Current Review

Can We Explain RNA-Mediated Virus Resistance by Homology-Dependent Gene Silencing?

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The use of viral sequences to produce virus-resistant plants is now almost a standard technique. A variety of sequences from a large number of viruses have been used but the mechanisms remain largely unknown. There are probably at least two distinct types of mechanisms operating: one requiring the expression of the viral protein(s) and the other dependent only on the presence of transgene-derived RNA. In this review, we will discuss this RNA-mediated resistance and its similarities with co-suppression, a recently described phenomenon leading to suppression of transgene expression and homologous sequences. We present a critical view of the current models available to explain this type of resistance.

Pathogen-derived resistance.

Viral epidemics can result in a large reduction in the yield of commercial crops and hence various methods for controlling them have been developed. Conventional methods include the use of virus-free germplasm and the eradication of infected plants, timing of planting so as not to coincide with the presence of the vectors, and the use of chemicals to control such vectors, especially insects. However, these approaches suffer from a number of disadvantages. The use of virus-free starting material and eradication of infected plants means that one must be able to detect viruses at an early stage. In addition, most of these methods are expensive and the chemicals to control the presence of virus vectors can be damaging to the environment and consumer health.

Another approach, which has the most potential in the long term, is breeding for resistance. The term resistance as used here does not only or necessarily imply absolute immunity, but also includes a delay in the appearance of symptoms and/or a milder form of infection. Unfortunately, there are few natural sources of resistance and, especially for RNA viruses, which constitute the majority of the plant viruses and have a high frequency of recombination, virulent strains might arise quickly to overcome the resistance. In addition, some virus resistance genes are recessive and/or inherited in a complex manner that further complicates breeding programs.

In 1985, the concept of parasite-derived resistance (since termed pathogen-derived resistance) was suggested (Sanford and Johnston 1985). It predicts that expressing genetic material of a pathogen in a host will disrupt the essential pathogenic processes and hence result in resistance to the pathogen. Because of the simplicity and accessibility of viral genomes compared with those of bacterial or fungal pathogens, the feasibility of this approach was first demonstrated with viruses. Pathogen-derived resistance has been obtained by expressing various forms of coat protein (CP) genes, the viral replicase genes (or parts thereof), and other sequences (for reviews see: Hull 1994; Lomonosoff 1995; Pappu et al. 1995; Baulcombe 1996; Palukaitis and Zaitlin 1997).

RNA-mediated resistance.

Initially, it was anticipated that pathogen-derived resistance would operate through expression of the protein, and such a mechanism has been suggested by various reports (Powell-Abel et al. 1986; Lapidot et al. 1993; Brederode et al. 1995). However, it was later shown that untranslatable constructs were also able to confer resistance (de Haan et al. 1992; Lindbo and Dougherty 1992; van der Vlugt et al. 1992). This type of resistance, based upon the presence of RNA, became known as RNA-mediated resistance and was characterized by a high level of resistance that was not easily overcome by a high inoculum dose, compared with, e.g., coat-protein-mediated resistance (see Lomonosoff 1995). However, this resistance seemed to be effective only against closely related viruses (for review see: Lomonosoff 1995; Baulcombe 1996; Prins and Goldbach 1996). In addition, an inverse correlation between steady-state RNA levels and protection was often found (Longstaff et al. 1993; Smith et al. 1994; Swaney et al. 1995).

Although not recognized as such, the characteristics of RNA-mediated resistance were first found in plants transformed with the 54-kDa sequence from tobacco mosaic virus (TMV) (Golemboski et al. 1990). These plants showed a high level of resistance, effective only against closely related viruses, in the absence of detectable 54-kDa protein. However, with a transient assay it was shown that the 54-kDa protein, rather than its mRNA, had a negative effect on TMV accumulation in protoplasts, thus suggesting the involvement of the protein rather than the RNA (Carr et al. 1992). It should be mentioned that the suggestion for protein involvement comes from a different system (protoplasts versus transgenic plants). In addition, it has been shown that one construct can give both

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protein- and RNA-mediated resistance in transgenic plants (Pang et al. 1994; Vaira et al. 1995).

Since the protein is not necessary to confer resistance, untranslatable constructs can be used, avoiding the accumulation of any foreign protein and eliminating the risks involved with transencapsulation. A disadvantage of RNA-mediated resistance, however, is the high sequence specificity. The resistance is only effective against viruses with a high sequence homology (up to 88%; Mueller et al. 1995) to the transgene.

