Update on Expression of RNA Interference Suppressors

Heterologous Expression of Viral RNA Interference Suppressors: RISC Management

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This Update reviews how virus-encoded suppressors of RNA silencing are currently being used in certain areas of plant research and biotechnology. Keeping within the scope of this special Focus issue, an emphasis is placed on the use of gene vectors for expression of suppressors in plants. Toward the end of this review, a discussion is presented on how the choice of vector system may influence the functionality of suppressors and the occurrence of side effects. The purpose of this brief review is to illustrate, for a general audience of biologists, how individual properties of viral-encoded suppressors might affect their performance in plants when expressed by heterologous vectors.

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. RNA silencing occurs in various organisms, including plants, single-celled algae, fungi, Caenorhabditis elegans, Drosophila melanogaster, and mammalian cells (Lindbo et al., 1993; Fire et al., 1998; Zamore et al., 2000; Bernstein et al., 2001; Baulcombe, 2004; Ding and Voinnet, 2007; Molnar et al., 2007). Many of the biochemical details remain to be determined for different organisms, but, as is presently understood, RNA silencing proceeds according to conserved principles (Fig. 1).

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. As shown for Arabidopsis (Arabidopsis thaliana), prominent, hierarchical, and perhaps redundant antiviral roles are played by several DICER-LIKE (DCL) enzymes, notably DCL2 and DCL4, and perhaps DCL3 (Deleris et al., 2006; Moissiard and Voinnet, 2006; Diaz-Pendon et al., 2007; Moissiard et al., 2007). The DCL-containing complex cleaves dsRNA into duplex short interfering RNAs (siRNAs) of approximately 20 to 25 nucleotides (nt); most characteristic for antiviral RNAi are 21- to 22-nt siRNAs. Some of the siRNAs might play a role in amplification of dsRNA by plant-encoded RNA-dependent RNA polymerase (RdRp; Deleris et al., 2006; Moissiard and Voinnet, 2006; Moissiard et al., 2007; Fig. 1). Most importantly, siRNAs are key to the specificity of RNAi in that one of the strands of the siRNA duplex is incorporated into another effector unit referred to as the RNA-induced silencing complex (RISC; Fig. 1). The precise biochemical composition of RISC involved in antiviral RNAi in plants remains to be determined (Omarov et al., 2007), but most likely involves the participation of one or more Argonaute-like (Ago) proteins (Baumberger and Baulcombe, 2005). 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 (Fig. 1); in case of minor mismatches, translational repression might occur instead of RNA degradation (Voinnet, 2005).

VIRAL-ENCODED SUPPRESSORS OF RNAi

A number of reviews were recently published on RNA silencing in plants and on the identification and characterization of viral-encoded suppressors (Roth et al., 2004; Qu and Morris, 2005; Scholthof, 2005; Voinnet, 2005; Bisaro, 2006; Li and Ding, 2006; Ding and Voinnet, 2007). A now commonly accepted interpretation of the major role of RNA silencing is to provide antiviral defense. As a defensive countermeasure, viruses have evolved the capacity to encode suppressors of RNAi. This was first shown to be the case for plant viruses, and eventually invertebrate and vertebrate animal-infecting viruses were also found to encode suppressors of RNAi (Baulcombe, 2004; Li and Ding, 2005). The number of available plant virus-encoded suppressors is increasing (see aforementioned reviews), but this Update will focus on select examples of proteins with known differential biochemical properties.

In the late 1990s it was found that the HC-Pro protein encoded by members of the Potyviridae suppresses RNAi (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Since HC-Pro is a pathogenicity determinant, it seemed a reasonable hypothesis that other viral pathogenicity factors, with
Carmovirus suppressors are as follows: P38, not considered in this scheme. The virus genera encoding the selected chromatin modification and miRNA-mediated translation, but these are target. In general terms, RNA silencing also includes siRNA-mediated out the scheme with the same color and position as the protein-complex interact with proteins of the silencing machinery are indicated through-

AC4 has preference for single-stranded siRNA. Suppressors that (also) interference for siRNAs for those suppressors toward the bottom of the list. AC4 and (A) L2, (b) C1, satDNA (Qu and Morris, 2005; Voinnet, 2005; Bisaro, 2006; Li and Ding, 2006). Recently it was also observed that suppressors may prevent key siRNA methylation steps (Vogler et al., 2007). Irrespective of the precise mechanism, all virus-encoded suppressors appear to share the overall ability to compromise RNAi-mediated degradation of viral RNA genomes or transcripts (Scholthof, 2005; Voinnet, 2005; Bisaro, 2006; Li and Ding, 2006).

