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Viral Counter Defense X Antiviral Immunity in Plants: Mechanisms for Survival

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1. Introduction

The history of plant virology dates to the late 19th century, when Iwanowski and Beijerinck, who were investigating the cause of a mysterious disease of tobacco, independently described an unusual agent that caused tobacco mosaic disease. This agent was later named *Tobacco mosaic virus* (TMV) [1]. During this period, viruses including *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Lettuce mosaic virus* (LMV) were described. These viruses could be distinguished based on their transmission and method of disease induction. In addition, numerous techniques for the study of viruses were developed.

Viruses are among the most agriculturally important groups of plant pathogens, causing serious economic losses in many major crops by reducing yield and quality. A virus can be defined as a set of one or more nucleic acid template molecules, often encased in a protective coat of protein or lipoprotein, which is able to organise its own replication only within suitable host cells [1]. Because the genetic information encoded by viral genomes is limited, viruses depend entirely on host cells to replicate their genome and produce infectious progeny. Both plant and animal viruses can be classified according to the type of nucleic acid that makes up their genome. In plants, the vast majority of viruses have positive-sense (+) RNA genomes (i.e., the RNA genome has the same polarity as cellular mRNA), although negative-sense (–) RNA and double-stranded RNA viral genomes also exist. Other plant viruses have DNA genomes; the DNA can be double-stranded (caulimoviruses) or single-stranded (geminiviruses) [1, 2]. Cell-to-cell movement of plant viruses occurs through cytoplasmic “bridges” between cells

called plasmodesmata, and viruses are able to move systemically throughout plants via the phloem [1].

Viruses depend on other organisms (vectors) to transmit them from diseased to healthy plants. These vectors are often sap-sucking insects such as aphids, thrips, whiteflies, leaf-feeding beetles, plant-feeding mites, soil-inhabiting nematodes or fungal pathogens. Some viruses can be mechanically transmitted, on pruning knives or gardeners hands; or by grafting material, and a relatively small number of species can pass through infected seed [3]. Viruses use a variety of strategies that frequently induce disease in the plants they infect. Different viruses induce distinct diseases, and this can be true even for different strains of the same virus. Virus infection can profoundly alter the physiology of the host due to the interaction with cellular components. In plants, the severity of viral diseases varies considerably depending on the host genotype, the stage of infection and the environmental conditions. Diseases caused by viruses can vary broadly in intensity, from very mild symptoms observed in tolerant plants up to very severe symptoms and plant death [1, 4, 5].

Each plant virus encodes an average of 4-10 proteins necessary to coordinate the complex biochemical and intermolecular interactions required for viral infection cycles. The cycle of infection includes viral genome replication, cell-to-cell and systemic movement and transmission [6]. For efficient viral infection to occur, viral proteins must be able to interact with factors in the host cell, thereby manipulating metabolic pathways and coordinating biochemical interactions that promote infection. Thus, during co-evolution between viruses and their hosts, a variety of complex interactions have developed that involve several distinct mechanisms of plant defence and virus attack.

During evolution, plants have developed diverse defence mechanisms that are activated during viral infection. One of these is the hypersensitive response (HR), which activates initial defence responses that prevent the infection from spreading further and then kills the cells within the infected zone. The onset of a second mechanism, systemic acquired resistance (SAR), then protects the plant together with HR against new attacks by the same pathogen. SAR is induced by a variety of agents after initial infection and can provide resistance to a wide range of pathogens for days [7-10]. The HR and SAR responses are accompanied by changes in gene expression that include the production of pathogenesis-related (PR) proteins and of several proteins involved in cell signalling [11].

Plants also possess other antiviral defence mechanisms such as RNA silencing, a remarkable type of gene regulation based on sequence-specific targeting and degradation of RNA [12], and the more recently described ubiquitin/26S proteasome system (UPS), which plays a central role in the degradation of proteins. The latter system is involved in almost all phases of the defence mechanisms of plants, regardless of the type of pathogen [13]. In addition to its proteolytic activity against ubiquitinated pathogen proteins, which directs their degradation by the 20S proteasome, the degradation of viral RNA can also occur via the ribonuclease activity of the 20S proteasome [14, 15]. While the proteasome as a structure, and RNA silencing as a mechanism, are two conserved features among eukaryotes, several lines of evidence suggest that the proteasome-linked RNase activity is most likely not directly related to RNA silencing. The selective degradation of viral RNAs by the 20S complex can represent an

alternative pathway of host defence that occurs in parallel to RNA silencing and reinforces cellular antiviral defence in plants [14].

Over time, the strategies used by the virus to overcome these elaborate host defences can lead to a number of fundamental changes in the plant's physiology. Such changes, including structural modifications in the host cell, may give rise to intranuclear inclusions of various types and may affect the nucleolus or the size and shape of the nucleus. Within the mitochondria, abnormal membrane systems may develop [16, 17]. In plants infected with *Turnip yellow mosaic virus* (TYMV - Tymovirus), abnormalities such as clumping of chloroplasts and abnormal size and number of starch grains in leaf cells may occur, and small vesicles near the periphery and chloroplasts may become greatly enlarged and filled with starch grains [18]. These abnormalities were also observed in squash infected by the *Zucchini yellow mosaic potyvirus* (ZYMV - Potyvirus) [17]. In plant cell walls, three types of abnormality have been observed: abnormal thickening due to the deposition of callose near the edges of virus-induced lesions; cell wall protrusions involving the plasmodesmata (these protrusions may have one or more canals and may be quite short or of considerable length); paramural bodies, which are depositions of electron-dense material between the cell wall and the plasma membrane, may appear and extend over substantial areas of the cell wall, or be limited in extent occurring in association with plasmodesmata. Moreover, in the cytoplasm of an infected cell, virus particles may accumulate in sufficient numbers to form three-dimensional crystalline arrays. The ability to form crystals within the host cell cytoplasm depends on properties of the virus itself, and is not related to the overall concentration reached in the tissue or to the ability of the purified virus to form crystals [1].

Intriguingly, in carrot plants (*Daucus carota* L.) infected by *Cucumber mosaic virus* (CMV) some cytological and physiological changes were observed due to alterations in various host metabolites. With respect to cytological changes, scattered metaphase was observed in the diseased plant cells. The mitotic index of the diseased cells was decreased, while the nucleus / cytoplasm ratio was increased. Chromatin bridges were also observed at anaphase I and II. Physiological changes resulting in decreased carbon, nitrogen and protein content and increased phosphorous content of the virally infected plants have been observed [19].

Other viral counterattack mechanisms involve changes in the plant cell cycle. In plants, as in all eukaryotes, the four phases of the mitotic cell cycle (G1, S, G2 and M) are conserved. During development, plant cells leave the cell division cycle, and in mature plants, DNA replication and the corresponding enzymes are confined to meristematic tissues [20]. Geminiviruses are good models for the study of the relationship between the cell cycle and viral DNA replication because they replicate in differentiated cells, such as mature cells of the leaves, stems and roots, in which most of the cellular factors required for viral DNA replication are normally absent. These cells have left the cell division cycle and no longer contain detectable levels of plant DNA replication enzymes necessary for geminivirus replication [21]. Due to the requirement for cellular factors, geminiviral DNA replication must be coupled to a special state of the infected cell, suggesting that the virus may have evolved mechanisms that affect the expression of cellular genes involved in S-phase progression and G1/S transition [22]. One such a mechanism involved in regulating changes of the host cell cycle appears to be the inactivation

of the retinoblastoma protein (pRb), which negatively regulates the G1/S transition in cells. The Rep protein, which is encoded by all geminiviruses and is the only viral protein necessary for viral DNA replication, has been found to induce expression and also interacts with the host “proliferating cell nuclear antigen” (PCNA), an auxiliary protein of DNA polymerases required during replication and repair in non-dividing plant cells. This observation suggests that Rep protein can provide the necessary stimulus to induce the dedifferentiation process. Mechanisms other than sequestering plant pRb most likely contribute to the multiple effects of geminivirus proteins on cellular gene expression, cell growth control and cellular DNA replication [21, 22].

Ultimately, RNA silencing suppressor (RSS) proteins are able to block or attenuate plant host defence mechanisms, particularly post-transcriptional gene silencing and efficiently inhibit host antiviral responses by interacting with the key components of cellular silencing machinery, often mimicking their normal cellular functions [23]. Viral suppressors of RNA silencing have been identified from almost all plant virus genera; these VSRs are surprisingly diverse, exhibiting no obvious sequence similarities. Most identified VSRs are multifunctional; in addition to being RNA silencing suppressors, they often perform essential roles, functioning as coat proteins, replicases, movement proteins, helper components for viral transmission, proteases or transcriptional regulators. The first viral RNA silencing suppressor identified was the helper component-proteinase (HC-Pro) of potyviruses [24]; currently, many different suppressors are known.