RNA-mediated resistance, obtained through the use of untranslatable constructs, was almost simultaneously described for three different viruses, i.e., tomato spotted wilt virus (TSWV; de Haan et al. 1992), tobacco etch virus (TEV; Lindbo and Dougherty 1992), and potato virus Y (PVY; van der Vlugt et al. 1992). The phenotype for each of these three systems was one of extreme resistance. The report on TEV described a CP gene that was untranslatable (Lindbo and Dougherty 1992). Some of the plant lines showed extreme resistance but others had the so-called recovery phenotype. In such plants, a systemic infection initially occurred; however, each new leaf that subsequently appeared displayed fewer symptoms. Eventually, virus-free leaves emerged that were completely resistant to superinfection.

Furthermore, it was reported that the TEV resistance was functional at the single-cell level and was highly sequence specific, being effective only against closely related viruses. In inoculated leaves, steady-state mRNA levels had dropped about 5- to 8-fold, compared with uninoculated tissue at the same developmental state (Dougherty et al. 1994). Nuclear run-on experiments to assess levels of transcription revealed that there was an unaltered rate of transcription, suggesting a post-transcriptional mRNA suppression. It was suggested that a sequence-specific RNA degradation mechanism was triggered that affected both the mRNA of the transgene and the replicating viral RNA. The differences between the two phenotypes, recovery and resistance, appeared to correlate with the number of integrated copies (Goodwin et al. 1996). One to two copies resulted in recovery whereas three or more copies resulted in resistance. Recently, it has been shown that plants showing the immunity phenotype did display a recovery phenotype when infected early in development, suggesting that immunity and recovery are based upon the same phenomenon (Tanzer et al. 1997).

A double haploid family of transgenic plants, containing an untranslatable PVY CP gene, showed differences in their response to PVY inoculation (Smith et al. 1994). These genetically identical plants were shown to have differences in steady-state mRNA levels related to the methylation state of the transgene. The authors suggested that some undefined host factor would be partially responsible for this difference.

Developmental regulation of RNA-mediated resistance has also been observed. Homozygous transgenic plants, containing the nucleoprotein of TSWV, were silenced earlier in development than hemizygous plants (Pang et al. 1996). The induction of the resistance phenotype was shown to depend on environmental conditions.

When plants were transformed with randomly chosen sequences (sense or antisense) of the TSWV ambisense genome, sequences from only two genes, the *N* gene (a virus-derived protein regulating transcription-to-replication) or *NS_M* gene (putative movement protein), were able to induce gene si-

lencing (Prins et al. 1996). The authors suggested that this might be due to the fact that the viral genomic RNA remains encapsidated during the replication cycle of this virus or that sequences, in order to induce silencing, must suit some as yet unknown criteria. Subsequent work showed that it is likely that the silencing mechanism is targeted to the mRNAs of the virus, supporting the hypothesis that only accessible viral sequences can confer resistance (Prins et al. 1997).

Resistance to cowpea mosaic virus (CPMV), after transformation with the movement protein (MP) gene, has also been shown to be RNA mediated (Sijen et al. 1996). Transcripts of recombinant potato virus X (PVX) clones, containing as little as 60 nucleotides of the CPMV MP gene sequence, were shown to be suppressed on the CPMV MP transgenic plants. For recombinant PVX transcripts containing small MP gene sequences, there was, in addition to complete immunity, another phenotype observed in some plants. These plants would show a delay of 3 to 4 days, compared with nontransgenic plants, before becoming systemically infected. When plants were transformed with a direct repeat of the MP gene, the number of resistant lines increased from 20 to 60%, indicating a causal role for transgene structure in silencing.

The induction of RNA-mediated resistance has been shown to depend upon transgene length. When plants were transformed with small, untranslatable segments (110 to 235 nucleotides) of the nucleoprotein gene of TSWV, no resistance was observed. However, when these same segments were fused to the nontarget, green fluorescent protein (GFP) gene, resistance was observed (Pang et al. 1997). This suggests that a critical length of the transgene is necessary to induce RNA-mediated resistance. There was a reduced accumulation of small transcripts, compared with large transcripts, and it was suggested that fusion to the GFP gene led to an increased accumulation of transgene-derived mRNA.

