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RNA as a target and an initiator of post-transcriptional gene silencing in trangenic plants

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Contents

Abstract

Post-transcriptional gene silencing in transgenic plants is the manifestation of a mechanism that suppresses RNA accumulation in a sequence-specific manner. The target RNA species may be the products of transgenes, endogenous plant genes or viral RNAs. For an RNA to be a target it is necessary only that it has sequence homology to the sense RNA product of the transgene. There are three current hypotheses to account for the mechanism of post transcriptional gene silencing. These models all require production of an antisense RNA of the RNA targets to account for the specificity of the mechanism. There could be either direct transcription of the antisense RNA from the transgene, antisense RNA produced in response to over expression of the transgene or antisense RNA produced in response to the production of an aberrant sense RNA product of the transgene. To determine which of these models is correct it will be necessary to find out whether transgene methylation, which is frequently associated with the potential of transgenes to confer post-transcriptional gene silencing, is a cause or a consequence of the process.

Introduction

It is now well established that transgenes in plants may suppress expression of homologous endogenous genes or transgenes (reviewed recently in [5, 15, 27, 28, 35, 36, 46]). This homology-dependent gene silencing represents one of the most puzzling and potentially important phenomena in transgenic plants. It is puzzling because the current paradigms of gene regulation do not explain the mechanisms that could cause gene silencing. It may be important because these unknown mechanisms could represent genetic controls involved in plant growth, development and response to environmental factors. Gene silencing is also important because, potentially, it is a powerful mechanism of inactivating genes that reduce yield or quality of

products from plants. Conversely, gene silencing is a complication for the exploitation of transgenic plants because uncontrolled it could suppress the expression of host genes and transgenes that are necessary for efficient crop production.

Gene silencing may operate at the level of transcription if the transgene shares homology with the promoter of the silenced gene. Homology in transcribed regions leads to post-transcriptional gene silencing (PTGS), which is the subject of this review. In the examples of PTGS discussed here the silencer transgene and the silenced genes are transcribed in the same orientation although, as discussed elsewhere [5], it is possible that PTGS and antisense suppression are related processes.

There are two phases of the process of PTGS. One phase is the suppression of RNA accumulation. RNA is involved at this phase as a target molecule. There is also an initiation phase which determines whether PTGS will be active. This initiation phase may also involve RNA. Before assessing the current information about these two phases it is first necessary to define how a gene silencing phenomenon is placed into the post-transcriptional rather than transcriptional category.

Examples of post-transcriptional gene silencing (PTGS)

The known and likely examples of PTGS in plants involve many different genes, plants and constructs (Table I). It is therefore likely that PTGS is a general phenomenon that could be produced in all plant species with most genes. The most reliable indicator of a post-transcriptional rather than a transcriptional mechanism is from transcription run-off analysis with isolated nuclei. In the absence of run-off data it may be inferred that the gene silencing is post-transcriptional, rather than transcriptional, if the silencer transgene and the silenced genes share homology in the transcribed region. However, if there is homology in both the promoter and the transcribed region there could be either transcriptional silencing or PTGS. For example, with the maize *A1* cDNA expressed in transgenic petunia, there was transcriptional silencing of the *A1* transgenes [39]. The silencer and silenced transgenes in these lines were identical in the promoter region as well as in the transcribed region and it is likely that this promoter homology led to the transcriptional gene silencing. The converse of this example is provided by tobacco plants carrying transgenes with 35S promoter of CaMV (35S) coupled to the *uidA* reporter gene of *Escherichia coli* (GUS). These transgenes had PTGS activity despite the sequence identity in the 35S promoter region of the silencer and silenced loci [14].