In hindsight, it seems plausible that the contribution of many pathogenicity factors to systemic transport is an indirect consequence of suppressor-mediated protection of viral transcripts. In the absence of the suppressors, the plants recover from infection, but in their presence viral load is sustained to permit an ensuing full systemic infection to become established and/or maintained (Scholthof, 2005).

GENE REGULATION AND PROTEIN BIOCHEMISTRY

A large number of different positive-sense RNA viruses encode an RdRp with common characteristics. These include a 5‘ proximal positioning of the RdRp open reading frame (ORF) on the RNA genome, a similar biochemical catalytic enzymatic activity, and conservation of specific protein motifs, which is indicative of common ancestry (Hull, 2002). However, this is not apparent when comparing those same virus families with regards to the ORFs for suppressors. Their ORFs are located or overprinted on various positions on the genome, and they are expressed by a plethora of gene regulatory mechanisms, including proteolytic processing of polyproteins, leaky scanning of ribosomes, synthesis of subgenomic mRNAs, and translational read-through. This suggests that the performance of suppressors is specifically adapted and optimized for each virus.

It is likely that the specific type of suppressor gene regulation for individual viruses is associated with the biochemical mode of action of the product (Fig. 1). For instance, a number of suppressors presumably aim to prevent the generation of siRNAs or to impede their incorporation into RISC (Lakatos et al., 2006; Merai et al., 2006; Fig. 1). Still others are known to operate upstream or downstream by compromising the activity of effector complexes, such as DICER or RISC (Deleris et al., 2006; Zhang et al., 2006; Fig. 1). It is also quite important to note that some suppressors are known to inactivate RNAi after its induction, while others can only prevent the onset of RNAi because once the process is initiated they are no longer effective (Li and Ding, 2006). Therefore, the gene regulation of suppressor then undetermined biochemical functions, would also suppress RNA silencing. The first evidence for this idea was reported in 1999 (Voinnet et al., 1999), and since then many plant RNA and DNA viruses have been found to encode one or more suppressors of RNAi (Voinnet, 2005; Bisaro, 2006; Li and Ding, 2006). Several of these newly discovered suppressors were previously documented to either directly promote long-distance virus transport or to enhance systemic invasion by some other unknown means (Scholthof, 2005). Many of the suppressors identified to date can be considered multifunctional because they also perform additional roles during the virus life cycle, for example, by functioning as a component of the replicase complex, as coat protein (CP), as movement protein (MP), or as an insect transmission factor (Scholthof, 2005). Recently it was found that specific plant viral RNA domains (rather than proteins) can also suppress RNA silencing (Takeda et al., 2005).

Experiments to determine the biochemical mode of action of virus-encoded suppressors are currently a highly active area of research. Thus far it is known that suppressors can target distinct processes in the silencing pathway, as illustrated for a few suppressors in Figure 1. Some seem to strictly and highly effectively target a specific process, like the sequestration of 21-nt siRNAs by P19 (Vargason et al., 2003; Ye et al., 2003). Others may target more than one event; for instance, 2b interacts with dsRNA and Ago1 (Fig. 1; Anandalakshmi et al., 2000; Lakatos et al., 2006; Merai et al., 2006; Zhang et al., 2006; Diaz-Pendon et al., 2007). Recently it was also observed that suppressors may prevent key siRNA methylation steps (Vogler et al., 2007). Irrespective of the precise mechanism, all virus-encoded suppressors appear to share the overall ability to compromise RNAi-mediated degradation of viral RNA genomes or transcripts (Scholthof, 2005; Voinnet, 2005; Bisaro, 2006; Li and Ding, 2006).

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expression during the life cycle of each plant virus may be related to the biochemical mode of action. This might ensure the correct timing and level of suppressor expression necessary for optimum performance in blocking one or more key steps in the RNAi pathway for a successful infection to occur.

Regardless of the precise gene regulatory and biochemical properties used to protect viral RNA from RNAi-mediated degradation during infection, the suppressors seem to share a general role in avoiding the programming of an antiviral RISC or to interfere with its activity (Fig. 1).