The aim of this chapter review is to discuss the current status of knowledge regarding various components of host silencing machinery and viral suppression. We also pretend to describe how the defence response in plants is directed against the virus and, in particular, how the virus can sidetrack the host's defence response. Relevant topics on the molecular bases of the induction and suppression of the RNA silencing mechanism, as well as the applications and perspectives of the use of silencing suppression in plant biotechnology, will be emphasised.

2. The importance of silencing pathways

A major breakthrough in the history of biology was the discovery of an RNA-induced silencing response in the nematode *Caenorhabditis elegans* [25]. Prior to that discovery, this RNA-induced silencing had been interpreted as a defence mechanism against viruses and other invading nucleic acids; the discovery of endogenous small regulatory RNAs in many species led to the realisation that gene silencing is a fundamental genetic regulatory mechanism in eukaryotic organisms [26].

Several studies in genetics, biochemistry and the development of novel techniques in molecular biology have helped identify different components of the RNA silencing machinery and have confirmed that RNA interference (RNAi), co-suppression and virus-induced gene silencing share mechanistic similarities [27]. RNA silencing is a conserved eukaryotic pathway involved in the suppression of gene expression via sequence-specific interactions that are mediated by 21–23 nucleotide (nt) RNA molecules [28]. Organisms utilise RNA silencing for

three purposes: 1) creating and maintaining heterochromatin at repetitive DNA and transposons; 2) regulating development, stress responses and other endogenous regulatory functions; 3) defending against viral and bacterial infections [29]. Silencing is utilised in developmental pathways and in cellular differentiation to repress genes whose products are not required at specific stages of development or in specific cell types; in plants silencing is also used to respond to internal and external stresses by changing the expression of specific genes involved in the response. In some situations, tissue- or cell-specific silencing is desirable.

Initially discovered in transgenic plants, especially in those created to acquire virus resistance, RNA silencing is now also believed to be responsible for various epigenetic effects and their maintenance; the silencing of transgenic loci in plants most likely results from the activation of defence mechanisms. A number of silencing studies with different plant systems have explored transgenes as indicators of silencing pathways; these works have received important attention in part because silencing reduces the reliability of transgenic approaches in biotechnology of several agriculturally important cultures.

RNA silencing can be classified into two major categories: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS is defined as inhibition of transcription, whereas PTGS involves the post-transcriptional degradation of RNA species but does not affect the transcription rate [30]. TGS occurs when double-stranded RNA molecules (dsRNA) containing promoter sequences are present, and PTGS occurs when dsRNA comprise open reading frames (ORF). Together, TGS and PTGS depend on small interfering RNAs (siRNA) or microRNAs (miRNA) that are produced from dsRNA precursors [31]. Because RNA silencing, mainly PTGS, also contributes to antiviral immunity in plants, fungi and invertebrates, it is an important part of innate immunity [32]. The silencing may persist over many cell divisions or plant generations [33].

The basic steps in common to all RNA silencing pathways (Figure 1) include: (i) formation of a dsRNA; (ii) processing of the dsRNA by an RNase III-like enzyme named Dicer (DCL) to shorter (20-30 nucleotide) dsRNA duplexes, the so called siRNA (iii) binding of the small RNA duplexes to a protein from the Argonaute (AGO) family; and (iv) targeting of the RNA-induced complex to mRNA (or DNA) guided by strand complementary to the small dsRNA, which is called the guide [34]. At present, there is good evidence for the existence of at least four different types of RNA silencing pathways in plants. These pathways involve different types of small RNA molecules, specially siRNA and miRNA. Heterochromatin-associated siRNA (hc-siRNA), trans-acting siRNA (tasiRNA) and viral siRNA (primary and secondary) are also important in silencing [35]. A better understanding of silencing pathways is very important because of the potential usefulness of silencing as a powerful tool for gene function studies and crop improvement.

2.1. Post-transcriptional gene silencing

As already mentioned, PTGS is essential to antiviral immunity in plants, thus our focus will be concentrated in this phenomenon. PTGS was first observed in 1990, and initially referred to as 'co-suppression', it was first discovered in transgenic petunia plants in which the

introduction of the gene for chalcone synthase created a block in anthocyanin biosynthesis, resulting in variegated pigmentation [36].

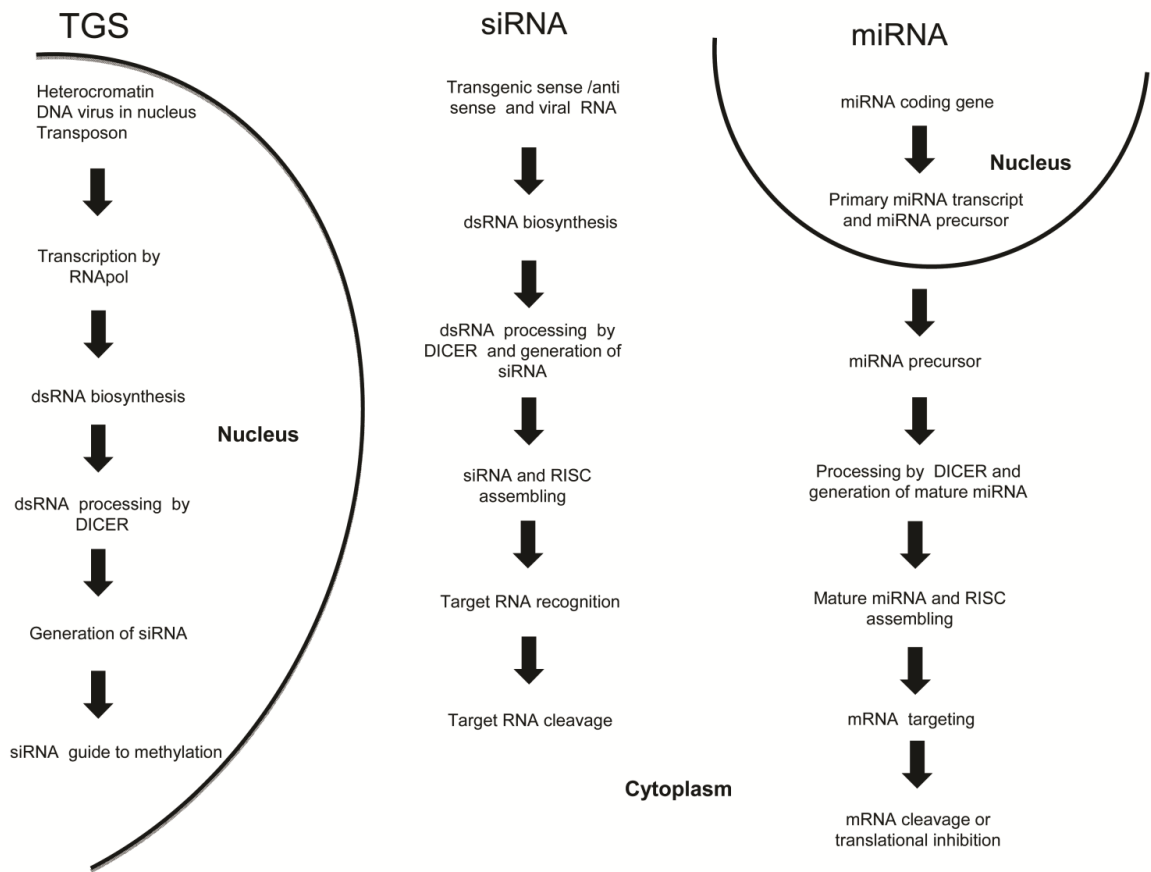


Figure 1. Overview of the transcriptional gene silencing (TGS), siRNA and miRNA pathways. The important steps of each pathway are depicted.

PTGS was also detected in transgenic plants engineered for virus resistance, and associated with the phenomenon of recovery of a host plant from viral infection. This recovery process was soon understood to be associated with the plant’s inherent RNA silencing mechanism, which is an evolutionarily conserved antiviral system. The first report related to virus-induced gene silencing was published as long ago as 1929 by McKinney, who reported that tobacco plants infected with the “green” strain of TMV were protected against infection by a closely related second virus (TMV “yellow” strain). This phenomenon was later described as “cross-protection” [37]. However, a remarkable explanation of cross-protection was provided when it was shown that virus infection prevents infection by a second virus if the two viruses possess homologous sequences. Importantly, this viral-RNA-mediated cross-protection was functionally equivalent to post-transcriptional gene silencing [38].

Viruses are not only targets of transgene induced RNA silencing but also elicit silencing themselves. Transgenic plants expressing a truncated version of the coat protein of *Tobacco etch*

virus (TEV) were initially susceptible to infection and showed symptoms. However, a few weeks after the transgenic plants recovered from the TEV infection, newly developed leaves were symptomless and virus-free. Remarkably, the recovered leaves were resistant against a second TEV infection but were susceptible to infection by the heterologous PVY [39].

PTGS is a mechanism that has been preserved among eukaryote kingdoms. It is a genetic regulatory mechanism that is involved in several processes including defence of the genome against mobile DNA elements, establishment of heterochromatin, control of plant and animal development, and downregulation of gene expression by specific cleavage and translational repression of target mRNAs that contain complementary sites to miRNAs or siRNAs [40].