It is also possible to use viruses to determine whether a gene silencing mechanism is transcriptional or post-transcriptional. This approach to the analysis of gene silencing is based on the finding that the mechanism of PTGS has the potential to suppress RNA viruses as well as RNA from nuclear genes. The antiviral potential of PTGS was established by transgenic expression of viral cDNAs in the sense orientation relative to the virus genome. In many instances the lines carrying these viral cDNA transgenes were specifically resistant to the virus from which the transgene was derived [33, 40, 49]. The resistance in these lines was associated with low level accumulation of the transgene RNA and the potential of the transgene to silence homologous loci at the post transcriptional level. Production of virus encoded proteins was not required for the resistance of silencing phenotypes [32, 40, 50]. Transgenic resistance associated with PTGS is referred to as homology-dependent resistance to reflect the specificity of the resistance mechanism for viruses with extreme sequence similarity to the sense RNA product of the transgene [40].

The association of PTGS and virus resistance was established initially in lines transformed with fragments of viral cDNA. Subsequently, using constructs based on the genome of potato virus X (PVX), it was shown that PTGS of non viral genes had the potential to suppress virus accumulation provided that there was sequence homology of the virus and the transgene. For example, tobacco plants displaying PTGS of GUS or neomycin phosphotransferase (NPT) were specifically resistant to PVX.GUS or PVX.NPT respectively and tomato plants with PTGS of polygalacturonase (PG) were specifically resistant to PVX.PG [14]. These data have implications for the analysis of the mechanism of PTGS that are discussed in more detail below. In addition these data illustrate how virus constructs may be useful for the diagnosis of PTGS in situations where it is difficult to carry out a nuclear run-off assay or where independent confirmation of the run-off data is required.

Table 1. Examples of post-transcriptional gene silencing in transgenic plants

Target genes	Plant	Construct[2]	Transcription assay[3]	References
Non-viral				
Chalcone synthase	petunia	nos	yes	[41, 51, 52]
Dihydroflavonol-4-reductase	petunia	nos	no	[52]
Homeotic gene-fbp1	petunia	nos	no	[1]
Polygalacturonase	tomato	nos	no	[47, 48]
Phytoene synthase	tomato	CaMV	no	[16]
Nopaline synthase	*Nicotiana*	CaMV	no	[18]
Neomycin phosphotransferase	*Nicotiana*	CHS	yes	[26]
β-glucuronidase (GUS)	*Nicotiana*	nos	yes	[13, 14, 25]
β-1, 3-glucanase	*Nicotiana*	nos	yes	[11]
Chitinase	*Nicotiana*	chitinase	no	[23]
S-adenosyl-L-methionine synthetase	*Nicotiana*	SAM	no	[6]
Nitrate reductase	*Nicotiana*	CaMV	no	[9]
Nitrite reductase	*Nicotiana*	NiR	no	[54]
Acetohydroxyacid synthase	*Nicotiana*	AHA	no	[7]
rolB	*Arabidopsis*	nos	yes	[12]
Viral[1]				
Potato leafroll virus	potato	nos	no	[29]
Tobacco etch virus	*Nicotiana*	CaMV, TML	yes	[17, 32, 33]
Potato virus X	*Nicotiana*	nos	yes	[40]
Tomato spotted wilt	tomato	nos	no	[44]
Potato virus Y	*Nicotiana*	nos	yes	[49]

[1] It is likely that there are many other examples of PTGS in transgenic plants expressing viral cDNAs [4]. The diagnostic features of these examples are that there is homology-dependent resistance and an irregular relationship between virus resistance and the accumulation of the viral transgene RNA.
[2] All constructs have the 35S promoter although there is PTGS of chalcone synthase in petunia transformed with promoterless constructs. The abbreviations refer to the transcriptional terminator/poly (A) addition site as follows: nos, nopaline synthase of *Agrobacterium tumefaciens*; CaMV, cauliflower mosaic virus; CHS, chalcone synthase; SAM, *S*-adenosyl-L-methionine synthase; NiR, nitrite reductase; AHA, acetohydroxyacid synthase; LMT, large tumour morphology gene of *Agrobacterium tumefaciens*.
[3] Indicates whether a transcription run-off assay has been carried out.

Mechanisms of PTGS

A role for antisense RNA

A transgene locus conferring PTGS can suppress host and viral RNAs in a sequence specific manner [33, 40] depending on sequence homology of the transgene and its RNA target. In principle, this specificity could result from a direct interaction of the transgene and the target RNA or because the transgene influences accumulation of a factor that interacts with the target RNA. That factor could be either protein or RNA.