**SUPPRESSORS AS TOOLS IN BIOTECHNOLOGY**

An interesting and attractive property of suppressors for biotechnological applications is that their protective effect is not limited to their corresponding viral genome or mRNA transcripts. In fact, a key observation that led to the discovery of suppressors was that the *Potyvirus* HC-Pro protected a coinfected *Potato virus X* (PVX) from RNA silencing, resulting in enhanced PVX titers and thus providing a mechanism for a phenomenon long known as synergism (Rochow and Ross, 1955; Anandalakshmi et al., 1998). Moreover, it was found that suppressors not only protect viral genomes or transcripts, but also might offer general protection for any RNA against RNAi inside cells; some suppressors are even active in cell lysates (Qu and Morris, 2005; Voinnet, 2005; Li and Ding, 2006).

During the past two decades much effort in plant biotechnology has been focused on generating plants that transiently or transgenically express foreign proteins. However, the attained level of expression is often negatively influenced by the activation of gene-silencing circuits (Lindbo et al., 1993), including RNA silencing. It turns out that plants effectively employ this mechanism as a protection against foreign invading RNA, in this case mRNA expressed by transgenes. Since suppressors interfered with RNA silencing in a sequence-nonspecific manner, the question arose whether suppressors could be used to enhance foreign gene expression in plants. In support of this possibility, pioneering experiments revealed that viral-encoded suppressors effectively prevented RNA silencing in plants to allow high levels of transiently or transgenically expressed reporter genes (Voinnet and Baulcombe, 1997; Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Voinnet et al., 1998, 1999, 2003). The following sections highlight examples of how different plant gene vector systems have since been, or potentially could be, used for the heterologous expression of suppressors to alleviate the problems associated with RNA silencing against foreign gene expression in plants.

**VIRUS VECTORS**

**Viruses Expressing Homologous Suppressors**

Since viruses express suppressors in a regulatory manner that is likely evolutionarily optimized for their RNA-silencing activity, it seems logical to infect transgenic plants with a suppressor-encoding virus to avoid the negative effects of RNA silencing on expression of transgenes. For instance, it has been shown for members of the *Potyviridae*, *Bromoviridae*, and *Tombusviridae* that infection of plants with these viruses compromises the onset of RNA silencing (Hull, 2002). However, as indicated before, for certain viruses their suppressors only function when the virus is inoculated at the same time or prior to the introduction of the RNA-silencing inducer because they cannot reverse RNA silencing once it has been activated (Li and Ding, 2006). In addition, for suppressors that function to sequester dsRNAs or siRNAs, their effect on maintenance of transgene expression can be suboptimal when these proteins become saturated with viral-derived dsRNAs (e.g. siRNAs).

Regardless of these mechanistic limitations, from a more practical point of view, purposefully infecting plants with pathogenic viruses is often deemed undesirable for those working in plant research or biotechnology because it may result in severely diseased plants. Consequently, this type of homologous virus vector-mediated expression of suppressors is not generally used. Nevertheless, it could have future potential, for instance, if a (recombinant) virus with a broad host range is identified that causes minimal symptoms but expresses one or more virus proteins in a highly coordinated manner to achieve very potent suppression to nullify activated RNA silencing in transgenic plants.

**Virus Gene Vectors Expressing Heterologous Suppressors**

There are a number of plant viruses that have been used to various levels of success to vector foreign genes in plants (Pogue et al., 2002; Scholthof et al., 2002). Briefly, one needs a full-length version of the viral genome that can be amplified by routine molecular biology cloning methods and this amplified material should be infectious (either directly as DNA or upon transcription into RNA). The two most common strategies used to express foreign genes are gene replacement and gene insertion (Scholthof et al., 1996). In gene replacement, a viral ORF is identified whose encoding sequences and resulting product are both dispensable for infection and thus suitable for substitution by the ORF specifying the foreign gene product. Expression relies on the remaining intact endogenous gene regulatory mechanism, such as the transcription of subgenomic mRNAs, or proteolytic processing of a fusion protein. With gene insertion, an extra gene expression module is inserted encompassing the foreign ORF and regulatory sequences, such as a promoter for synthesis of an additional 3′ coterminal subgenomic mRNA, or additional cleavage sites for proteolytic processing. Consequently, with gene insertion the vector will express the foreign product in addition to all the endogenous viral proteins.