The involvement of short RNAs in PTGS was discovered when ~25 nt RNA molecules with sequence homology to a transgene were detected only in plants in which the corresponding transgene was silenced [41]. These molecules are also generated against replicating viruses. They are loaded in an active silencing complex called RISC (RNA-induced silencing complex). RISC shows slight variability in composition from one RNA silencing pathway to the next and from species to species; however, all RISCs contain a guide RNA strand bound to an AGO protein. AGO imparts endonuclease activity to the RISC (the so called slicer activity) being responsible for target RNA cleavage.

During PTGS, RNA is degraded predominantly in the cytoplasm. The strongest evidence in support of this comes from study of PTGS-based degradation of RNA viruses that are expressed exclusively in the cytoplasm [42]. However, recent work has demonstrated that the enzymes involved in PTGS are localised in both the cytoplasm and the nucleus. Overall, the available data suggest that PTGS activity in plant cells occurs in both the cytoplasmic and nuclear compartments. Nuclear PTGS would allow regulation of a potentially larger set of endogenous targets that cannot be regulated through cytoplasmic PTGS [31].

Some aspects of genomic silencing remain unknown. For a more complete understanding of genomic silencing, supplementary approaches are needed. This is especially important because gene silencing has the potential for use as a potent sequence-specific gene inactivation tool in functional genomics and plant biotechnology.

3. RNA silencing suppression

RNA silencing is known to serve as a mechanism for plant defence against pathogens. To counteract this mechanism, viruses have evolved the ability to avoid or suppress RNA silencing. Using this strategy, viruses protect their genomes from degradation through the production of proteins that act as suppressors of RNA silencing. These viral proteins act through a variety of molecular mechanisms including, particularly, blockage of the intercellular and systemic spread of mobile small silencing RNA molecules. The ability of viruses to infect cells can have a profound impact on host endogenous RNA silencing regulatory pathways and can result in alterations in endogenous short RNA expression profile and gene expression [43]. A general overview of the RNA silencing pathway discriminating the different steps targeted by different VSR is provided in Figure 2.

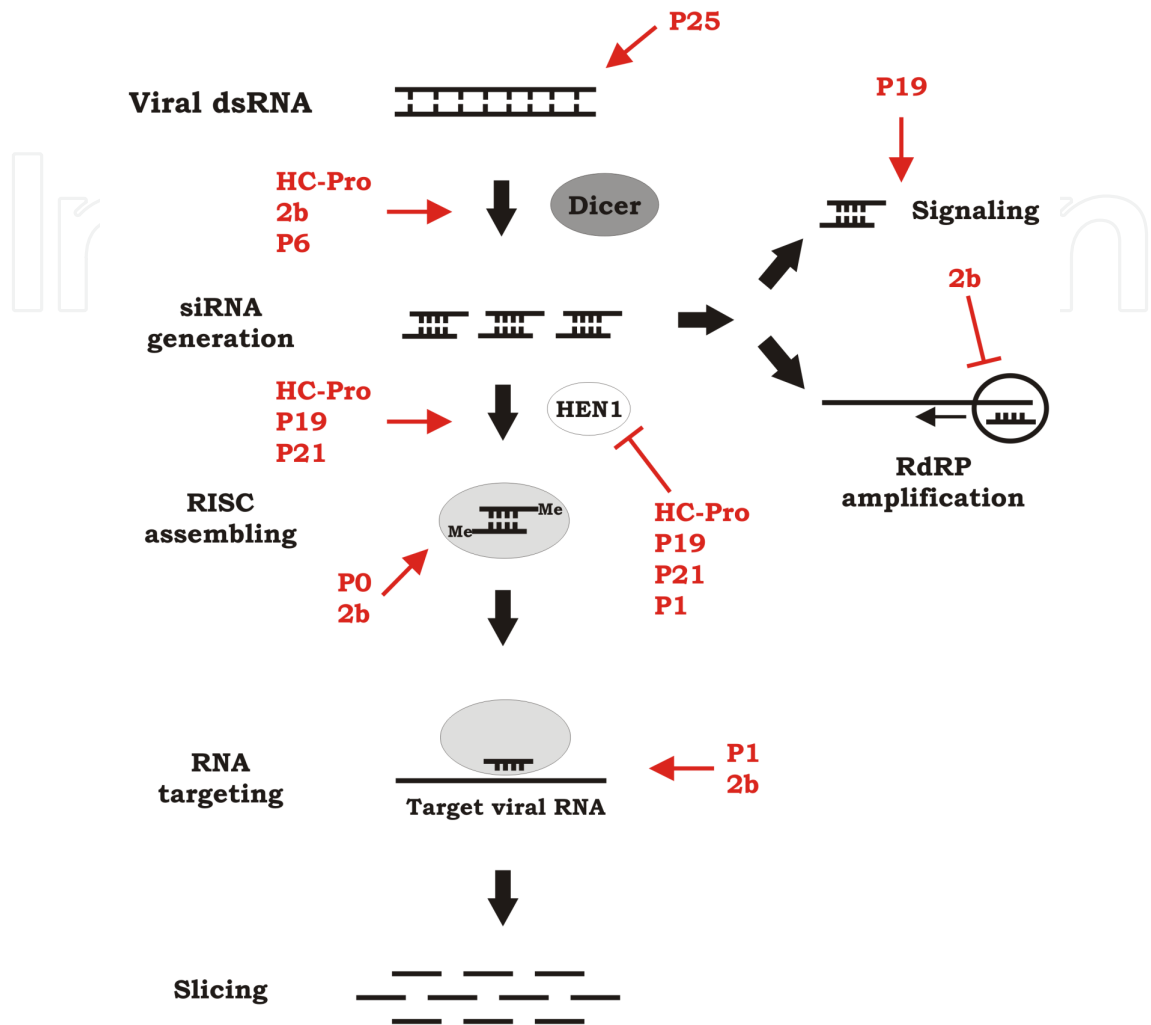


Figure 2. Schematic representation of the RNA silencing pathway triggered by double stranded RNA molecules (dsRNA) of virus origin. The steps of the pathway that are targeted by different viral suppressor proteins are depicted. Amplification of the silencing signal requires an RNA-dependent RNA polymerase (RdRP). Both strands of the small RNA duplexes (3' termini) are methylated by HEN1 before RISC loading.

VSRs were first evident from the analysis of potyviral synergistic interactions with other viruses. It was shown that this synergism is the result of suppression of a host defence mechanism by the potyviral HC-Pro [44]. Subsequent studies established that HC-Pro is a suppressor of PTGS and provided a link between PTGS and antiviral defence [24, 45]. At the same time, analysis of a second viral protein, the 2b protein of CMV, identified this protein as a suppressor of PTGS in *Nicotiana benthamiana*. Intriguingly, HC-Pro and 2b do not target the silencing mechanism in the same way; HC-Pro suppresses silencing in tissues in which it is

already established, whereas the 2b protein only affects silencing initiation [45]. In 1999, a seminal study [46] showed definitively that suppression of RNA silencing is an anti-defence strategy commonly used by plant viruses with DNA or RNA genomes, such as Geminivirus (through protein AC2), Sobemovirus (through protein P1), Tombusvirus (by means of the “19K” protein) and others (Comovirus, Tobamovirus and Tobravirus).

Silencing suppression has also been documented in virus capable of infecting other organisms such as insect and fungus, including flock-house virus (FHV), cricket paralysis virus *Drosophila* C virus, and *Cryphonectria parasitica* hypovirus [47]. In insect cells, functional similarities between the CMV 2b protein and the suppressor B2 protein from FHV were described. Deletion of the B2 ORF from FHV results in a drastic loss of virus accumulation in *Drosophila melanogaster* S2 cells, and this loss can be rescued by decreasing the cellular content of AGO2. B2 therefore seems to suppress the effect of the AGO2-dependent silencing response that normally restricts FHV accumulation [48].

Viral suppressors are considered to be of recent evolutionary origin, and they are often encoded by out-of-frame ORFs within more ancient genes. They are surprisingly diverse within and across kingdoms, with no obvious sequence homology [47]. VSRs are variously positioned on the viral genome and can be expressed using different strategies such as subgenomic RNAs, transcriptional read-through, ribosomal leaky-scanning or proteolytic maturation of polyproteins. Due to their molecular evolution, many of the viral suppressors identified to date are multifunctional, *i.e.*, besides being RNA silencing suppressors; they also perform essential roles in other steps of the viral life cycle [23].