It is unlikely that the transgene DNA has the potential to interact with the target RNAs directly because, at least with the viral RNAs, they are not in the nucleus [33, 40]. It is similarly unlikely that the transgene influences a protein that mediates the silencing mechanism because PTGS is conferred by transgenes encoding untranslatable RNAs [32, 40, 50]. It is also fundamentally unlikely that every sequence with the potential to confer PTGS encodes a protein that can bind to its RNA template. This leaves the RNA product of the transgene as the most plausible mediator of PTGS.

If this RNA mediator has the same polarity as the target RNA there would be a potential for sequence-specific interaction in regions of RNA secondary structure. The RNA mediator could displace the base-paired RNA. However there is no evidence that the target sequences of PTGS are those with RNA secondary structure. An antisense RNA mediator with polarity opposite to that of the target RNA could easily explain the specificity of PTGS of nuclear and viral genes. Oth-

ers have also come to the conclusion that an antisense RNA is a necessary cofactor in PTGS [20, 33, 40].

There have been some attempts to detect antisense RNA associated with PTGS. In some instances there was evidence for antisense RNA but not, so far, correlated with PTGS [51].

There are gaps in the reported searches for antisense RNA which do not account for the possibilities that the molecules may be small, heterodisperse or covalently associated with proteins. Given the almost inescapable conclusion that an antisense RNA is needed to confer sequence specificity on PTGS, a major research priority should be a search for these molecules. The subsequent sections of this review assess and interpret the known information about PTGS in terms of this proposed involvement of antisense RNA.

How antisense RNA might mediate PTGS

Antisense RNAs and oligonucleotides are known potent inhibitors of gene expression at several levels [42, 55]. These molecules anneal to the complementary sense RNA or DNA. Depending on the target sequence the antisense RNAs can inhibit translation or stability of corresponding sense RNAs. In principle, PTGS could be due to inhibition at either of these levels. A block at the level of translation could cause reduced accumulation of the target RNA because translation has an indirect effect on RNA stability [19]. A direct destabilization of sense RNA could be the consequence of duplex formation with the antisense RNA because double-stranded RNA may be a substrate for a double-stranded RNAase [42].

The effect of PTGS on accumulation of viral, as opposed to cellular, RNAs could be the consequence of antisense inhibition at either of these levels. However it might be expected that viral RNAs would be suppressed to a greater extent than cellular RNA because the duplex of viral and antisense RNA inhibits several processes specific for accumulation of viral RNAs. For example, an inhibition of translation could affect RNA stability indirectly, as discussed above. It could also have a direct effect on virus accumulation if the target gene encodes a protein required for accumulation of virus RNA. In addition, a region of duplex sense/antisense RNA could prevent a replicase enzyme having access to the viral genome and thereby interfere directly with the process of virus replication. Data showing that viral RNAs may be more sensitive to PTGS than cellular RNA was generated in the analysis of tobacco plants transformed with the RNA-dependent RNA polymerase (RdRp) gene of PVX. These lines displayed PTGS of the transgene and were extremely resistant to PVX [40]. In one of these lines the transgene had the potential to reduce accumulation of cellular RNA by a factor of 8. In striking contrast, the suppression of viral RNA accumulation was at least ten thousand fold [34].

There is currently very little direct information about the target mechanisms of PTGS although there is evidence consistent with targeted degradation of RNA. For example, in tomato exhibiting PTGS of PG [48] and tobacco displaying PTGS of a tobacco etch virus (TEV) transgene [17] there were discrete-sized sense RNAs that were homologous to, and smaller than, the normal transgene RNA. These RNAs may be degradation products produced during the PTGS mechanism. From the discrete size of these RNAs it was suggested that the degradation mechanism preferentially cleaves defined sequence motifs or structures within the target region determined by the silencer transgene [17, 48]. However these putative motifs or structures could not be identified from the sequence at or around the cleavage sites [17].