One of the first virus vectors used to express heterologous suppressors was a PVX gene insertion vector (Chapman et al., 1992; Voinnet and Baulcombe, 1997;
Expression of RNA Interference Suppressors

AGROINFILTRATION WITH BINARY VECTORS

Although plant cell expression vectors can be introduced by particle bombardment, presently the most commonly used technique for transient expression of suppressors in plants is agroinfiltration. In this Focus issue several transfer DNA (T-DNA) derivative binary plant gene expression vectors are described. In essence these represent plasmids that harbor a segment (referred to as T-DNA) located between two sequences (termed the left border and the right border) that is transferred from Agrobacterium tumefaciens into the plant nucleus (Tzfira and Citovsky, 2006). To enable expression of foreign genes, the T-DNA contains a convenient site for introducing a foreign insert (i.e. restriction enzyme, T/A, or TOPO sites, or recombination-compatible sequences). To ensure transcription of mRNA, a eukaryotic promoter is used that is recognized by the plant RNA polymerase II. For dicots this is often the 35S promoter of Cauliflower mosaic virus, while for monocots other promoters, such as a maize ubiquitin promoter, are preferred (see elsewhere in this issue). Transcription is terminated by means of a polyadenylation signal. Agrobacterium cultures containing the T-DNA vector are grown and treated with acetosyringone to activate the bacterial T-DNA transfer machinery and simple syringes are used to force aliquots of the cultures into the intracellular spaces of leaves. Through a process that is not yet precisely understood, either the bacteria or T-DNA/protein complexes penetrate into symplastic areas to enable Type IV secretion of the T-DNA for its transfer to the plant nucleus, followed by expression of the cargo gene encoding the suppressor in the infiltrated area.

Initial experiments to test for suppressor activity involved agroinfiltration of T-DNA/GFP-expressing constructs onto GFP-transgenic plants. In these tests, the expression of the extra GFP transcripts activated RNA silencing-mediated degradation of GFP mRNA, visible by disappearance of green fluorescence in noninfiltrated tissue (Voinnet and Baulcombe, 1997). Coinduction of suppressor-expressing constructs prevented systemic RNA silencing and, consequently, green fluorescence was maintained (Voinnet et al., 1999). However, since then it has been shown that GFP-transgenic plants are not necessary to illustrate the beneficial effect of suppressors on foreign gene expression. It was found that agroinfiltration of T-DNA/GFP onto normal nontransgenic plants initially yielded readily detectable transient levels of green fluorescence, but over a time span of 7 to 10 d the green fluorescence disappeared, as illustrated in Figure 2. Apparently the transiently expressed GFP mRNA is subject to RNA silencing-mediated degradation even in absence of extra transgenically expressed copies (Voinnet et al., 2003). Consequently, coinfiltration with a functional suppressor-expressing construct results in maintenance of green fluorescence (Fig. 2) because the GFP mRNA is not
degraded (Voinnet et al., 2003). Considering its straightforward application, co-agroinfiltration of T-DNA/GFP with T-DNA/suppressor vector constructs has become the most common technique to assess the suppressor activity of proteins. Likewise, this method can also be conveniently adapted to obtain elevated levels of transient gene expression for foreign proteins other than GFP.

Advantages of agroinoculation include the speed of the assay because results are obtained within approximately 10 d and the experiments can be performed on various plant species as long as they are amenable to Agrobacterium-mediated T-DNA transfer. Disadvantages involve the property that some of the more commonly used Agrobacterium strains can be severely symptomatic on specific hosts (Wroblewski et al., 2005). Occasionally it may be desirable to obtain transient expression of the suppressor throughout the whole plant rather than only in the infiltrated area. For this purpose, vacuum-infiltration of whole plants (i.e., magnification) can be attempted similar to how this method was used to express recombinant proteins (Gleba et al., 2005).

Agroinfiltration of suppressor-expressing constructs can also be effectively used in combination with plant virus vector technology. For instance, when agroinfiltrating leaves with cultures containing T-DNA expression cassettes to launch RNA virus infection, the number of foci can be substantially increased upon coinfiltration with cultures containing suppressor-expressing T-DNA constructs (Chiba et al., 2006). Similar approaches using virus vectors in combination with suppressors can yield substantially elevated levels of transient expression of value-added proteins that is not attainable with T-DNA vectors (Sudarshana et al., 2006).

TRANSGENIC EXPRESSION

During the past two decades, the development and use of transgenic plants has found increasing applications, although the enthusiasm for their use in human food crops is still hampered due to concerns of the public about ingesting genetically modified food. Nevertheless, the transgenic expression of foreign proteins in different plant species is a fairly rapidly achievable process to increase their agroeconomic value. It quickly became apparent in numerous laboratories worldwide where transformations were conducted that many of the initially selected transgenic lines (based on Southern blots) produced little or no foreign protein, presumably due to a process that became known as posttranscriptional gene silencing (Lindbo et al., 1993), now known as RNA silencing or RNAi. Fortunately, stable transgenic lines could often still be identified that produced a level of expression sufficient to obtain the desired traits, such as disease, insect, and/or herbicide resistance.