Viruses have developed three efficient silencing suppression strategies to counter host antiviral immunity. The first is related to the inhibition of key components of RNA silencing pathways; the mechanisms involved in this strategy have already been well characterised for some viral proteins and will be described forward. A second silencing-suppression strategy, which will also be described later, involves the recruitment of endogenous negative regulators of RNA silencing. For example, yeast two-hybrid system results showed that HC-Pro is able to interact with the tobacco calmodulin-related rgs-CaM, a cellular suppressor of PTGS [49]. The third strategy relies on modification of the host transcriptome and is supported by studies of the geminivirus transcriptional-activator proteins (TrAPs), which have been identified as silencing suppressors (50).

VSRs are essential for viruses to replicate in host cells and to achieve systemic infection [47]. Although they do not share any obvious sequence or structural similarity across viral families and groups, they have having been initially identified as pathogenicity determinants causing developmental defects in host plants, or as host range determinants [51, 52]. Viral silencing suppressors could cause developmental defects in plants because they act in miRNA and siRNA pathways that are mechanistically similar to developmental pathways. In a study involving transgenic expression of the HC-Pro of *Turnip mosaic virus* (TuMV) in *Arabidopsis thaliana*, this protein was shown to alter the accumulation of miRNAs and to prevent the endonucleolytic cleavage of a number of their cellular targets. This effect coincided with the occurrence of morphological defects resembling those of Dicer-like partial mutants called *dcl-1*. Remarkably, similar defects were observed

upon TuMV infection, providing support for the idea that some of the symptoms caused by this virus are actually the result of alteration of miRNA-guided functions by HC-Pro [53]. Analyses of *N. benthamiana* and *Nicotiana tabacum* plants expressing well characterized silencing suppressors derived from 6 different virus genera: P1 of *Rice yellow mottle virus* (RYMV) and *Cocksfoot mottle virus* (CfMV), P19 of TBSV, P25 of PVX, HC-Pro of PVY, 2b of CMV and AC2 of *African cassava mosaic virus* (ACMV) were performed. Interestingly, some of the silencing suppressors promoted specific phenotypic effects. HC-Pro caused a severely distorted growth habit in both *Nicotiana* spp., while the P25 protein of PVX caused a specific flower malformation and an early senescence phenotype in the *N. benthamiana* plants, although not affecting *N. tabacum*. Moreover, P19 expressing *N. benthamiana* plants had blistered leaf epidermis, hairy and serrated leaves in one of the lines and occasional bending of the flower stalks while in *N. tabacum* caused occasional malformation of flowers [54].

From almost all virus genus that infect plants, over 50 individual VSRs have been identified, strongly suggesting that successful virus infection requires their expression [47, 55]. The data available in the literature suggest that almost all viruses encode at least one suppressor, but in many cases, viruses encode more than one [47]. Virus-encoded suppressors seem to have primordial RNA-binding properties and often show preference for a specific RNA molecule [56, 57].

Studies comparing the activities of three distinct RNA silencing suppressors (P19, P21 and HC-Pro) *in vitro* and *in vivo* showed that all three silencing suppressors are dsRNA-binding proteins that interact physically with siRNA duplexes [57]. These three suppressors inhibit siRNA-directed target RNA cleavage in a *D. melanogaster in vitro* RNA silencing system. Moreover, P19, HC-Pro and P21 uniformly inhibit the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation through siRNA sequestration. None of these silencing suppressors inhibit pre-assembled RISC activity *in vitro* or *in vivo*.

Suppression can vary in degree and spatial detail ranging, for example, from suppression in all tissues of all infected leaves to suppression only in the veins of newly emerged leaves. This suggests that different suppressors might be targeted to different parts of the gene silencing mechanism [58] such as viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification [20]. For example, DCL function was indirectly blocked by the *Cauliflower mosaic virus* encoded P6 protein (viral translational transactivator protein). Transgenic P6 expression in *A. thaliana* reduced levels of DCL4-dependent 21-nt siRNAs (DCL4 converts non-coding RNA precursors into 21-nt tasiRNAs controlling developmental timing and organ polarity), similar to the effect of inactivating *A. thaliana* DRB4 (dsRNA-binding protein that physically and specifically interact with Dicers). Moreover, immunoprecipitation assays demonstrated that P6 physically interacts with DRB4 and that the ability of P6 to move within cellular compartments (nucleus and cytoplasm) was important for its silencing suppression activity [59].

Direct interaction between the V2 protein of *Tomato yellow leaf curl virus* (TYLCV) with SISGS3, the tomato functional homolog of the *A. thaliana* SGS3 protein (AtSGS3), which is a coiled-coil protein involved in siRNA signal amplification, interferes with RNA silencing. Furthermore,

the fact that a V2 mutant is unable to bind SGS3 loses its ability to suppress silencing indicates that the V2-SGS3 interaction may represent one of the key events in V2-induced RNA-silencing suppression in TYLCV-infected plant cells [60]. The HC-Pro protein can also act in a different manner as a viral suppressor of RNA silencing and might additionally be involved in sequestration of RNA duplexes. It was demonstrated that the FRNK amino acid motif in the central domain of HC-Pro is a probable point of contact involved in siRNA and miRNA duplex sequestration [61]. Mutations of FRNK (severe strains) to FINK (attenuated strains) caused attenuation of symptoms in squash leaves upon infection by *Zucchini yellow mosaic virus* (ZYMV). A decrease in miRNA accumulation was also observed. This raises the hypothesis that interactions of the FRNK box with different plant miRNAs directly influences their accumulation and endogenous regulatory functions, thereby contributing to symptom development.

Viral suppressors can interfere with the activity of methyltransferase HEN1. Studies performed with transgenic *A. thaliana* expressing P21 of BYV, P19 of tombusviruses, or P1/HC-Pro of TuMV demonstrated that, in addition to affect miRNA duplexes, these proteins interfere with short RNA stabilisation by blocking HEN1 methylation. Because miRNA precursors are supposedly cleaved in the nucleus, the fact that they are blocked for methylation by cytoplasmic viral suppressors could be explained in three ways: first, they may compete with HEN1 for substrate miRNA/miRNA* duplexes (sequestration by the suppressors could prevent HEN1 from interacting with duplexes or prevent HEN1 access to the 2' OH of the 3' terminal nucleotide); second, the viral silencing suppressors may bind directly to HEN1 and inhibit its activity, or interact with other factors required for HEN1 function; and third, viral suppressors may affect the subcellular localisation of HEN1 [62].

Diverse VSRs have been shown to bind AGO proteins. The first protein identified that binds AGO1 and AGO4 *in vivo* was 2b protein encoded by CMV. AGO1 is the major effector in both miRNA-directed and virus-induced RNA silencing. The 2b protein co-localises with AGO1 both in the cytoplasm and in the nucleolus [63]. The direct interaction of 2b protein with the PAZ and PIWI domains of AGO1, leading to the inhibition of its slicer activity, was verified by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays [64]. The 2b protein also interacts directly with AGO4 in the nucleolus [65]. AGO4 binds to 24-nt long repeat-associated siRNA (ra-siRNA) to participate in RNA-directed DNA methylation (RdDM) [66]. In this case, 2b competes with AGO4 for binding to 24-nt ra-siRNA, suppressing the DNA methylation mediated by AGO4 [65]. However, the effects of inhibition by 2b of the RNA-dependent DNA methylation phenomena on virus replication and spread remain to be investigated.

RISC activity also undergoes interference by viral suppressors. In agro-infiltrated leaves of *N. benthamiana* containing a GFP transgene, the P0 protein encoded by *Beet western yellows virus* (BWYV) was identified as having strong silencing suppressor activity [67]. Further studies on two *A. thaliana* infecting poleroviruses revealed that P0 contains a conserved minimal F-box motif that interacts with homologues of S-phase kinase related protein 1 (SKP1), a core subunit of the multi-component SCF family of ubiquitin E3 ligases. Mutations in the F-box motif interrupt the interaction between P0 and a SKP1 homolog in *N. benthamiana*, causing a decrease

in virus pathogenicity. In transgenic *A. thaliana* plants, expression of P0 caused severe developmental defects similar to those observed in mutants affected in miRNA pathways. Downregulation of a SKP1 homolog in *N. benthamiana* resulted in plant resistance to polerovirus infection. These results support a model in which P0 acts as an F-box protein that targets an essential component of the host post-transcriptional gene silencing machinery [68]. The results of subsequent investigation of the molecular mechanism by which P0 impairs PTGS showed that P0 expression does not affect the biogenesis of primary siRNAs, but it does affect their activity. Furthermore, in transformed *A. thaliana* plants P0 expression leads to various developmental abnormalities reminiscent of mutants affected in miRNA pathways. In this system, P0 expression is accompanied by enhanced levels of several miRNA-target transcripts, suggesting that P0 acts at the level of RISC. It was also revealed that P0 physically interacts with AGO1 to trigger AGO1 protein decay in plants [69].

There are also viral suppressors with unspecified function, such as the triple gene block protein 1 (TGBp1) of PVX, cysteine-rich proteins encoded by hordeiviruses, tobnaviruses, furoviruses, pecluviruses and carlaviruses, and the b protein of *Barley stripe mosaic virus* (BSMV) [52].