The potential of cytoplasmic viruses of the potex- and poty-viral groups to be a target of PTGS implies that the mechanism is also cytoplasmic. Consistent with a cytoplasmic site of action is the finding that PTGS of β-glucanase in tobacco has no effect on the accumulation of the unspliced precursor of the β-glucanase mRNA [10]. However this analysis of pre mRNA is also consistent with suppression of maturation or transport of the nuclear pre-mRNA and it remains possible that the mechanism of PTGS is active in several compartments of the cell.

Initiation of PTGS

It is generally observed that PTGS is a feature of only a small proportion of lines transformed with any one construct although a modified 35S GUS construct did induce variable PTGS in all lines tested [13]. Typically the silencing was evident in 5–20% of lines. There are currently three models to account for the reason why the level of PTGS varies between lines (Fig. 1). All three models predict the formation of an antisense RNA to mediate the PTGS, as described above. The first proposes that a silencing transgene is integrated in the plant genome adjacent to endogenous promoters [20]. This endogenous promoter would direct transcription of the transgene directly into antisense RNA. The second model (Fig. 1) invokes a sensing mech-

anism that can detect high levels of transgene RNA. According to this model, once the mechanism senses accumulation of the transgene RNA above a certain threshold level, the transgene RNA would be copied into antisense RNA by an endogenous RdRp [33]. In the third model (Fig. 1) the distinctive feature of the silencing transgenes is that they produce an aberrant RNA which is a template for the host-encoded RdRp. The production of the aberrant RNA would therefore lead to accumulation of antisense RNA [14]. The following sections assess the evidence for and against these hypothetical mechanisms of PTGS.

Direct transcription of antisense RNA

It would be predicted, if PTGS is mediated by antisense RNA produced directly from promoters adjacent to the transgene, that transgenes designed to produce antisense RNA would confer PTGS of nuclear genes and virus resistance. Consistent with this prediction it has been found that nuclear genes are suppressed by antisense transgenes [42]. However virus resistance is conferred only rarely by transgenes designed to produce the antisense strand of positive strand RNA genomes [8, 22, 29]. It is more often found that antisense constructs do not confer resistance and therefore that antisense RNA produced by direct transcription from a transgene does not have the potential to confer the type of PTGS associated with virus resistance.

Further evidence against the direct transcription of antisense RNA is for transcription run-off analysis in petunia lines displaying PTGS of chalcone synthase (CHS) [51]. There was very little transcription of CHS antisense RNA and no relationship of these low levels of antisense CHS with the degree of PTGS. Other data that are not easily reconciled with the direct transcription of antisense RNA include the observations that PTGS may be transgene dosage-dependent [11, 17, 40, 54] or affected by expression of host genes with homology to the transgene [6, 9, 48]. Together, these various data indicate that direct transcription of antisense RNA is the least plausible of the three models.

Transgene methylation and PTGS

There are now several reports that transgene methylation is associated with PTGS [14, 21, 26, 49]. For example, in transgenic tobacco there was a correlation between the level of PTGS and the degree of methylation of an NPT transgene: the methylation was high in the progeny of a transformed plant that displayed the gene silencing phenotype [26]. Other examples in which there was an association of PTGS and transgene methylation were also in tobacco. These include lines displaying homology-dependent resistance and PTGS of a PVY transgene [49] or PTGS of a GUS transgene [14]. The methylation of the GUS transgene was in the 3′ part of the gene which, as RNA, was the target of the gene silencing mechanism. This spatial correlation of transgene methylation and the target of PTGS reinforces the notion that these are associated phenomena [14].

As yet there is no clear indication whether transgene methylation is a cause or an effect of PTGS. However, PTGS of a GUS transgene in tobacco is relieved by the application of 5-azacytidine [43]. This agent is an inhibitor of DNA methylation, suggesting that the loss of PTGS was due to suppression of transgene methylation.