A more complicated phenotype was obtained with plants resistant against cucumber mosaic virus (CMV). It was shown that transformation of tobacco plants with a truncated form of the replicase gene of the Fny strain of CMV (Anderson et al. 1992) led to two independent mechanisms of resistance, both targeting the viral RNA rather than acting against a protein of CMV (Carr et al. 1994; Hellwald and Palukaitis 1995). It was shown that the resistance was highly sequence specific and effective at the single-cell level, leading to a reduction of replication. However, it was also shown that there was a second, independent mechanism acting against viral movement. Injection studies with fluorescently labeled CMV RNA showed a correlation between the resistance phenotype and the suppression of viral RNA trafficking (Nguyen et al. 1996). The authors mentioned that in preliminary studies with transgenic plants containing untranslatable forms of the replicase protein gene, no resistance was observed. This suggests a protein involvement that is consistent with a mechanism acting against viral movement.

The mechanisms behind RNA-mediated resistance remain largely unknown; however, similarities have recently been shown with homology-dependent gene silencing, a process affecting the expression of transgenes and homologous sequences.

Homology-dependent gene silencing.

The stability of transgene expression for the expression of heterologous sequences is an important aspect in the devel-

opment of transgenic plants for commercial applications. Homology-dependent gene silencing, a phenomenon whereby the presence of (multiple) transgene(s) can lead to the inactivation of one or all of the transgenes (for reviews see: Dougherty and Parks 1995; Matzke and Matzke 1995; Baulcombe and English 1996; Meyer and Saedler 1996; Stam et al. 1997b), thus came as an unwelcome surprise to some. This gene inactivation is a great disadvantage in applied plant biotechnology and a survey held in 1994 among 30 biotechnology companies revealed that almost all of them had encountered problems associated with gene silencing (Finnegan and McElroy 1994).

Gene silencing can occur either at the transcriptional level (no transcription) or at the post-transcriptional level (transcription but increased degradation of transcript). When post-transcriptional silencing affects both the transgene and the endogenous gene the phenomenon is also termed co-suppression. Since RNA-mediated resistance is associated with post-transcriptional gene silencing (Lindbo et al. 1993; Mueller et al. 1995; Sijen et al. 1996), transcriptional silencing will not be dealt with here.

Co-suppression is perhaps one of the most interesting manifestations of the phenomenon. It was first discovered in 1990 when two different groups were trying to overexpress the chalcone synthase (*chs*) gene (a key enzyme in flavonoid biosynthesis, the process responsible for giving flowers their colors) in petunia (Napoli et al. 1990; van der Krol et al. 1990). Both groups found that the flowers of a small percentage of the transformed plants were white instead of the expected deep purple color. In addition to plants with completely white flowers, an intermediate phenotype was found in which flowers possessed white and purple sectors. Although the *chs* mRNA level in the white sectors was significantly reduced, the rate of transcription of the transgene and endogenous mRNA was not affected, suggesting a post-transcriptional mode of silencing (van Blokland et al. 1994). Furthermore, some plants that possessed white flowers developed new branches with either completely purple flowers or flowers that had both purple and white sectors. This indicates that the silenced state is reversible and somatically unstable.

Co-suppression has been shown to be developmentally regulated (Dehio and Schell 1994; Niebel et al. 1995) and meiotically reversible (Dehio and Schell 1994). When the *rolB* gene of *Agrobacterium rhizogenes* was expressed in *Arabidopsis thaliana*, the young seedlings expressed the gene at a high level but later in the development of the plant the expression was suppressed in a post-transcriptional manner. The induction of the silencing was shown to depend upon environmental conditions but once it was established it was maintained during subsequent development of the plant.

Kumagai et al. (1995) demonstrated that the presence of a nuclear transgene is not even a requirement for co-suppression. They placed the gene encoding phytoene desaturase, an enzyme involved in the carotenoid biosynthetic pathway, under the control of a tobamovirus subgenomic promoter in an infectious clone of TMV. Upon inoculation these plants initially developed symptoms typical of a TMV infection; however, 1 to 2 weeks after inoculation, endogenous phytoene desaturase mRNA could not be detected. The suppression of endogenous mRNA was visible as photobleaching, caused by a decrease in phytoene desaturase and subsequent lack of carotenoid protection, and was observed in the systemically

infected leaves of the plant. These experiments demonstrated that a transcript generated in the cytoplasm can suppress endogenous gene expression and that a DNA-DNA interaction is not essential for co-suppression, although the involvement of the latter remains to be shown.