A number of assays have been established to verify the silencing suppression activity of a given viral gene and/or to identify multiple VSRs encoded by a single virus. In the case of plants, two assays have been widely used. The first is based on the transient, mixed expression of two transgenes in leaves co-infiltrated with two *Agrobacterium tumefaciens* strains. One strain induces RNA silencing of a reporter gene such as the green fluorescent protein (GFP) in the infiltrated leaf (local silencing); the subsequent spread of silencing into upper non-infiltrated tissues in transgenic plants that carry a homologous, integrated transgene (systemic silencing) is measured. The potential silencing suppressor is identified by the ability of the transiently expressed viral gene to enhance and/or sustain visibly higher levels of expression of the reporter gene. However, this assay was not capable of identifying several viral suppressors, including the CMV 2b gene, P25 of PVX, and the coat protein (CP) of *Citrus tristeza virus* (CTV) because they display very low suppression activity in agro-infiltration assays. Thus, their suppressor activities were confirmed by alternative approaches [40, 51]. The second type of assay, which is based on the use of grafting experiments, enables the identification of VSRs that are active against systemic silencing but not local silencing. In these experiments, selected transgenic plants stably expressing a candidate VSR are genetically crossed with a transgenic plant line that carries an autonomously silencing reporter transgene, such as 35S-GUS (β -glucuronidase) in tobacco line such as 6b5. Spreading expression of the viral protein suppresses reporter transgene silencing in the resulting F1 progeny (6b5xVSR) and can be determined by grafting reporter scions onto rootstocks made from the F1 plants. The reporter scions are from another transgenic plant line that expresses the reporter GUS transgene at high levels. The reporter gene becomes silenced a few weeks after grafting onto 6b5 rootstocks owing to the importation of a sequence-specific silencing signal from the silencing rootstock. Silencing does not occur in the scions if the VSR can inhibit either the synthesis of the mobile silencing signal in the F1 rootstocks or its export from rootstock to scion. Analysis of expression of the reporter transgene in the F1 progeny can also reveal whether the VSR suppresses local silencing, DNA methylation of the reporter transgene, or both [40].

Viruses are able to spread through infected plant cells using two ways of movement: cell-to-cell movement and long-distance movement. To combat this distribution, plants emit a silencing signal that spreads between cells. Because the effect of spreading is nt-sequence specific, the nature of the signal is likely to incorporate a siRNA or other RNA species [70]. Long-distance spreading depends on an RNA-dependent RNA polymerase (RDR), whereas short-distance movement of the signal does not [70]. A large number of suppressors of RNA silencing, including some effectors of long-distance virus movement through the phloem, are involved in these movements. For example, the P19 of tombusvirus is a suppressor of silencing that is not required for virus replication in isolated cells but is required for extensive viral invasion of systemic leaves [46]. P19 blocks the intercellular movement of the silencing signal by binding DCL4-dependent 21-nt siRNA [40]. Likewise, the potyviral HC-Pro and cucumoviral 2b proteins are suppressors [24] required for systemic virus infection [71]. *Cucumovirus* 2b protein inhibits the systemic movement of RNA silencing by either binding dsRNA/siRNA or inhibiting the slicer activity of AGO1 [23]. A site-directed mutation strategy involving the HC-Pro protein of TEV showed a correlation between silencing suppression and the ability to mediate long-distance virus movement [72]. Reevaluation of the role of *Tomato bushy stunt virus* (TBSV) P19 in the systemic invasion of *N. benthamiana* by the virus revealed a silencing suppression role for the structural protein (CP) of *Turnip crinkle virus* (TCV). The authors showed that a TBSV P19 deletion mutant, while capable of systemic movement in the plants, accumulated progressively less viral RNA in the systemic leaves due to loss of silencing suppressor ability. When the TBSV structural protein was replaced with TCV CP to create a chimeric virus, it restored close to wild type levels of virus accumulation in systemic leaves. This result shows that both of these genes participate in efficient systemic TBSV infection and suggests that TCV CP not only provides structural protein but also complements the silencing suppressor function of TBSV P19. Moreover, it is also suggestive that assembled virions are likely important for the effective unloading of viruses from the vascular system into the leaf mesophyll. This work provides direct evidence that P19 primarily enhances systemic invasion by suppressing the host PTGS responsible for eliminating viral RNAs in the infected plants [51]. The P25 protein of three 'triple gene block' (TGB) proteins of potexviruses is another example of suppressor that is required for cell-to-cell movement of the virus; it is an RNA helicase that moves cell-to-cell and modifies plasmodesmata [73, 74].

As previously mentioned, in many cases viruses encode more than one VSR. A good example is the closterovirus CTV, which encodes three different silencing suppressors [P23, coat protein (CP) and P20] and exhibits distinctive features related to silencing suppression. CTV has a plus-strand RNA genome of approximately 20 kilobases (kb) in length. Its P20 and P23 proteins, but not CP, suppressed RNA silencing in an agro-infiltration assay and were able to reverse transgene silencing. In addition, P20 and CP, but not P23, prevented intercellular silencing spread. It was suggested that P23 appears similar to HC-Pro because, although both are potent suppressors of intracellular silencing, neither prevents intercellular silencing or DNA methylation of the target transgene. On the other hand, the suppressor activity of P20 shares features with silencing suppression mediated by CMV 2b, i.e., both are potent but incomplete suppressors of intercellular silencing, and suppression of intercellular silencing is not associated with reduced DNA methylation of a target GUS transgene. In the case of CP,

suppression of intercellular silencing spread is not associated with suppression of intracellular silencing, unlike P20, CMV 2b and P25 of PVX, which are known to interfere with intercellular silencing [75].

Silencing suppressors may confer biased protection against viral RNA and subviral parasites. It was shown that the P1/HC-Pro proteins of TEV caused an increase in the accumulation of the negative strand viral RNA of PVX [44], suggesting that negative-strand viral RNAs are more susceptible to the RNA silencing based host defence while positive-strand viral RNAs are better protected.

Viral infection is greatly influenced by changes in environmental temperature. A general explanation for this phenomenon is that RNA silencing-mediated plant defence is temperature dependent. Generally, at low temperature (15°C), both virus- and transgene-induced RNA silencing is inhibited; the level of virus- or transgene-derived siRNAs is dramatically reduced, leading to enhanced host susceptibility to virus infection and loss of silencing-mediated transgenic phenotypes. In contrast, with increasing temperature (27°C), RNA silencing is activated, and the amount of siRNA gradually increases. However, accumulation of miRNAs, which play a critical role in developmental regulation is temperature independent [76]. Because the replication of viruses does not appear to be disproportionately inhibited by higher temperature, one can assume that the activity of viral silencing suppressors is relatively constant over the temperature range that permits viral systemic infection. Thus, the level of silencing suppression activity should be relatively constant over this temperature range and therefore more readily overcome at higher temperature due to enhancement of the RNA silencing pathway. Conversely, it can be predicted that at low temperatures, the weakened RNA silencing would be more readily overcome by viral silencing suppressors [51].

3.1. Mechanisms of suppression

3.1.1. Viral suppressors

HC-Pro

As described above, HC-Pro was one of the first viral proteins to be identified as a suppressor of transgene- and virus-induced RNA silencing [77, 78]. HC-Pro is produced by plant viruses of the Potyvirus genus, family Potyviridae, the most important group of plant pathogenic viruses. HC-Pro has attracted renewed attention in recent years due to its multifunctionality and involvement in different steps of the potyvirus life cycle [79, 80]. Potyviruses, like the majority of plant viruses, have a single-stranded, positive-sense RNA genome that consists of approximately 10,080 nucleotides and is polyadenylated on its 3' end and surrounded by a capsid [81]. The genomic RNA has a single ORF located between two noncoding regions, which are called 5'NTR and 3'NTR (non-translated region). Translation of the single ORF produces a polyprotein with molecular weight between 340 and 370 kilodaltons (kDa). This polyprotein is cleaved into functional proteins of the virus through the proteolytic activity of three proteases of viral origin (P1, HC-Pro, and NIa), resulting in 8-10 final products. HC-Pro and P1 act in *cis*, each carrying out its own cleavage, and NIa catalyses its own cleavage and that of six other polypeptides [80, 82].

A typical potyviral HC-Pro consists of approximately 460 amino acids and has a molecular weight of approximately 52 kDa, it performs a surprisingly large number of functions; in fact, among proteins produced by potyviruses, it is the protein for which the greatest number of features has been described [80]. Apart its role as silencing suppressor, HC-Pro plays several other roles as a proteinase, participates in aphid transmission, acts as an auxiliary viral replication factor and participates in virus cell-to-cell and long distance movement [44, 80, 83-88].

HC-Pro can be divided into three functional regions, a N-terminal region that is essential for transmission, a C-terminal region that is responsible for its proteolytic activity and a central region involved in all other functions described. However, recent studies show that most functions overlap along its primary amino acid sequence [89].

