc., have been tested for silencing suppression activities in animal cell cultures (Dunoyer et al., 2004; Lakatos et al., 2004).
2.10.2 RSS can affect silencing pathway globally

Silencing suppressors not only affect the viral induced RNA silencing pathway but can suppress the general RNA silencing machinery. RSS like P19, HC-Pro and P21 have been found to interfere with the HEN1 mediated methylation of miRNA (Yu et al., 2006). The overlapping biogenesis pathway of siRNA and miRNA could be reasons for such common suppression (Chapman et al., 2004). The suppression effect on both siRNA and miRNA mediated pathways has also been elucidated by the developmental abnormalities associated with the suppressor transgenics (Voinnet, 2005).

2.10.3 RSS promotes synergism

Synergism is a common phenomenon in viral diseases of higher plants and is characterized by dramatic increases in symptoms and in accumulation of viruses. It is caused by the interactions of two or more independent viruses in the same host. One of the classical examples is potato virus X (PVX)/potyviral synergism, where the increased pathogenicity and accumulation of PVX are mediated by the expression of HC-Pro, a RSS of potyvirus (Pruss et al., 1997).

2.11 Mechanism of RNA silencing suppressors

Most of the suppressors act using different mechanisms and no ‘suppression motifs’ have yet been found in these molecules (Voinnet et al., 1999; Anandalakshmi et al., 2000; Llave et al., 2000; Mallory et al., 2001; Voinnet et al., 2000; Guo and Ding, 2002). However, on the basis of the existing knowledge, the mechanisms of suppression by RSS are discussed below.

2.11.1 Suppression activity at double stranded (ds) RNA level

dsRNAs serve as a key mediator of silencing pathway, where long dsRNA serves as a major inducer and small dsRNA acts as a major effector of RNA silencing pathway. Double stranded RNA binding is a common strategy for many of the viral encoded suppressors (Merai et al., 2006) (Figure 2.3). RSS encoded from phylogenetically and evolutionarily divergent viruses like tombusvirus P19, closterovirus P21, carmovirus CP, pecluvirus p15, hordeivirus yB, potyvirus HC-
Pro, etc., show double stranded RNA binding activity (Figure 2.3). In these cases RSS masks the long dsRNA and thus protects it from the attack of DICER, which subsequently processes long dsRNA into siRNA, even though a few RSS can bind to both long and short dsRNA, like p14, FHV-B2 (Singh et al., 2009).

2.11.2 Suppression activity at DICER level

DICER/DCL is an important component of RNA silencing pathway and many RSSs have been found to interact with and inhibit DICER/DCL activities. P38, a RSS of TCV (*Turnip crinkle virus*), suppresses DCL4 activities (Deleris et al., 2006). Singh et al. (2009) have shown that FHV-B2 interacts with the PAZ domain of DICER to suppress siRNA biogenesis (Figure 2.3). Hepatitis C virus core protein also directly interacts with DICER to antagonize RNA silencing (Chen et al., 2008). Interference with the dicer activity directly affects the siRNA biogenesis and hence the silencing of the target gene.

2.11.3 Suppression activity at single stranded (ss) RNA level

Some RSSs are found to have great affinity for siRNA duplexes which are important effectors for silencing pathways. By binding with siRNA, RSS sequesters them to make a complex with RISC. RSSs like P19, P21, γB, HC-Pro and FHV-B2 bind to siRNA duplex and prevent them from entering into RISC (Figure 2.3) (Vargason et al., 2003; Ye et al., 2003; Ye and Patel, 2005; Lakatos et al., 2006; Singh et al., 2009). Binding of RSS with siRNA either makes it biologically inactive by demethylation or sequester it to get associated to form complex like RISC which, in turn, protects the viral RNA from sequence specific endonucleolytic cleavage of RNA silencing. The crystal structure of RSS further indicates that the mechanism of RNA binding also varies with RSSs (Chen et al., 2008). TAV2b adopts an all α-helix structure and forms a homodimer to measure siRNA duplex in a length-preference mode. TAV2b recognizes siRNA (19nt) duplex by a pair of hook-like structures, while P19 protein uses an extended β-sheet surface and a small α-helix to form a caliper-like architecture for binding and measuring the characteristic length of siRNAs.
2.11.4 Suppression activity at RISC level

Another important protein involved in RNA silencing pathway is argonaute, which has been shown to be targeted by various RSS to suppress RNA silencing. Polerovirus encoded P0 suppresses RNA silencing by destabilizing the argonaute1 (Bortolamiol et al., 2008). Ruiz-Ferrer and Voinnet (2007) have shown that CMV-2b directly interacts with argonaute to inhibit its slicing activity (Figure 2.3). Interestingly, similar slicing mechanism has also been observed in case of MYMIV-AC2 (unpublished data of our lab).