A role of transgene methylation as a causal factor in PTGS is consistent with the observations from tobacco and petunia that PTGS is more frequent in lines with multiple homologous transgenes than in lines with a single-copy insert [11, 17, 24, 25, 40, 54]. It is thought that multiple copies of homologous DNA at a single locus or at unlinked positions in the genome are able to interact through ectopic pairing [36]. The analyses of both plant and fungal systems indicates that ectopic pairing then leads to methylation of the interacting DNA [2, 3, 38, 39, 45]. An observation with a fungal system links this association of repeated DNA, transgene methylation and the aberrant RNA model of PTGS. It was shown in *Ascobolus immersus* that duplication of the 3′ part of the *met2* gene led to methylation of the corresponding region of the intact endogenous gene. This methylation of the transcribed region of *met2* disrupted transcription so that the accumulated RNA was truncated in the 3′ region [3].

English *et al.* [14] pointed out similarities in this fungal system and in transgenic tobacco with PTGS of GUS. There were multiple copies of the GUS transgenes in these lines and hypermethylation at the 3′ end of the transgenes [14, 24, 25]. The same 3′ region was the target, as RNA, of the mechanism of PTGS suggesting that the methylation of the transgene somehow influenced a sequence specific RNA degradation. The coincidence of the DNA methylation and target of PTGS could be explained if there was aberrant RNA produced from the methylated DNA, as in *Ascobolus immersus*, and if the aberrant RNA was the template for antisense RNA production [14].

The influence of endogenous gene transcription on PTGS

In some examples the initiation of PTGS is affected by transcription of host genes with homology to the silencer transgene. For example, constructs with the 35S promoter to transcribe PG and phytoene synthase (PE) sequences were not silenced until the endogenous PG and PE genes were activated during fruit ripening [47, 48]. After that stage both the endogenous genes and the homologous transgenes were subject to PTGS. Similarly in tobacco with nitrate reductase [9] or S-adenosyl methonine synthetase (SAM synthetase)[6] transgenes, the level of PTGS was increased under conditions in which host gene expression was enhanced. Clearly this effect of host gene expression is more easily reconciled with the threshold model than either of the alternative hypotheses.

The influence of viruses on PTGS

Homology-dependent resistance varies in strength between lines. In the most extreme examples there is no detectable accumulation of the target virus anywhere in the inoculated plant [31, 40, 44, 53]. In other examples the resistance is weaker and there may be virus accumulation at least on the inoculated leaf. One of these intermediate types of homology-dependent resistance involves TEV [17, 33]. Initially the plant was susceptible to the TEV and the viral cDNA transgene was expressed at a high level. The initially infected leaves showed the normal TEV symptoms. However, in the upper leaves of the plant the symptoms were attenuated or absent and there was little or no TEV accumulation. Accumulation of the transgene RNA was suppressed in this asymptomatic tissue and there was resistance to secondary infection provided that the inoculated virus was similar to the sense RNA of the transgene. This induced resistance was referred to as 'recovery' [33], although that term is not strictly accurate because the resistant tissue never showed signs of disease. It was suggested that the resistant state in these infected plants was related to PTGS mediated by an RNA threshold. The combined accumulation of homologous RNAs produced by the transgene and the virus would have contributed to this threshold.

Viral RNA accumulation may also influence endogenous plant gene (i.e. non-transgene) expression. When *Nicotiana benthamiana* was inoculated with a TMV genome carrying part of the *N. benthamiana* phytoene desaturase (PDS) gene there was evidence for suppression of the endogenous PDS expression [30]. On the inoculated leaf and the first leaves to be systemically infected there were mild mosaic symptoms typical of wild-type TMV. In contrast, in the upper parts of the plant there was photobleaching consistent with suppression of the host PDS sequence and consequent loss of carotenoid protection against photo-oxidation. This suppression of PDS was likely due to virus-induced PTGS of the homologous host genes and, like the recovery phenomenon, could be more easily explained by the threshold model than the direct antisense and aberrant RNA models.