Concerning its ability to suppress silencing, HC-Pro was shown to restore GFP expression in both old and new leaves of post-transcriptionally silenced transgenic plants (reviewed in [90]). HC-Pro suppresses PTGS via interaction with one or more cellular proteins that are either components of the silencing machinery or regulators of the silencing pathway. Studies have shown that HC-Pro interferes with the accumulation of the small RNAs associated with silencing. These small RNAs derive from the cleavage of dsRNA by Dicer and HC-Pro may target the process at this step [91, 92]. Dicer could be blocked by HC-Pro in several ways: HC-Pro can prevent the small RNAs from being produced by preventing the enzyme from binding to the dsRNA template, thus blocking the cleavage step; alternatively, it could block at a step downstream of cleavage, preventing incorporation of siRNAs and making the silencing unstable. A model in which HC-Pro suppression of PTGS occurs upstream of accumulation of small RNAs has been proposed [93]. Furthermore, HC-Pro has been shown to transactivate the replication, and enhance the pathogenicity, of a broad range of heterologous plant viruses [44].

Since HC-Pro prevents accumulation of siRNAs of silenced genes, it prevents silencing in a universal manner; however, in tobacco HC-Pro was shown to increase the *in vivo* accumulation of several miRNAs, namely, *miR167*, *miR164* and *miR156* [94]. In addition, HC-Pro is not able to inhibit the systemic silencing signal, suggesting that HC-Pro works downstream from production of the systemic signal. It was suggested that HC-Pro works at the point of RISC assembly and that it most likely unwinds miRNA duplexes [95]. The specificity of HC-Pro binding to small RNAs was tested by the use of synthetic 21-nt or 24-nt siRNA duplexes and 19-nt or 21-nt blunt-ended RNA duplexes. The results showed that HC-Pro binds with specificity to 21-nt siRNA duplexes. Moreover, it has higher binding affinity for duplexes with 2-nt overhangs than for small single-stranded RNAs or blunt-ended small RNA duplexes [57].

HC-Pro is often mentioned in conflicting reports in the literature that address the relationship between PTGS and DNA methylation. In some instances, a good correlation between HC-Pro suppression of PTGS and the decrease of DNA methylation is observed. When introduced in a GUS-silenced tobacco line, for example, HC-Pro affected the accumulation of small RNAs of the PTGS pathway and reduced methylation of the corresponding GUS locus [96], suggesting that silencing is directly related to DNA methylation. In contrast, another study showed that HC-Pro increased DNA methylation of the promoter sequence of a silenced DNA target gene

when silencing was induced by dsRNA directed against the promoter region [97]. In the same study, it was shown that the amount of promoter-derived siRNA molecules increased five-fold in the presence of HC-Pro.

P25

P25, the product of the first gene of the "triple gene block" (also known as TGBp1) encoded by PVX, is an RNA helicase that induces plasmodesmal gating. P25 promotes cell-to-cell movement of the virus and is also associated with suppression of RNA silencing [98]. P25 was one of the first VSRs to be identified and shown to inhibit transgene sense- or dsRNA-induced RNA silencing. The mechanism of action of P25 contrasts with that of HC-Pro, which acts at a downstream cellular signalling step. For this reason, mixed infections of PVX with any other potyvirus (which encode HC-Pro) normally result in synergistic disease [86]. Such diseases are common and often occur in plants as a result of the interaction between viruses that suppress silencing at various points of the silencing pathway [99, 100]. P25 is the only suppressor so far described that affects gene silencing but fails to recover silenced GFP expression post-transcriptionally. Surprisingly, P25 does not interfere with silencing of viral-induced sites [101].

Antiviral silencing suppression by P25 is required for cell-to-cell movement of the virus but has no apparent effect on viral accumulation in protoplasts, unlike most known VSRs such as cucumoviral 2b, tombusviral P19, and carmoviral P38. The analysis of a variety of random mutants of P25 showed that all produced defects in the suppression of silencing and in cell-to-cell movement. Some P25 mutants, defective in suppression activity, could be supplemented by heterologous viral suppressors. However, other mutants showed silencing suppression activity but were not functional as movement proteins. These results demonstrate a crucial role for P25 in cell-to-cell movement of the virus and also suggest the importance of an additional function of P25 in these activities [98].

P25 exhibits strong activity against silencing produced by both sense and inverted repeat transgenes in leaves of *N. benthamiana* and in transgenic *A. thaliana*. These observations indicate that P25 targets a downstream step in the synthesis of dsRNA [98, 101, 102]. As reported above, P25 inhibits systemic silencing but does not inhibit gene silencing induced by viruses in locally infected leaves. Moreover, it reduces the accumulation of both primary and secondary siRNAs but has no effect on the accumulation of endogenous miRNA and siRNA. It has been speculated that P25 does not interfere with programmed RISC [101, 102].

Co-immunoprecipitation assays indicate that P25 interacts with various members of the AGO family, including AGO1, AGO2, AGO3 and AGO4, but not AGO5 or AGO7. Furthermore, P25 promotes the proteasome-dependent degradation of AGO1 [103], indicating that its suppressor activity is dependent on AGO1 degradation. It is not currently known whether P25 inhibits the local motion silencing signal targeting AGO1 to promote movement of the virus [104].

P19

The tombusviral 19 kDa protein, P19, is one of the best studied viral silencing suppressors. The hypothesis that P19 is a viral suppressor arose in 1995 when Scholthof and col-

leagues reported that the 19 K protein of TBSV is a pathogenicity determinant. TBSV is a virus with a broad host range that induces a variety of symptoms in different hosts [105]. This virus contains a single copy of a positive-sense single-stranded RNA genome of 4800 nucleotides [106]. Five major ORFs are encoded by the TBSV genome. Two small nested genes located near the 3' terminus of the genome are expressed via a second subgenomic mRNA that directs synthesis of a 22 kDa protein (P22) and a 19 kDa protein (P19) [107]. P19 can act both as an elicitor of the HR response in *N. tabacum* or as an inductor of systemic necrosis in *N. benthamiana* [108]. Due to its activity as a host-specific symptom determinant, the P19 was suggested to play a role in overcoming host defence systems [108, 109]. This hypothesis was confirmed by the inoculation of silenced GFP tobacco plants with a recombinant PVX carrying the 19K coding region [48]. In these assays, plants infected with PVX-19K showed severe symptoms two weeks after inoculation while those already infected with PVX-m19K (with a nontranslatable P19 RNA) showed mild mosaic symptoms. Suppression of silencing occurred in PVX-19K infected plants but was manifested only in new emerging tissues and was most pronounced in the veins. However, symptoms of PVX-19K were visible on all areas of the leaves. Interestingly, P19 restores GFP expression in PTGS inactivated transgenic plants only around the veins of new emerging leaves [46], even though TBSV accumulates to a high concentration in the whole leaf [108].

Several recent studies report a breakthrough in understanding the molecular mechanism of the suppressor activity of P19. This suppressor prevents incorporation of siRNAs into effectors such as the RISC complex by binding specifically to 21-nt siRNAs *in vitro* and *in vivo* [110, 111]. This model was confirmed by three-dimensional structural resolution of the P19-siRNA complex showing that P19 acts as a clamp for dsRNA binding to the ends of the siRNA duplex [112, 113]. However, it was also reported that after the RISC complex is formed, P19 is no longer effective, being unable to bind to siRNA and miRNA [57].

P19 inhibits the onset of transgene-induced local and systemic silencing [110]. It does not interfere with the location of virus-induced silencing, but it can prevent systemic silencing. It was suggested that P19 depletes PTGS generated 21-25-nt dsRNAs, thus inhibiting the development of transgene-induced silencing and preventing the production of active signal complex. Interestingly, transgenic plants expressing biologically active P19 showed an altered phenotype, suggesting that the P19-targeted PTGS pathway might also have a role in developmental regulation. Low level expression of P19 altered leaf morphology in transgenic plants. In addition to leaf curling, some severely affected plants also showed delayed appearance of developed secondary stems. Although it is possible that developmental abnormalities in transgenic plants are not related to the silencing suppressor activity of P19, these findings are suggestive that the P19-targeted PTGS pathway plays a role in plant development.

The silencing suppressor activity of P19 is also observed in other hosts. Since siRNA binding by P19 does not require host factors *in vitro*, and that these short RNAs are specificity determinants of silencing effector complexes, P19 could be used to inhibit RNA silencing in heterologous systems, including *D. melanogaster*, worms and mammals [110].

The P19 protein of *Cymbidium ringspot virus* (CymRSV), a relative of the TBSV P19 protein, specifically binds to siRNAs *in vitro*, and two reports show co-crystallisation of P19 homodimers with siRNA [112, 113]. P19 also binds RNA duplexes with a blunt end and with a 2-nt 3' overhanging end. In animals, Dicer digests from the ends of long dsRNAs [114] and therefore might produce long dsRNAs with 2-nt 3' overhangs. Although it is possible that P19 competes with Dicer-related proteins for the 2-nt 3' overhanging ends of long dsRNAs, the high level of 21-25-nt RNAs in CymRSV infected cells suggests that P19 fails to suppress Dicer-like activity.