A similar conclusion can be drawn from the work of Elmayan and Vaucheret (1996). They showed that an integrated bacterial *UidA* gene (β -glucuronidase, GUS), cloned downstream of a double enhanced 35S promoter, was silenced in hemizygous plants and haploid plants derived from the homozygous plants containing a single copy of the transgene. The suppression was induced developmentally and showed a difference in the stage at which silencing was induced between homozygous and hemizygous plants. The homozygous plants showed the silenced state after 4 months of growth whereas the hemizygous plants did not show any silencing for up to a year.

Grafting experiments have shown that silencing can be transmitted from silenced stocks to nonsilenced scions containing a transcriptionally active homologous (trans)gene (Palauqui et al. 1997). The experiments suggested that post-transcriptional silencing involves a non-metabolic, transgene-specific, diffusible messenger. Voinnet and Baulcombe (1997) came to the same conclusion with plants expressing GFP. When these plants were locally infected with *Agrobacterium tumefaciens* carrying the GFP transgene, silencing of GFP expression was found in the upper leaves. Both groups suggested that a nucleic acid derived from the transgene might be the signal involved.

Models to explain co-suppression and RNA-mediated resistance.

Co-suppression and RNA-mediated resistance seem to have a lot of features in common. Both require sequence homology between, respectively, the transgene and endogenous gene or the transgene and incoming virus. Both have post-transcriptionally inactivated (trans)genes that, in turn, have trans-inactivating capacities on homologous sequences (see below). The two phenomena might therefore have a common mechanism.

The finding that silencing involves a diffusible signal (Palauqui et al. 1997; Voinnet and Baulcombe 1997) stands in contrast to the conclusion reached by Dougherty et al. (1994). The grafting experiments of the latter authors suggested that RNA-mediated resistance was "programmed" at the cellular level and did not involve a diffusible signal. However, the authors mainly used nontransformed target tissue that did not contain a transcriptionally active transgene. The results obtained with susceptible transformed tissue are, unfortunately, inconclusive due to a low number of experiments.

The strongest evidence for a link between co-suppression and RNA-mediated resistance comes from work of Mueller et al. (1995) and English et al. (1996). Two types of transgenic lines were available, both containing the RNA polymerase (RdRp) of PVX. The first showed highly strain-specific resistance against PVX and a low level of RdRp mRNA, and had properties associated with a post-transcriptional mechanism of transgene expression. The second type was susceptible to infection with PVX and displayed a higher level of RdRp mRNA. When the progeny of a cross between these two transgenic lines were analyzed it was shown that the

resistant line had trans-inactivating capabilities toward the homologous transgenes of the susceptible line (Mueller et al. 1995).

In addition to virus-derived sequences, nonviral sequences introduced into viral vectors can also induce resistance. Transgenic plants containing the GUS gene have been described and were shown to be post-transcriptionally silenced (Hobbs et al. 1990). When infectious transcripts of a recombinant PVX clone, containing the GUS gene cloned behind a duplicated PVX CP promoter, were inoculated on these plants a resistance phenotype was observed (English et al. 1996).

There are currently two models, not mutually exclusive, that attempt to explain post-transcriptional silencing. The first involves a threshold level of RNA (Jorgensen 1992; Meins and Kunz 1994; Baulcombe 1996; Prins and Goldbach 1996; Stam et al. 1997b). This model proposes that the plant is somehow able to sense the transcription level of the transgene and, if the level is too high, the plant targets the mRNA for degradation. This hypothesis came from work on transgenic tobacco plants containing an untranslatable TEV CP gene (Lindbo et al. 1993). As mentioned previously, two phenotypes were observed, resistance and recovery, that were related to the number of integrated copies of the transgene (Goodwin et al. 1996). Resistance was observed when three or more copies were present whereas one to two copies resulted in a recovery phenotype. The authors suggested that the induction of the resistance phenotype was dependent on the transcription level of the transgene. Three or more copies would lift the mRNA above a critical threshold to induce the resistance. One or two copies, initially, would not be sufficient to induce the resistance; however, the RNA of the replicating virus combined with the transgene transcription level would be sufficient to reach the threshold and hence induce the degradation mechanism. The authors further suggested that once the threshold level was reached, an RNA-dependent RNA polymerase, present in the cytoplasm of plants (Fraenkel-Conrat 1986), would produce short, antisense RNA molecules with sequence homology to the target RNA. Annealing of the antisense RNA molecules would lead to the formation of a duplex RNA structure that could be a target for degradation by double-strand, specific RNases.