The influence of transgene transcription on PTGS

The threshold hypothesis of PTGS leads to the prediction that high-level transcription of a transgene would be both necessary and sufficient for PTGS. Consistent with this prediction is the fact that most reports of PTGS involve the 35S promoter of CaMV which is transcribed at a high level throughout the plant (Table 1). Analyses of transcription in the examples of PTGS associated with homology-dependent resistance to TEV and PVY are also consistent with the threshold hypothesis. Transcription of the viral transgenes was higher in the resistant lines displaying PTGS than in susceptible lines in which the transgene RNA accumulated at high levels [17, 33]. The expression of PTGS in tobacco lines with single copy GUS transgenes is also consistent with the threshold model. In these lines the PTGS developed during later stages of plant growth [13]. There were high levels of GUS and GUS mRNA in the juvenile plants and it was suggested that these high levels of transgene expression exceeded the threshold necessary for activation of PTGS.

One or more mechanisms to initiate PTGS?

It is difficult to reconcile these various observations with any one of the three models of the mechanism for the initiation of PTGS. The direct production of antisense RNA does not fit the run-off analysis of antisense RNA production, methylation data or the influence of host gene transcription, virus infection or transgene transcription on PTGS. The threshold model does not obviously tie in with the transgene methylation data. It is also difficult to reconcile the threshold model with the finding that there is PTGS of chalcone synthase in petunia due to a transgene construct that is transcribed at a very low level, if at all [51]. Nor does the threshold model fit easily with several examples of PTGS with

35S constructs in which the silencer transgenes are transcribed at a similar or lower levels than loci that do not confer PTGS [12].

The aberrant RNA model can accommodate the methylation data and can explain why there is not necessarily a correlation of transgene transcription and PTGS. However this model is not consistent with the influence of endogenous genes on PTGS. Nor does it explain PTGS of GUS in tobacco lines with single copies of the GUS transgene [13]. There is no potential for ectopic pairing of homologous DNA in these lines and it is not obvious why these single-copy transgenes would produce aberrant RNA.

The failure of any one model to accommodate all of the experimental data must mean there are several mechanisms or that the models need further refinement. Two refinements to the aberrant RNA model have been recently described which may remove some of the inconsistencies referred to above and which may also unite aspects of the aberrant RNA model with the threshold model [5].

The first of these refinements addresses the initiation of PTGS when the genome carries multiple homologous sequences. According to the original model, the production of aberrant RNA follows from ectopic pairing of homologous DNA and the consequent methylation of transcribed regions [14]. The proposed refinement to the model is that the ectopic pairing would be a non-reciprocal interaction of homologous sequences. Only one of the interacting DNAs need be transcribed and methylated consequent to the ectopic pairing. This locus, referred to as the receptor locus, would be the template for abberant RNA production. The second silencer locus need not be transcribed or methylated by the interaction. The only requirement of the silencer locus is that it has the potential to pair ectopically with the receptor locus. There is a precedent for non-reciprocal ectopic pairing of DNA from the analysis of transcriptional gene silencing of the nopaline synthase promoter [37].

This refinement to the model was proposed to account for the potential of a non-transcribed chalcone synthase transgene to silence the endogenous gene. The modification could also accommodate the influence of endogenous genes on PTGS of PG [48] and PE [47] in tomato and SAM synthetase [6] and nitrate reductase in tobacco [9]. According to the modified model the transgenes in these examples would carry out the silencer role and the endogenous homologues the receptor function. The model thus allows for PTGS independently of transcription of the transgene.

The second refinement to the aberrant RNA model is similar to the first but proposes that, in certain situations, the role of the silencer locus could be carried out by RNA. This variation is proposed to account for the influence of viruses on PTGS. It would require that there is the potential for the viral RNA or the transgene RNA to interact with the homologous sequence in the nucleus. This RNA:DNA interaction would lead to DNA methylation and would initiate production of the aberrant RNA as proposed originally.

A precedent for DNA methylation due to an RNA:DNA interaction is provided by the analysis of plants carrying transgenes based on viroid cDNA [56]. Whenever those plants were infected and produced high levels of the viroid RNA there was methylation of the viroid cDNA transgenes. The transgenes were not methylated in the uninfected plants.