![Figure 2.3. Schematic representation of mechanisms used by RSS on RNA silencing pathway](image-url)
2.11.5 Suppression activity through microRNA (miRNA) binding/inhibition

miRNAs are small (~21nt), noncoding RNAs that negatively regulate target mRNAs at the posttranscriptional level that are involved in developmental regulations. In plants, virus-induced disease symptoms often result in developmental abnormalities resembling perturbation of miRNA-mediated function. Moreover, several studies have now shown that transgenic expression of suppressors can alter the accumulation and/or functioning of miRNAs leading to developmental abnormalities related to the action of miRNAs (Siddique et al., 2008). It has been proven that RSSs interfere with miRNA biosynthesis in plant and inhibit the cleavage translational repression of target genes by specific miRNA in the plant developmental pathway (Figure 2.4) (Alvarez et al., 2006; Chapman et al., 2004; Dunoyer et al., 2004; Jacobsen et al., 1999; Kasschau et al., 2003; Llave et al., 2002; Mallory et al., 2002, 2004; Millar and Gubler, 2005; Park et al., 2002; Ray et al., 1996; Vazquez et al., 2004). Tombusvirus encoded P19 and P21 of Beet yellow virus bind to duplex forms of miRNAs (miRNA–miRNA*) and thus interfere with miRNA and siRNA pathways. P1/HC-Pro (potyvirus) inhibits miRNA-mediated cleavage of target mRNAs, but the exact mode of action of this protein in the silencing pathway is not known. Capsid protein (CP) of Turnip crinkle virus (TCV) has been implicated to interfere with DICER. AC4 protein of ACMV is a unique RNA-silencing suppressor because of its ability to bind to single-stranded forms of miRNAs and siRNAs and thus interferes with both miRNA-mediated function and suppression of siRNA-mediated PTGS (Figure 2.4).

2.12 Some well known RNA silencing suppressors

2.12.1 Suppressor P19 of Tombusviruses

In the beginning P19 was recognized as a RSS based on its ability to reverse the expression of a silenced GFP transgene in the systemic leaves of plants infected with either TBSV or PVX carrying a P19 insert (Voinnet et al., 1999). Subsequently, several groups have independently demonstrated the potent silencing suppressor
activity of P19 from a number of different tombusviruses using the agro-infiltration assay (Qu and Morris, 2002; Qiu et al., 2002; Silhavy et al., 2002). P19 binds single-stranded siRNAs, long dsRNAs, or blunted 21 nt dsRNAs (Silhavy et al., 2002). Notably, it was the first protein demonstrated to directly bind siRNAs, functioning presumably to prevent the siRNAs from entering the RISC complex (Silhavy et al., 2002). The crystal structure of P19-siRNA complex elegantly established a structural explanation about the dimerization of P19 which is essential for binding of P19 with siRNA (Vargason et al., 2003; Ye et al., 2003). Studies have verified that the degree of P19-siRNA binding in vivo correlates with the severity of pathogenicity (Dunoyer et al., 2004; Chapman et al., 2004; Lakatos et al., 2004). P19 of Beet yellow virus bind to duplex forms of miRNAs (miRNA-miRNA*) and thus interfere with miRNA pathway (Chellappan et al., 2005).

Figure 2.4. Schematic representation of mechanisms used by RSS on miRNA pathway in plants (Adapted from Chellappan et al., 2005)
2.12.2 Suppressor HC-Pro of Potyviruses

HC-Pro is one of the first viral RSS characterized by the studies using potyvirus as a model system. HC-Pro was also known to act as pathogenicity enhancer causing increased viral RNA accumulation and severe viral symptoms (Pruss et al., 1997). Later it was shown that HC-Pro strongly suppresses RNA silencing, most likely by acting on a maintenance step affecting the assembly and/or targeting of the RISC complex (Chapman et al., 2004; Mallory et al., 2001). Recent studies have demonstrated that it also plays an important role in genome amplification and its long distance movement correlates with its silencing suppressor activity. A breakthrough discovery regarding possible mechanism of silencing suppression was the demonstration of interaction between P1/HC-Pro of TEV and rgs-CaM, a tobacco calmodulin-like protein (Anandalakshmi et al., 2000) showing that rgs-CaM suppresses RNA silencing.