A study using mutants of CymRSV demonstrated that lack of P19 suppressor did not affect most basic viral functions, including genome replication, cell-to-cell movement and phloem long-distance transport [109]. In contrast, the systemic infection of plants inoculated with a silencing suppressor mutant of CymRSV was seriously compromised and led to the development of a recovery phenotype, suggesting that P19 suppressor targets a non-cell-autonomous step of RNA silencing [110].

In CymRSV infected plants, siRNAs are present in P19–siRNA complexes, while in plants infected with the P19-defective mutant in which P19 was inactivated (termed Cym19stop), siRNAs were found as free molecules. P19 apparently does not affect virus-induced cell-autonomous silencing because CymRSV and Cym19stop viral RNAs, as well as siRNAs derived from these viruses, accumulate to the same levels in transfected single cells [110]. In addition, the P19 protein was shown to repress the accumulation of all size classes of siRNA produced in agroinfiltration assays [110, 115]. While CymRSV infects *N. benthamiana* systemically and typically kills the host within two weeks, infection with the mutant virus results in a recovery-like phenotype showing mild symptoms and low virus levels in the upper leaves [116]. Moreover, P19-deficient and wild type CymRSV accumulate at similar levels in both protoplasts and inoculated leaves, indicating that this protein does not prevent RISC from degrading viral RNAs by sequestering viral derived siRNAs (vsiRNA) [37]. In systemic leaves, P19-deficient CymRSV accumulates only in the vascular bundles and exhibits defects in invading the surrounding tissues suggesting that blocking the local movement of RNA silencing by P19 is essential for systemic virus infection [117].

In this context, studies have shown that P19 specifically sequesters the DCL4-dependent 21-nt siRNAs derived from transgene RNAs; these siRNAs normally move into the neighbouring recipient cells and act as a silencing signal [118]. These results imply that P19 promotes systemic virus infection by sequestering vsiRNA, thus preventing the signal for RNA silencing from spreading out of vascular bundles into neighbouring cells [104]. Therefore, when P19 is absent, the systemic signal moves faster than the virus in the infected plant, thereby establishing antiviral silencing in cells ahead of the infection front. As a result, any virus entering these cells is immediately controlled by silencing-mediated RNA degradation. In conclusion, the presence of the silencing suppressor is essential for the development of systemic virus infection [37].

3.1.2. Endogenous suppressors

In addition to the numerous viral suppressors of RNA silencing, endogenous RNA silencing suppressors have also been reported in eukaryotes. The endogenous suppression of RNA

silencing negatively controls the presence of siRNAs and miRNAs in different ways. The generation and control of such siRNAs and miRNAs are essential for normal development of plants and animals [119-121].

The first endogenous RNA silencing suppressor was identified in *N. tabacum* and was named rgs-CaM (regulator of gene silencing CaM). This protein was found in a screen for proteins interacting with the viral suppressor HC-Pro. Expression of rgs-CaM can be induced in leaves of *N. tabacum* when HC-Pro is expressed either from a transgene or from infection with a virus that encodes HC-Pro. When expressed at high levels in *N. benthamiana*, rgs-CaM suppresses both PVX-induced gene silencing and sense transgene-mediated PTGS (S-PTGS) [49]. A recent study, however, demonstrated that rgs-CaM is not an endogenous suppressor of silencing [122]. In fact, this protein acts as an endogenous pattern recognition receptor able to bind to several viral silencing suppressors through their RNA-binding domains. Thus, rgs-CaM activity confers a countermeasure against viral suppressors.

In addition, an inhibitor protein of *A. thaliana* RNase L activity, called RLI2, was also described as having a silencing suppressor activity when expressed at high levels in transgenic *N. benthamiana* [123]. Another known endogenous suppressor, the *A. thaliana* exoribonuclease XRN4, suppresses silencing by promoting the degradation of aberrant, uncapped RNAs that constitute possible templates for an RNA dependent RNA polymerase (RdRP) pathway involved in silencing. These aberrant molecules represent important activators of silencing, serving as templates for the production of new dsRNAs by the action of the RdRP. Indeed, mutations in the gene *xrn4* promote RdRP-dependent silencing [124] and lead to over-accumulation of miRNA-generated cleavage products [125]. Three other suppressor proteins, the exoribonucleases XRN2, XRN3 and FRY1, were identified in *A. thaliana*, thus complementing existing knowledge of the suppression of silencing involving XRN4 [126]. While XRN4 is cytoplasmic, XRN2 and XRN3 are nuclear exoribonucleases [127]. XRN2 and XRN3 contribute to the suppression of RNA silencing by degrading miRNA-derived loops excised during miRNA maturation in the nucleus. In contrast, XRN4 acts exclusively in the cytoplasm, promoting degradation of uncapped messages such as miRNA target cleavage products [124-126]. Fry1 acts as a fine-tuning modulator of the activities of XRN2, XRN3 and XRN4 [126]. Interestingly a family of exoribonucleases known as small RNA degrading nucleases (SDN) degrades mature miRNA molecules in *A. thaliana*, acting specifically on single-stranded miRNAs [121].

3.1.3. Modifications of the host transcriptome

Viruses can counterattack RNA silencing immunity not only by acting directly on gene products that are required for silencing *per se* but also by inducing stress and plant defence responses that interfere with antiviral silencing [128, 129]. An interestingly example include the RAV2/EDF2 protein, which belongs to the RAV/EDF family of transcription factors. This protein is required for suppression of silencing by potyvirus HC-Pro and carmovirus P38, two viruses that belong to unrelated families. RAV2 is required for suppression of silencing in a direct way that involves blocking the activity of primary siRNAs as well as indirectly by its effects on upregulation of some stress and defence response genes [128].

The induction of biotic or abiotic stress activates other defence responses that can divert the host from antiviral silencing [129]. Therefore, RAV2 is a critical control factor for carmovirus and potyvirus suppressors [128].

Other viruses make use of alternative mechanisms for suppression of silencing. The TrAP geminiviral protein AC2 upregulates a gene coding for the cold- and abscisic acid-inducible protein KIN1 as well as five additional known or putative cold-regulated genes [50]. As already mentioned, the efficiency of RNA silencing is dependent on temperature; at low temperatures, inhibition of silencing occurs and the plant becomes susceptible to viral infection [76]. The inhibition of silencing at low temperature is a pathway used by AC2 to accomplish the suppression of silencing [50]. Another strategy exploited by geminivirus is up-regulation of an endogenous RNA silencing suppressor, Werner exonuclease-like 1 (WEL1), which is mediated by AC2. Interestingly, the related proteins MUT-7 (mutate 7) and Werner syndrome-like exonuclease (WEX) have been identified as positive regulators of RNA silencing in *C. elegans* and *A. thaliana*, respectively [130, 131]. Thus, AC2 up-regulation of Wel-1 results in interference with, or competition for, factors that are required for normal WEX function. Transient expression of a WEL-1 transcription unit is sufficient to suppress RNA silencing in *N. benthamiana* [50].

4. Applications

4.1. Virus-Induced Gene Silencing (VIGS)

Virus-induced gene silencing (VIGS) is a technique derived from the knowledge of RNA silencing. It uses recombinant viruses to specifically reduce or knock-down endogenous gene activity; it is based on post-transcriptional gene silencing (PTGS) [132]. When used to infect plants, recombinant viral vectors carrying segments of host genes produce siRNAs that are specific to host mRNA. The RISC complex mediates the degradation of target host mRNAs, leading to downregulation of gene expression. Thus, the infected plant has a phenotype similar to a loss-of-function mutant of the gene of interest [133].

VIGS is used as a tool for turning down host gene expression, especially in plants. In principle, a plant gene of interest can be silenced by infecting the plant with a viral vector that has been modified to express a nucleic acid sequence homologous to the host gene. As a proof of concept, several endogenous genes have been silenced using VIGS. The plant gene *phytoene desaturase* (PDS), a regulator of carotenoid biosynthesis, was silenced in *N. benthamiana* plants by the use of a recombinant TMV vector. As a result, degradation of the host PDS mRNA and resultant alterations in the pigment synthesis pathway were observed [133].

There are four main reasons for the popularity of VIGS. First, the methodology is simple, often involving agroinfiltration or biolistic inoculation of plants. Second, the results are obtained rapidly, typically within two to three weeks of inoculation. Third, the technology bypasses transformation steps and hence is applicable to a number of plant species that are recalcitrant to transformation. Fourth, the method has the potential to silence multi-copy genes [134].