Thus, from a practical point of view, the obstacles imposed by RNA silencing do not necessarily always create insurmountable problems in generating transgenic plants. Nevertheless, even though high levels of foreign gene expression may not always be a strict necessity, in plant biotechnology efforts it is frequently desired to express and isolate the highest amounts possible of the transgenically expressed value-added protein. This goal is often compromised by RNA silencing. Therefore, 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. In case the suppressor is able to cancel the effects of already activated RNA silencing (e.g., HC-Pro), one could potentially vacuum-infiltrate the plants with Agrobacterium harboring the suppressor-encoding T-DNA vector to relieve the restrictions imposed by RNA silencing. However, this may not be a practical solution on a large scale.

Most suitable 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 foreign protein expression. Transgenically expressed suppressors can indeed alleviate the negative impact of RNA silencing on transgenic expression of a foreign protein, as was shown for HC-Pro (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). Yet, a potential problem with this approach coincides with the above-mentioned property that many of the more potent suppressors are pathogenicity factors that often contribute significantly to the onset of symptoms upon infection of plants (Silhavy and Burgyan, 2004; Scholthof, 2005, 2006; Voinnet, 2005; Li and Ding, 2006). It remains to be determined whether suppressors can be identified that have minimal developmental or symptomatic effects while retaining their ability to suppress RNA silencing with a potency that is attractive for plant biotechnology.

VECTOR SYSTEM-DEPENDENT SIDE EFFECTS

The gene regulation and mode of action of suppressors are important matters for consideration when expressing suppressors in nonhost (for the parental virus) systems and from heterologous gene vectors. For the latter, endogenous viral gene regulatory circuits are lost, and thus the chosen expression vector and its time of application can critically influence whether meaningful results are obtained. For instance, the Tombusvirus P19 protein is expressed at high levels during infection of plants (Scholthof et al., 1999; Qiu et al., 2002), whereas the Polerovirus P0 suppressor protein is expressed at virtually undetectable levels (Pfeffer et al., 2002; Pazhouhandeh et al., 2006). These properties could be important when selecting vectors for heterologous expression of these suppressors. Indeed it has been found that optimum performance of P19 as a suppressor requires its expression at high levels (Qiu et al., 2002). These levels are generally readily achieved using virus gene vectors, but require a strong promoter on T-DNA vectors, such as the cauliflower mosaic virus 35S promoter in dicots and ubiquitin promoters in monocots. In any case, when expressing candidate suppressors by heterologous T-DNA or viral vectors, it is important in most cases to measure that the protein is expressed at detectable levels, preferably using standard immunoblotting (western) techniques.
Furthermore, not all suppressors are equally effective or “strong,” and it may be advantageous to test a few of the more commonly applied suppressors (e.g. P19, HC-Pro, yb, 2b; Fig. 1) or combinations thereof.

When using a heterologous vector (virus or T-DNA), it is imperative that the introduced viral suppressor operates independently of the other virus proteins encoded by the parental virus. Many suppressors that are currently used were found to have this property, but that finding should be considered within the context of the methodology used to identify suppressors. For instance, it is generally accepted that suppressor activity is correlated with the ability to prevent RNA silencing (e.g. of GFP expression; Fig. 2) upon agroinfiltration using T-DNA vectors. As such, the technology selects for proteins that function independently of other proteins. Closteroviruses and geminiviruses encode more than one suppressor (Lu et al., 2004; Bisaro, 2006), and it is likely that their coordinated activity is required for optimum RNA-silencing suppression during infection. Along these lines, there may be examples of viruses for which suppression entirely depends on the expression of two or more proteins. Therefore, when assaying potential suppressors by implementing T-DNA or heterologous viral vectors, a negative result may not be meaningful because it is possible that one or more additional virus proteins are required to obtain suppression. Likewise, it must be kept in mind that the agroinfiltration approach only works for suppressors that affect local silencing, not for suppressors that mostly compromise systemic silencing. In that case, the use of GFP-transgenic plants as a tester for systemic silencing would be preferred. At the same time, when using heterologous virus vectors, the possibility has to be considered that the expressed foreign product (candidate suppressor) may be compromised in its functionality through interference by an endogenous viral protein expressed by the vector.