2.12.3 Suppressor 2b of Cucumoviruses

CMV-2b protein was identified as RSS at about the same time as P1/HC-Pro of potyviruses (Brigneti et al., 1998). CMV-2b was found to enhance the long distance movement of CMV in a host-dependent manner (Ding et al., 1995). Later, it was shown that the systemic infection of plants containing a silenced GFP transgene by either CMV or a PVX vector expressing 2b restored GFP expression in the leaves emerging after infection, but not in the leaves where GFP silencing had already been established (Brigneti et al., 1998). Thus, CMV-2b functions to prevent the systemic spread of RNA silencing signals (Guo and Ding, 2002). CMV-2b has been found to interfere with the slicing activity through direct interaction with AGO (Ruiz-Ferrer and Voinnet, 2007). 2b has also been found to interact with siRNA, indicating that CMV-2b might suppress RNA silencing by binding directly to siRNAs (Goto et al., 2007). The crystal structure analysis unraveled that TAV-2b recognizes siRNA duplex by a pair of hook-like structures by fitting the protein backbones inside the major groove and wrapping around both faces of the dsRNA to recognize the major groove (Chen et al., 2008)
2.13 Geminiviruses are also the inducers and targets of gene silencing

Geminiviruses are characterized by small geminate particles (18x20 nm) containing either one or two single-stranded circular DNA molecules of ~2.7 kb length (Stanley and Gay, 1983). Geminiviruses infect a wide range of economically important crops worldwide (Mansoor et al., 2003). In many cases a huge economic losses are caused due to infection of both monocotyledonous and dicotyledonous plants by a large number of geminiviruses. Geminiviral caused important diseases include: leaf curling disease of cottons, golden mosaic disease of tomatoes, yellow mosaic disease of pulses, etc. Based on genome organization, host-range and vector specificity, the members of the family Geminiviridae are classified into four genera: Begomovirus, Mastrevirus, Curtovirus and Topocuvirus (Fauquet et al., 2003).

The majority of begomoviruses have two genomic components, referred to as DNA-A and DNA-B, both of which are essential for infectivity. DNA-A contains six ORFs: AC1 (AL1) encodes a replication-associated protein (Rep) essential for viral DNA replication in association with host DNA polymerase (Hanley-Bowdoin et al., 2000), AC2 (AL2) encodes a transcription activator protein (TrAP) (Sunter and Bisaro, 1992), AC3 (AL3) encodes a replication enhancer protein (REn) (Sunter et al., 1990), AV1 (AR1) and AV2 (AR2) encode coat protein and pre-coat protein, respectively (Padidam et al., 1996), but no function has been attributed to AC4 (AL4) encoded protein in relation to virus multiplication. However, Raghavan et al. (2004) have shown that mutation in AC4 causes 30-fold reduction of Mungbean yellow mosaic India virus (MYMIV) replication in yeast model developed for studying geminiviral DNA replication, emphasizing that AC4 plays an important role during rolling circle mode of replication. On the other hand, DNA-B contains BR1 and BL1 genes that encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively (Sanderfoot and Lazarowitz, 1995), which are important for viral cell to cell and long distance movements.
Monopartite begomoviruses in isolates of various geminiviruses e.g. *Tomato yellow leaf curl Sardinia virus* (TYLCSV) with a single genomic component equivalent to DNA-A have been identified (Kheyr-Pour et al., 1991). In addition, ssDNA satellite DNA-β molecules identified in association with many monopartite begomoviruses are involved in symptom enhancement (Mansoor et al., 2003). In many monopartite begomoviruses, DNA replication is accomplished through a rolling circle mechanism with a dsDNA intermediate, and gene transcription is bi-directional from the common region (Hanley-Bowdoin et al., 2000).

Most plant RNA viruses replicate through the formation of dsRNA intermediates, the potential inducer of PTGS. Unlike the positive single-stranded RNA (ssRNA) viruses, geminiviruses do not use a dsRNA intermediate in their replication cycle. Geminiviruses have ssDNA genomes which replicate through dsDNA as the replicative intermediates in the infected plant cell nucleus (Hanley-Bowdoin et al., 2000). In 2004, Chellappan and co-workers (2004) have demonstrated the ability of isolates of five distinct species of cassava-infecting geminiviruses, viz., *African cassava mosaic virus* (ACMV-[CM], isolate from Cameroon), *East African cassava mosaic Cameroon virus* (EACMCV, isolate from Cameroon), *East African cassava mosaic virus* (EACMV-[UG], isolate from Uganda), *Sri Lankan cassava mosaic virus* (SLCMV, isolate from Sri Lanka), and *Indian cassava mosaic virus* (ICMV, isolate from India) to induce PTGS in two plant hosts, tobacco (*N. benthamiana*) and cassava (*Manihot esculenta*, Crantz) in terms of their ability to produce virus-specific siRNAs.