For RNA-directed methylation of DNA there must be the potential for the RNA to interact directly with the homologous DNA. Viroid RNAs accumulate in the nucleus and this type of interaction could occur throughout the plant. In contrast, viral RNAs accumulate in the cytoplasm of infected cells. Consequently, the potential for an interaction of viral RNA with homologous DNA would be restricted to dividing cells during the phase of nuclear membrane breakdown. This proposed requirement for an interaction of viral RNA with homologous DNA means that viruses would not activate PTGS in the inoculated tissue in which there would be little or no cell division. The PTGS should be manifest only in cells that undergo division and emerge from the meristem after the inoculated virus has spread through the plant. The development of the recovery phenotype in young leaves of plants displaying the induced form of homology-dependent resistance to TEV is consistent with this prediction [33]. Similarly, the virus-induced silencing of the host PDS gene is restricted to tissue that develops after inoculation and is also consistent with the proposed interaction of viral RNA and DNA in dividing cells [30].

An RNA:DNA interaction could also account for PTGS in the absence of virus infection when there is only a single transgene present in the plant genome and therefore no opportunity for ectopic pairing of homologous DNA [13]. In this situation the RNA:DNA interaction would involve the transgene and its RNA product instead of viral RNA. The suggestion of this type of interaction brings together the threshold and aberrant RNA hypotheses of PTGS because the likelihood of the RNA:DNA interaction would increase with the accumulation of the silencer RNA. It is likely