The involvement of antisense RNA is an attractive hypothesis since it is known that antisense nucleic acids can inhibit gene expression (Frischmuth and Stanley 1993; Hawkins and Nellen 1994), and it would account for the high sequence specificity of gene silencing and RNA-mediated resistance. However, although antisense mRNA has been found for the petunia plants containing the *chs* gene, no correlation was found between the transcription levels of antisense RNA and the efficiency of silencing (van Blokland et al. 1994).

Further support for the threshold level model comes from other reports describing the relationship between silencing and virus resistance (Smith et al. 1994; Goodwin et al. 1996). Developmental timing differences between homozygous and hemizygous plants can also be explained by the threshold level (Elmayan and Vaucheret 1996). The homozygous plants would have a higher transcript level, and thus induce the silenced state earlier in development, compared with hemizygous plants.

However, there are a number of reports that cannot be explained by the threshold model. Van Blokland et al. (1994)

found that the expression levels of suppressed and non-suppressed *chs* genes were similar. A similar result was reported with virus resistance (Mueller et al. 1995; English et al. 1996), which led these authors to suggest another model that involved a qualitative feature of the mRNA also known as aberrancy (Baulcombe and English 1996).

This aberrant mRNA would be distinct from the normal mRNA. This distinction in turn would somehow be recognized, which would then lead to degradation of the aberrant and "normal" mRNA. An ectopic pairing model has been suggested to account for the production of aberrant RNA. This would involve a silencer locus and a receptor locus. Transcription of the silencer locus is not essential. If a DNA-DNA pairing between the two loci takes place, the transcription of the receptor locus would be changed, leading to the production of an aberrant mRNA. It is this aberrant RNA that would induce a sequence-specific degradation mechanism affecting all RNA with sequence homology.

Support for the role of aberrant RNA in gene silencing comes from work of English et al. (1996). As has been described earlier, replication of a recombinant of PVX carrying the GUS gene is suppressed in plants transgenic and post-transcriptionally silenced for the GUS gene expression. This suppression was also post-transcriptional and it was shown that the target for resistance was in the 3' region of the GUS gene, which correlated with the methylation pattern of the transgene. These authors suggested that transgene methylation could be directly responsible for the production of a truncated (aberrant) mRNA because of transcript termination in the 3' methylated region. Alternatively, DNA methylation could result from an interaction with the aberrant RNA, leading to methylation of the transgene. RNA-directed DNA methylation has been shown for potato spindle tuber viroid (PSTVd) transgenes, but only after autonomous replication of PSTVd (Wassenegger et al. 1994). The production of an aberrant RNA could also be caused by features of the transgene itself (e.g., untranslatable, no introns, or other unusual characteristics). Once this aberrant RNA is produced, it serves as a preferred template for an RNA-dependent RNA polymerase and targets the RNA for degradation as described above.

It is difficult to see, however, how this model could explain the post-transcriptional silencing of a single-copy gene reported by Elmayan and Vaucheret (1996), who showed that haploid and hemizygous plants derived from transformed plants containing one copy of T-DNA show silencing. Furthermore, these plants had a high transcription rate before silencing was induced, suggesting a threshold model rather than the production of aberrant RNA through DNA-DNA pairing.

Both models assume the presence of an RNA degradation mechanism. Support for such a mechanism comes from work on silenced petunias containing a *chsA* transgene (Metzlaff et al. 1997) and tobacco plants containing a TEV CP gene (Tanzer et al. 1997). In both systems it has been shown that silencing correlates with the presence of low molecular weight RNA fragments derived from degradation of, respectively, *chsA* mRNA and TEV CP mRNA.