How geminiviruses trigger PTGS has been a mystery since they are nuclear replicating single-stranded, circular, DNA viruses with no known dsRNA form present in their replication cycle. However, transcription in geminiviruses is bidirectional with the production of polycistronic mRNAs occurring from the CR, which contains the promoter sequences. These polycistronic mRNAs of opposite polarity could overlap at their 3' ends. In DNA-A of bipartite geminiviruses, two major polycistronic mRNA transcripts have been identified for ACMV-[KE]
(Townsend et al., 1985) and Tomato golden mosaic virus (Sunter and Bisaro, 1989): one on the virion sense strand encompassing the AV1 and AV2 genes (AV2-CP transcript) and a second on the complementary-sense strand encompassing AC1, AC2, AC3, and AC4 genes (AC1-AC3 transcript). In ACMV-[CM], the virion-sense AV1 (CP) gene and the complementary-sense AC3 gene overlap by 4 bp at their 3' ends. Using strand-specific probes, it was elucidated that both AV2-CP (AR2-CP) and AC1-AC3 (AL1-AL3) transcripts extended at their 3' ends by more than the 4-bp overlapping region by a number of nucleotides, since the probes used were devoid of the overlapping nucleotides. The resulting overlap region can form a dsRNA, which would be sufficient to potentially induce the plants' PTGS system (Chellappan et al., 2004). Alternatively, the early and abundant AC1-transcript of these viruses could serve as the template for the host RdRP to dsRNAs to induce the RNAi machinery leading to the production of siRNAs (Dalmay et al., 2000). Yet another possibility is that the strong fold-back structure of geminivirus transcripts could simply become a template for DICERs to cleave at specific locations and produce siRNAs (Vanitharani et al., 2005). In plants, some virus-host interactions naturally lead to host recovery. The natural recovery responses induced by a nepovirus (Ratcliff et al., 1997) and a caulimovirus (Covey et al., 1997) have been shown to be similar to RNA-mediated virus resistance. The symptom recovery phenomenon observed by Chellappan and co-workers (2004) was unusual for geminiviruses. Moreover, this recovery phenomenon in both N. benthamiana and cassava was associated with the production of virus derived siRNAs beginning one week post-inoculation and becoming abundant in the newly developed symptom-less recovered leaves. This increase in virus-derived siRNA accumulation was accompanied by a reduction in the levels of both viral DNA and mRNA accumulation.

In addition, host-virus interactions are strongly modified by environmental factors, particularly by temperature. Molecular analysis of Cymbidium ring spot virus (CymRSV) has revealed that low temperature (15°C) inhibits RNA silencing-mediated defense by controlling siRNA generation. By contrast, RNA silencing was activated with an increase in siRNA accumulation with rising temperature.
(27°C) (Szitty et al., 2003). In ssDNA viruses, irrespective of the recovery (ACMV-[CM] and SLCMV) and non-recovery (EACMCV, EACMV-[Ug], and ICMV) type viruses, there is a general trend for symptom decrease with high temperature, indicating that ssDNA viruses might be following the same trend as RNA viruses (Chellappan et al., 2005).

2.14 AC2 protein of Geminiviruses

AC2 (AL2) is one of the multifunctional proteins encoded by all members of the genus Begomovirus (formerly subgroup III). The protein is encoded by the complementary strand of DNA-A component, hence the name AC2. The protein possesses transactivation potential and is required for the expression of late viral genes AV1 and BV1 in at least some geminiviruses, thus also known as TrAP (transcriptional activator protein) (Sunter and Bisaro, 1991 and 1992; Jeffrey et al., 1996). Interestingly, TrAP function is not virus specific, as the AC2 gene products of Tomato golden mosaic virus (TGMV), African cassava mosaic virus (ACMV), Texas pepper geminivirus (TPGV), Squash leaf curl virus (SqLCV), and Tomato yellow leaf curl virus (TYLCV) complement a TGMV al2 mutant in tobacco protoplasts (Sunter et al., 1994). TGMV DNA-A also complements ACMV and Potato yellow mosaic virus (PYMV) al2 mutants in planta (Saunders and Stanley, 1995; Sung and Coutts, 1995), reinforcing the conclusion that TrAP is functionally interchangeable among begomoviruses. This absence of functional specificity suggests that either all begomovirus late promoters contain a common sequence element recognized by TrAP and/or that TrAP interacts with cellular proteins common to all begomovirus plant hosts to effect transcriptional activation.