Efficient silencing depends mainly on the choice of VIGS vector. There are many factors to be considered when choosing the virus to be used for VIGS. Among the factors to be considered are (1) the virus must produce few or no symptoms during infection, thereby facilitating easy visualisation and interpretation of the mutant phenotype; (2) it must induce persistent silencing, thus viruses with strong silencing suppressors are to be avoided because they can interfere with the establishment of silencing; (3) it is advantageous to have infectious cDNA clones of the virus for cloning purposes; and (4) the virus must retain infectivity after insertion of foreign DNA. The virus should also show uniform spread, infect most cell types including the meristem, and preferably show a broad host range [133].

Several RNA and DNA viruses have been modified to create VIGS vectors. The gene to be silenced is cloned in an infectious derivative of a viral DNA (DNA virus-based vectors) or cDNA (RNA virus-based vectors) derived from viral RNA. Plant inoculation with viral vectors is most commonly achieved via *A. tumefaciens* infection, but can also be achieved by mechanical inoculation of *in vitro* synthesized transcripts, or for DNA-based vectors, by biolistic delivery methods. During the course of viral infection, either double-stranded RNA or RNA with a high degree of secondary structure is often produced; both of these are efficient initiators of RNA silencing directed against the infecting viral RNA. Other factors that play an important role in gene silencing in VIGS are the orientation of the insert (inverted repeats are more efficient than antisense orientation, which, in turn is more efficient than same sense orientation) and systemic spread of the silencing effect (the silencing signal is believed to spread independently of the VIGS vector to other parts of the plant) [134]. More than 30 VIGS vectors have been developed, and these vectors have been widely used to study the functions of genes involved in basic cellular functions, metabolic pathways, development, plant-microbe interaction, and abiotic mechanisms [132].

The first viral vector used for VIGS was TMV. Shortly thereafter, another vector was produced based on another RNA virus, PVX carrying a cDNA fragment derived from the *PDS* gene [135]. However, although these first vectors were effective, they have intrinsic disadvantages. First, the VIGS phenotype is superimposed and sometimes complicated by chlorosis, leaf distortion and necrotic symptoms of virus infection. A second disadvantage of these viral vectors is their inability to invade every cell, such that cells in which the target gene is not silenced may obscure VIGS phenotypes [136]. A novel VIGS vector based on TRV was then established. TRV was shown to induce more efficient silencing of transgenes and endogenous genes. It could spread more vigorously throughout the entire plant, including meristem tissue, and the symptoms induced by TRV are much milder than those produced by other viruses [136].

A primary limitation of VIGS technology is that a viral vector can be used only in plants that are hosts of the virus used. The first VIGS vectors (e.g., PVX) do not infect the model plant *A. thaliana*. Therefore, new vectors such as the TRV-based vector [136] were developed to overcome this difficulty. TRV is also one of the few viruses that have been modified into a highly efficient cloning and expression system for use in large-scale functional genomics screening. TRV vectors can induce VIGS in a number of solanaceous hosts like *N. benthamiana*, tomato, potato, pepper, petunia, poppy (Eudicot species), and the model system *A.*

thaliana (family Brassicaceae) [133, 137]. VIGS vectors have been applied not only in dicotyledonous plants but also in monocotyledonous plants. For this, a modified VIGS vector based on *Brome mosaic virus* (BMV) was developed and validated in barley, rice and maize [138].

The VIGS system can be helpful in assessing gene function, especially for genes that cause zygotic/embryonic lethality when mutated and in species that are recalcitrant to genetic transformation. As aforementioned, it can be designed to silence multiple members of a gene family, thereby circumventing the problem of functional redundancy of genes [133].

4.2. Use of viral suppressors

The discovery of RNA silencing, and its derived technology (RNA interference; RNAi), has increased our knowledge of gene regulation and function. RNAi opened up novel avenues in biology, making it possible to develop fascinating strategies for application in genetic analysis, plant protection, and many other areas related to crop improvement [139]. In this context, a large number of silencing suppressor proteins have been described, and the discovery of the molecular basis of silencing suppression has inspired new concepts about the molecular basis of symptoms caused by viruses in plants [37].

Many biotechnological applications involving plants require high levels of protein expression. Generally, stably transformed plants are the preferred platform for large-scale production. To try to increase expression levels, transgenic lines that encode a replicating RNA virus vector carrying a gene of interest, a technology coined 'amplicon', have been exploited. The rationale of this method involves increasing the accumulation of the product of interest through transcription of an amplicon transgene that initiates viral RNA replication and gene expression. However, the strategy failed because the transformants consistently exhibited RNA silencing of the amplicon transgene [140]. The viral dsRNA replication intermediates produced in every cell of the transgenic plants were recognised as potent triggers of the silencing-based defence mechanism that is normally elicited in the course of natural infections. Based on those findings, it was subsequently reasoned that co-expression of viral suppressors might prevent this adverse response and permit the high levels of gene expression initially envisioned with the use of amplicons [141].

To test this idea, in reference [94] crossed transgenic tobacco plants expressing TEV HC-Pro with amplicon lines designed to express a GUS reporter gene from the PVX genome. Pairing the suppressor and the amplicon locus resulted in a dramatic increase in virus accumulation and gene expression such that the leaves of mature plants accumulated the GUS protein up to 3% of total soluble protein. Remarkably, in spite of high virus accumulation, the plants did not suffer from viral disease and remained symptomless.

As opposed to stable, transgenic expression, transient expression is of interest for achieving expression of useful proteins. In plants, recombinant strains of *A. tumefaciens* can be used for transient gene expression. In principle, this system could allow high levels of gene expression; however, its utility has thus far been limited because ectopic protein expression usually ceases after 2–3 days [141]. RNA silencing is, in fact, a major cause of this lack of efficiency. It was therefore anticipated that co-delivery of *A. tumefaciens* cultures with silencing suppressors

would enhance expression of the genes of interest [101]. Studies with the P19 protein of TBSV were among those that provided the best results. Expression of a range of proteins was enhanced 50-fold or more in the presence of this suppressor, and experiments with GFP indicated that the co-infiltrated tissues accumulated the protein up to 7% of total soluble protein [142]. Due to its simplicity and rapidity, the P19-enhanced expression system is currently used in industrial production as well as used as a research tool for the isolation and biochemical characterisation of a broad range of proteins without the need for the time-consuming regeneration of stably transformed plants [141].

5. Perspectives

The molecular basis of the silencing suppression of VSR proteins is quite complex and is currently incompletely understood. By the way, the discovery of the mode of action of different viral suppressors has demonstrated the existence of a complex interaction between VSR and plant silencing-regulated networks. For example, in addition to sequestering siRNA duplexes, the P19 protein of tombusviruses specifically controls antiviral AGO1 expression through enhanced miR168 expression, which arrests AGO1 translation [23]. It is likely that many other VSRs interact in diverse ways with RNA-silencing pathways. Many of these interactions remain to be discovered, and there are several gaps in our knowledge regarding the effectors of plant silencing machinery. Until very recently, the mechanisms of plant si/miRNA RISC assembly or the components of the plant RISC, which may also be potential targets of VSRs, were little known. The recently developed system of plant *in vitro* RISC [143] will likely accelerate the exploration of plant RISC assembly and RNA-targeting mechanisms mediated by this effector. This system will enable exploration of the mechanisms by which VSRs interact with one or more of the RISC components and prevent its assembly.

More information about the replication, subcellular localisation and regulation of the expression of viral genes, including VSRs, is required so that we may better understand the molecular mechanisms of VSR-mediated silencing suppression for the many plant viruses for which they are still not known. Because many VSRs have multiple functions in the virus life cycle, separate analysis of their silencing suppressor activities can lead to misinterpretations; thus, it is essential that VSRs be studied in their natural virus backgrounds [23].

Although common mechanisms of silencing suppression exist, there is also great variation in suppression mechanisms, likely driven by evolution and fitness, this variation has yielded viral strains with different properties. It is likely that additional differences will be found when plant viruses and their suppressors are tested in several plant species. This will provide us with a greater understanding of the parameters associated with the natural host range of a virus and may possibly lead to new strategies for crop protection [52].

Some of the already well described VSRs can be used as powerful tools for better understanding silencing pathways because they target specific steps of silencing machinery. Indeed, the P19 protein was recently used to demonstrate that siRNA duplexes function as mobile

silencing signals between plant cells, in addition that P1, P38 and P0 proteins may prove to be powerful tools for studying the still unknown components of RISCs [118].

6. Conclusions

The discovery of the mechanisms involved in RNA silencing and in silencing suppression by virus has helped researchers to investigate several aspects of the plant-virus co-evolution. Our understanding of the underlying mechanisms served as a basis for the development of different technologies aiming plant manipulation to generate novel traits or pathogen resistance. Elucidation of the mode of action of different plant viral suppressors provided fundamental contributions to the comprehension of the RNA silencing phenomena. In this context, strategies exploiting viral suppressors to prevent the occurrence of RNA silencing in genetically engineered plants have been developed [144]. High levels of transgene expression should be expected through the implementation of such strategies. This review has addressed some important topics in the areas of RNA silencing and silencing suppression. Ongoing studies aimed at further clarification of the main points of these processes are currently being conducted by many different groups.

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