An important question is why co-suppression usually occurs only in a fraction of transgenic plants. It is therefore interesting that tobacco plants transformed with a cDNA representing the genome of PVX behind a 35S promoter showed a post-transcriptional gene silencing phenotype in all of the lines

tested (Angell and Baulcombe 1997). It is unclear exactly why the replicating PVX genome is such a potent activator of gene silencing. The threshold model is insufficient to explain this consistent phenotype since an infection in nontransgenic plants does not lead to a recovery phenotype (although it is unclear to what level a replicating PVX genome behind a 35S promoter accumulates, compared with an infection of PVX in nontransgenic plants). Another possibility is the production of aberrant RNA. This RNA could be structurally different, or it could be that the timing or location is responsible for the induction of this phenotype. Alternatively, the strong expression of PVX could have led to a strong selection for silenced lines.

Both models are unable to explain all cases of co-suppression that could operate through a combination of the mechanisms proposed by the two models. The use of different constructs with different promoters makes comparison of all the different systems rather difficult. However, a correlation was found between promoter strength and frequency of co-suppression (Que et al. 1997). The authors also mentioned that there is an interesting correlation between promoter strength and the model proposed. Researchers using cauliflower mosaic virus 35S promoters under the control of one upstream activation region (1*UAR) and looking at transgenic plants containing inverted repeats rejected the threshold model in favor of other models (English et al. 1996; Stam et al. 1997a). In contrast, those models using 2*UAR 35S transgenes generally resulted in co-suppression with single-copy transgenes consistent with the threshold model (Elmayan and Vaucheret 1996; Goodwin et al. 1996).

It is unlikely that the underlying mechanism has evolved solely for the purpose of transgene suppression so it is perhaps not surprising that some natural virus defense systems have been found to resemble gene silencing. Nontransgenic kohlrabi (*Brassica oleracea* var. *gongylodes*) infected with cauliflower mosaic virus shows a recovery phenotype. This recovery was shown to correspond to a post-transcriptional decrease of viral polyadenylated RNAs (Covey et al. 1997). A similar phenomenon was observed for nontransgenic *Nicotiana glauca* infected with tomato black ring virus strain W22. These plants recover from infection and are resistant to superinfection. It was shown that this resistance is strain specific and is targeted against the RNA of the virus (Ratcliff et al. 1997).

It is possible that there are two different mechanisms operating or that there is a threshold level of aberrant RNA required to trigger co-suppression. However, a lot more work needs to be done before any model can be devised that can explain all cases of co-suppression.

Concluding remarks.

It now seems likely that co-suppression and RNA-mediated resistance are based upon the same basic phenomenon (see van Kammen 1997). However, the mechanism actually responsible for co-suppression is still unknown. Elucidation of the mechanism requires, for example, that the genes and their products involved in the phenomenon are analyzed. A start has been made by Dehio and Schell (1994), who have identified (some of the) plant genes that are involved in the silencing process. In addition, future experiments should look for a function, if any, for the host-derived RNA-dependent RNA polymerase, its role in the production of antisense RNAs, and

the role of transgene structure, and try to find the proposed aberrant RNAs, since these form a central role in the supposed mechanisms.

The use of co-suppression for creating virus-resistant plants has advantages and disadvantages. The resistance is not easily overcome and does not suffer from the presence of foreign proteins. However, the high sequence specificity makes it questionable whether the resistance is durable, due to selection of resistance-breaking strains, and also the resistance spectrum is rather narrow. The use of multiple constructs to overcome this latter problem (Pang et al. 1994; Prins et al. 1995; Prins and Goldbach 1996) could offer a solution (Prins et al. 1995) but can cause problems associated with transcriptional silencing because of homologous sequences present in transformation vectors. However, the observation that linking viral sequences to nontarget sequences can reduce the necessary length of homologous transgene to induce resistance (Pang et al. 1997) suggests that linking several viral sequences together might be an effective way of inducing broad spectrum resistance, as has been suggested by the authors.

If virus resistance, based on co-suppression, is to be commercially exploited then the developmental dependence could lead to further complications since it means that one cannot be sure if the plants are resistant at any given time of their life cycle. In addition, a field trial of transgenic petunias has shown that there was significantly more variation in the field than in the greenhouse (Meyer et al. 1992). Clearly, these are traits that will be further investigated during field trials.

Overall, co-suppression seems to be a useful phenomenon to combat viral infections and, therefore, it should come as no surprise that it is not restricted to transgenic plants but that similar mechanisms exist in nature.

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