In general, the AC2 protein has a modular structure consisting of three conserved domains: a basic domain with a nuclear localization signal at the N-terminus, a central DNA-binding Zn-finger motif and C terminal acidic activator domain (Hartitz et al., 1999). It binds to ssDNA in a non specific manner and only weakly to dsDNA, suggesting that it is not a canonical transcriptional factor, but probably interacts with host plant cellular proteins to trigger transcriptional activation (Hartitz et al., 1999). In addition, the TGMV TrAP was shown to be
phosphorylated and to bind zinc, two properties that facilitate its optimal association to DNA in a sequence nonspecific manner (Hartitz et al., 1997). Sequence alignments revealed that a zinc-finger motif and four potential phosphorylation sites are highly conserved among TrAP proteins from diverse monopartite and bipartite geminiviruses (Van Wezel et al., 2002). Analysis of the *Tomato yellow leaf curl virus* (TYLCV) TrAP showed that point mutations affecting any of the three conserved cystein residues of the zing-finger motif abolished its zinc- and DNA-binding properties (Van Wezel et al., 2002 and 2003). By contrast, mutations affecting the conserved, putatively phosphorylated residues had no effect. AC2 of bipartite geminiviruses shares a considerable sequence homology (56–88%), and they cluster according to a geographical distribution of 92 begomoviruses in a similar manner to the rest of the genome.

Geminivirus AC2 is one of the major pathogenicity factors. It has been reported that the most striking reactions induced by AC2, e.g. systemic necrotic lesions, necrotic veins and petioles leading to leaf collapse and subsequent severe stunting (Hong et al., 1997; Voinnet et al., 1999; van Wezel et al., 2001; Selth et al., 2004). Furthermore, AC2 interacts and inactivates SNF1 and adenosine kinases, enzymes which appear to be involved in defense response (Hao et al., 2003; Wang et al., 2003). Finally AC2 has also been implicated as a suppressor of RNA silencing (Bisaro, 2006; Selth et al., 2004; Trinks et al., 2005; Van Wezel et al., 2001; Vanitharani et al., 2004; Voinnet et al., 1999; Wang et al., 2005). The point mutation study with TYLCV-AC2 has shown that abolishing zinc- and DNA-binding properties eliminates silencing suppression and symptom accentuation mediated by the protein (Van Wezel et al., 2002 and 2003). By contrast, mutations affecting the conserved, putatively phosphorylated residues had no effect. These results strongly suggest that zinc- and DNA-binding properties are necessary for the suppression of silencing by TrAP proteins. Although the exact significance of this finding awaits further experimentation, it is tempting to speculate that *de novo* transcriptional activation of endogenous genes may be involved in TrAP-mediated suppression (Trink et al., 2005). Moreover, there are reports, where it has clearly been shown that the loss of transactivation potential would not
necessarily lead to the compromise in the PTGS suppression activity. Interestingly, AC2 protein of Mungbean yellow mosaic India virus, MYMIV, a member of the begomoviridae genus of the geminiviridae family has been established to function as a RSS (Karjee et al., 2008; Singh et al., 2007a).

2.15 RSS promotes transgene expression

During the past two decades, the development and use of transgenic plants has found increasing applications. However, optimized and stable levels of transgene expression has been considered as vital requisites in modern plant transformation technology because of low or even undetectable transgene expression reported from many labs (Alvarez et al., 2008). Moreover significant differences in the transgene expression level of introduced transgenes are observed among transgenic plants generated under identical conditions and using the same transgene construct (Bennet 1993; Birch, 1997; Bhat and Srinivasan, 2002; Butaye et al., 2004). Several strategies have been adopted in an attempt to minimize variation in transgene expression including, most recently, the use of viral genes that suppress gene silencing (Anandalakshmi et al., 1998). Implementation of the current knowledge about the RNA-silencing mechanism in transformation technologies has emerged as a potential alternate way of tackling undesired low transgene expression as well as to obtain and maintain substantially elevated levels of the foreign proteins in plant (Ma et al., 2009; Alvarez et al., 2008).