The above section relates to the possibility that a viral encoded suppressor may not be active when expressed out of the context of virus infection. However, the opposite is also a possibility. For instance, the Turnip crinkle virus (TCV) CP (P38) is a strong suppressor when expressed from a T-DNA vector during agroinfiltration experiments, but it may not be active as such when it is assembled into virions (Qu et al., 2003; Thomas et al., 2003). It appears that the N-terminal portion of the TCV CP that is normally embedded in virions and not exposed is important for optimum suppression (Qu et al., 2003; Thomas et al., 2003), and might be more and longer accessible when expressed out of context of a TCV infection. Nevertheless, the suppressor activity of viral-expressed P38 is sufficient to sustain viral RNA load in Arabidopsis (Deleris et al., 2006).

Considering that many suppressors are RNA-binding proteins (Lakatos et al., 2006; Merai et al., 2006), as are most RNA virus-encoded proteins, the possibility exists that quite a few virus proteins have the potential to act as suppressors when expressed out of context from the highly coordinated gene regulation that occurs during infection. In fact, we have observed that TBSV mutants that do not express the P19 suppressor retain some ability to protect viral RNA from RNA silencing (H. Scholthof, unpublished data), whereas this has not been observed for mutants expressing a defective P19 protein (Omarov et al., 2006). Although several explanations are possible, it is suggestive that in absence of a suppressor (but not in presence of certain dominant-defective versions), another virus-encoded RNA-binding protein gains access to protect the viral RNA from degradation to a certain degree.

Some of the aforementioned symptom effects associated with suppressors (Voinnet et al., 1999; Hull, 2002; Roth et al., 2004; Qu and Morris, 2005; Scholthof, 2005; Voinnet, 2005; Li and Ding, 2006) could be related to their interference with normal siRNA or microRNA (miRNA) pathways. The miRNAs originate from host-encoded hairpin RNAs that are cleaved by DICER for programming of RISC to control specific developmental processes (Voinnet, 2005; Li and Ding, 2006). In addition, suppressors may have functions or activities independent of their interference with RNA silencing. For instance, some suppressor proteins are known to induce defense-signaling pathways, such as a hypersensitive response, which might be genetically linked or unlinked in a system-dependent manner to the ability to suppress RNA silencing (Li et al., 1999; Chu et al., 2000). It is important to be aware of any suppressor activity-independent phenotypic effects when expressing these proteins outside the context of virus infection with (in) heterologous systems.

Knowledge about the modus operandus of the suppressor (Fig. 1) can be crucial for designing experiments. For instance, if the objective is to avoid the generation of siRNAs, suppressors should be used that target the pathway upstream of this process. If, on the other hand, the objective is to deplete the siRNA and miRNA pool of specifically sized RNAs for further analysis, suppressors that sequester such molecules would be preferred. Furthermore, some suppressors, like Tombusvirus P19, sequester siRNAs in a system- or organism-independent manner, but this is not necessarily the case for other suppressors. Since suppressors have evolved to perform optimally in one or more natural viral hosts (ancestors of current crop species), it is not unlikely that suppressors, like other viral proteins, enlist the participation of host factors (Scholthof, 2005). Therefore, when expressing suppressors with heterologous vectors in plant species that are not hosts for the parental suppressor-encoding virus, it is not axiomatic that they perform adequately. In this context, it might be helpful to know in advance whether the plant species that is used for expression of the suppressor (either transiently or transgenically) is a suitable host for the original virus encoding the suppressor.

CONCLUSION

Virus-encoded suppressors of RNA silencing are turning out to be very interesting and useful tools for
studying RNA silencing in plants and for biotechnology. Depending on the precise application and the intrinsic properties on the suppressor, one can select from a number of virus-based or Agrobacterium T-DNA-mediated transient or transgenic plant gene vector systems to express suppressors. An important issue to consider when using vectors for expression of heterologous (candidate) suppressors is to ensure that the protein is expressed at levels needed for activity. Moreover, it is possible that negative results could be due to inactivity or interfering side effects associated with the type of suppressor under study in a particular plant species. An obstacle that may limit a more precise implementation of suppressors thus far characterized is their nonspecificity regarding the RNA sequence that is protected. Future plant biotechnology efforts might benefit from protein-engineering strategies to create suppressors that sequence-specifically prevent RNA silencing of target genes and that do not interfere with the developmentally important RNAi pathway. For instance, a suppressor that sequence-specifically sequesters only those siRNAs that correspond to the introduced value-added foreign gene could be advantageous.

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