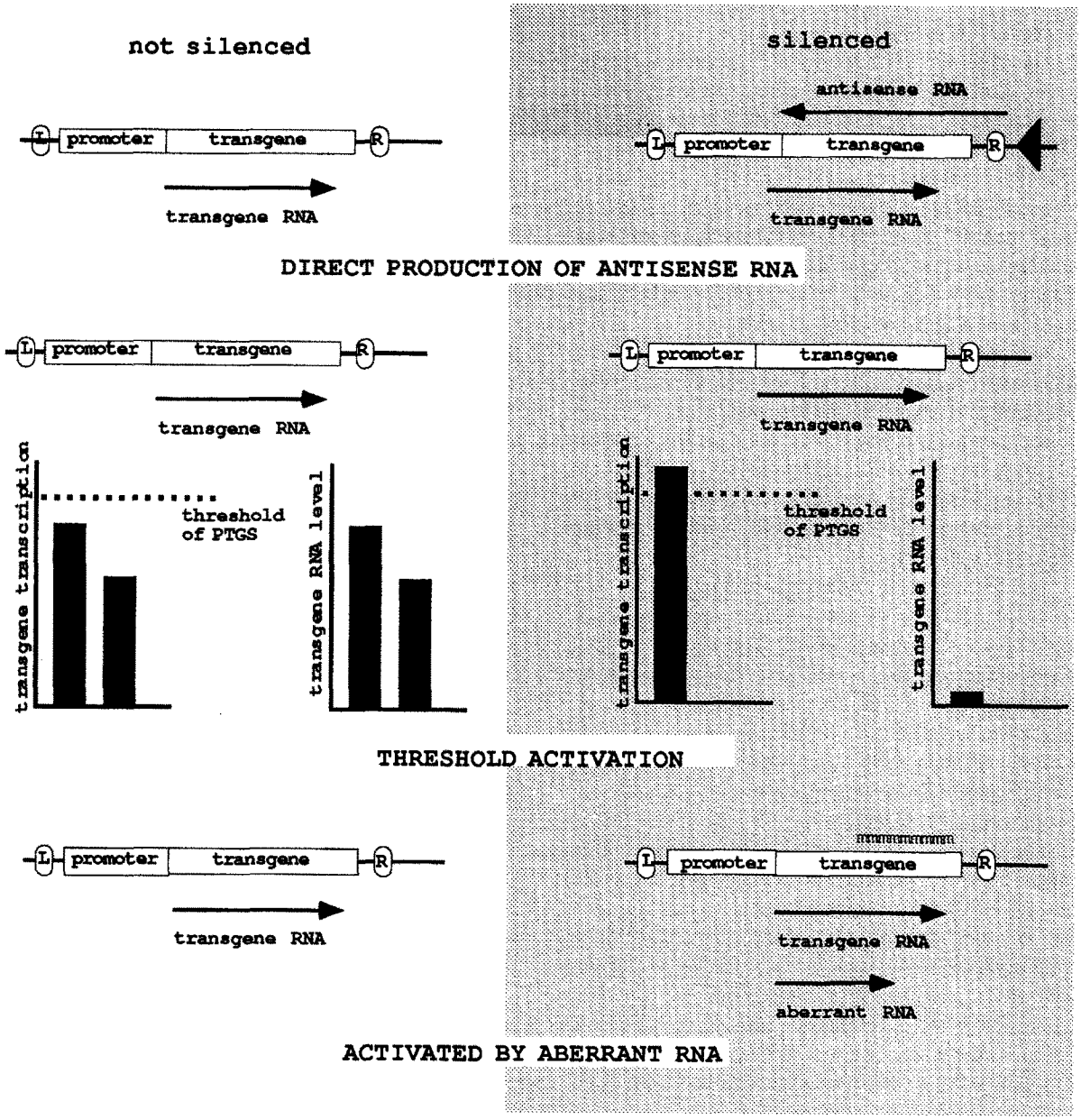

Figure 1. Hypothetical mechanisms of PTGS and homology-dependent resistance. These models are based on a transgene construct in which a promoter coupled to a transgene between the left (L) and right (R) borders of the T-DNA of *Agrobacterium tumefaciens* Ti plasmid is transformed into a plant genome. The direct production of antisense RNA model proposes that there is no PTGS when the T-DNA is inserted in the plant genome unless the insert is adjacent to a plant promoter (triangle). This promoter would produce the antisense RNA that could feature in the mechanism of cosuppression and gene silencing. The threshold model proposes that PTGS is activated by high level transcription of the transgene. If transcription is below this threshold the final RNA accumulation may be either high, medium or low corresponding to the transcription rate. However if the transcription is above the threshold the final accumulation of transgene RNA (and other RNAs with homology to the transgene) would be very low. The transcription rate may be affected by features of the genome or chromatin at or close to the transgene insert. In the aberrant RNA model it is proposed that the transgene in the silenced state produces an aberrant RNA which could activate the cosuppression mechanism. A factor that could influence production of the aberrant RNA is methylation (m) in the transgene. If the transgene is not silenced there may be either no methylation or methylation that does not affect production of aberrant RNA.

that accumulation of silencer RNA in dividing cells, as for the virus-induced PTGS, and for the same reasons, would lead to stronger silencing than RNA accumulation in differentiated cells.

Somatic instability of PTGS

One of the more striking features of PTGS is somatic instability. Flowers of petunia, in which there is PTGS of CHS, are not usually uniformly white as would be expected if the anthocyanin pigmentation pathway was blocked [41, 52]. There is often a variegated phenotype in which white and pigmented tissues are mottled or display spectacular patterns. The PTGS associated with homology-dependent resistance to PVY is somatically unstable and was lost when the transgenic plants are cultured *in vitro* and allowed to regenerate back into mature plants [50]. This instability, and the consequent unpredictability of a transgene phenotype, is a serious constraint to the practical application of transgenic plants.

The only clue to the basis of the instability of PTGS is from the transgene methylation data. Instability of transgene methylation could lead either to loss or to suppression of PTGS. There would be the potential for loss of PTGS if there is a failure to maintain the methylation of the transgene during cell division and if, as predicted by the aberrant RNA model, methylation is required for PTGS. There is also the potential for an increase of transgene methylation to suppress PTGS. The increased methylation could lead to transcriptional suppression of the transgene [39, 45]. This transcriptional suppression would lead to inhibition of PTGS if, as in the aberrant RNA and threshold models, sense RNA production is necessary for the silencing phenotype.

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