2.15.1 RSS boosts the transgene expression in transient expression system

In Agrobacterium based transient expression system, recombinant strains of Agrobacterium tumefaciens are generally used for transient expression of transgenes that have been inserted into the T-DNA region of the binary-Ti plasmid. A bacterial culture is vacuum-infiltrated into leaves, and upon T-DNA transfer, there is ectopic expression of the gene of interest in the plant cells. The use of RSS proteins has proven as an efficient strategy to boost the transgene expression in transient expression system. One such example involves the production of
transgenic tobacco lines that contain a replicating potato virus X (PVX) vector carrying a gene of interest (Mallory et al., 2002). The idea was that the transcription of these ‘amplicon’ lines would initiate viral RNA replication and gene expression, resulting in very high levels of the gene product of interest. This approach failed, however, because every amplicon transgene was subject to PTGS. However, high-level transgene expression was achieved by combining the amplicon approach with the use of a viral suppressor for PTGS, more specifically the Tobacco etch virus (TEV) helper component-proteinase (HC-Pro). Leaves of mature tobacco plants co-expressing HC-Pro and the PVX-amplicon accumulated heterologous protein to 3% of TSP (total soluble protein), termed as ‘the amplicon plus system’ (Mallory et al., 2002). It should be noted, however, that HC-Pro transgenic plants often suffer from severe phenotypic abnormalities, including stunting and altered leaf shape (Pruss et al., 2004). Hence, this approach might be more appropriate for in planta production of heterologous proteins rather than application in phenotypic analyses.

Similar strategies to increase the transgene expression involve a transient expression system based on the co-expression of a transgene and the P19 suppressor of gene silencing of the Tomato bushy stunt virus. Heterologous protein production was enhanced 50-fold or more in the presence of P19 due to prevention of PTGS in the infiltrated tissues (Voinnet et al., 2003). As such, co-expression of a transgene and a viral suppressor gene might be an attractive option to reduce variation of transgene expression caused by RNA silencing. The use of the P19 silencing suppressor gene from Artichoke mottled crinkle virus (AMCV) increased the expression level of human antibody against the tumour-associated antigen tenascin-C (TNC) by an order of magnitude (yields of purified H10, 50-100 mg/kg fresh weight) when co-infiltrated with Agrobacteria in six weeks old N. benthamiana plants (Villani et al., 2009). In another study, Ma et al. (2009) showed that GFP production was enhanced threefold or more in the presence of P1/HC-Pro in an Agrobacterium mediated transient expression system.
2.15.2 RSS boosts the transgene expression in stable transgenic expression system

Although transient expression system is a fast, flexible and reproducible approach to yield high levels of gene expression of useful proteins, however, its utility has thus far been limited because the ectopic protein expression usually ceases after 2-3 days. Therefore, stable transgenic system would constitute the alternative for transgene expression. Most suitable for the purpose would be the utilization of plants that are not only transgenic for the desired foreign value added gene, but that also constitutively express a suppressor. This would enable consistent high levels of expression of foreign protein. Remarkable example of the utilization of RSS to boost the transgene expression came from the studies of Anandalakshmi et al. (1998) and Mallory et al. (2001) in which P1/HC-Pro suppressor was used to reverse the posttranscriptionally silenced uidA reporter gene encoding the reporter enzyme GUS in tobacco. HC-Pro expressing transgenic tobacco lines were crossed with a GUS silenced transgenic lines. After crossing with HC-Pro suppressor transgenic lines, the expression of GUS was reversed from silenced state to expression state in the F1 hybrids. These results indicate that the expression of P1/HC-Pro interferes with transgene-induced posttranscriptional gene silencing in stable transgenic system. Another example of exploitation of RSS in stable transgenic system for production of high valued protein is overexpression of Yersinia pestis F1-V antigen fusion protein in tomato by utilizing P19 suppressor of Tomato bushy stunt virus, TBSV (Alvarez et al., 2008). The expression of the F1-V gene was undetectable in leaves and fruit by ELISA, even though they contained multiple copies of F1-V (as evidenced by Southern blot analysis) in some transgenic tomato lines. These lines were retransformed with P19 suppressor and almost a three-fold increase in F1-V protein levels in fruits was observed than that previously reported for the non-silenced F1-V elite tomato lines. These results confirmed the high potential of P19 suppressor of TBSV to reverse the effects of gene silencing and increase the expression of value-added proteins in stable transgenic system.