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The effect of endogenous mRNA levels on co-suppression in tomato

Yuanhuai Han[a,b], Allen Griffiths[a,1], Hongying Li[a,b], Don Grierson[a,*]

[a] *Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK*
[b] *School of Bioengineering, Chongqing University, Sha Zheng Jie 174#, Sha Ping Ba Qu, Chongqing 400044, PR China*

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Abstract Introduction of truncated polygalacturonase (PG) transgenes into tomato plants caused the production of small interfering RNAs (siRNAs) and co-suppression of both the endogenous and PG transgenes in ripening fruits by post-transcriptional gene silencing. In order to test the possible effect on co-suppression of the endogenous PG mRNA level, we transferred the PG transgenes from a PG-silenced line (wild type background) by crossing to two ripening regulatory mutants with reduced PG: *Never-ripe* (*Nr*, ~10% endogenous PG mRNA compared to wild type) and *ripening-inhibitor* (*rin*, ~1% endogenous PG mRNA) and to wild type (as a control). The PG transgenes caused strong co-suppression of the transgenes and the endogenous PG gene in cells with high PG mRNA background (wild type) and silencing appeared to be linked with higher transgene copy number and/or a particular transgene locus. In cells with low endogenous PG mRNA accumulation (*Nr*), the endogenous PG gene was preferentially suppressed compared to the transgenes, whose expression was not reduced significantly. The expression of the transgenes was also not reduced in the very low PG background (*rin*), in which endogenous PG was barely detectable. In all the analysed lines with all three PG background levels, siRNAs accumulated in leaves and green fruits, in which the endogenous PG gene is not transcribed. The relatively abundant production of siRNAs in most of the lines was linked with a particular transgene insert. These results suggest that a certain threshold level of endogenous PG mRNA is required for the co-suppression of the truncated PG transgenes and the endogenous PG gene or for extensive silencing of the transgenes.

Key words: RNA interference; Post-transcriptional gene silencing; Co-suppression; Threshold

1. Introduction

The phenomenon of post-transcriptional gene silencing (PTGS), initially discovered in transgenic plants [1–3], has been extended to fungi (quelling in *Neurospora crassa*) [4] and animal cells (RNA interference or RNAi) such as *Caenorhabditis elegans*, *Drosophila* and mammalian cells [5–7]. PTGS has been used as an important tool to study gene function in plants and RNAi is proving itself a remarkable tool for reverse genetics [8,9]. Biochemical analysis of the post-transcriptional degradation process in *Drosophila* cells has helped to gain some insights into the mechanism behind the phenomenon. Long double-stranded RNAs (dsRNA) applied exogenously into the cells are cleaved into 21–25 nucleotide (nt) small interfering RNAs (siRNAs) [10]. This process is ATP-dependent and catalysed by the enzyme Dicer, a member of the RNase III family of dsRNA-specific endonucleases [6,11]. These siRNA duplexes are then incorporated into a protein complex, RNA-induced silencing complex (RISC), which is guided by the siRNA to the target mRNA to be cleaved [6,10,12,13]. Long dsRNA formed within a transgene transcript or between sense and antisense transgene mRNA also causes strong silencing of cognate target genes in plants, which is probably based on the same mechanism as RNAi [14–19]. In plant PTGS caused by sense transgenes, RNA complementary to the sense transgene RNA is suggested to be generated by RNA-dependent RNA polymerase (RdRP) and forms dsRNA with the transgene mRNA, from which siRNAs are generated [20,21]. SiRNAs from the polygalacturonase (PG) transgenes that caused silencing of the endogenous genes [2] were shown to be associated with co-suppression, resulting in the cleavage of the endogenous PG mRNA, and accumulation of intermediate RNA degradation fragments [22]. These findings suggest that the various types of silencing systems in animals, plants and fungi share common mechanisms.

A key question remains, however, as to what triggers gene silencing in transgenic plants. If the transgene itself is constructed to express mRNA that can form hairpin structures [15,19], this is sufficient to give a high level of silencing. In addition, normal transgene mRNA without secondary structures may form dsRNA with its antisense RNA generated via RdRP, thereby initiating silencing. How can plant cells recognise the transgene mRNA as abnormal and start to make antisense RNA using RdRP? One hypothesis is that RNA over a threshold level switches on the gene silencing machinery. In most cases of gene silencing in plants, it has been found that multiple copies of the transgenes were integrated into the genome [23,24]. However, if only a single copy of a transgene is highly expressed it can cause gene silencing. A single insertion of a chalcone synthase transgene driven by the cauliflower mosaic virus (CaMV) 35S promoter with a double enhancer triggered gene silencing, whereas the same gene driv-

*Corresponding author. Fax: (44)-115-951 3298.
E-mail address: donald.grierson@nottingham.ac.uk (D. Grierson).

[1] Present address: Unilever Research and Development, Colworth, Sharnbrook, Bedford MK44 1LQ, UK.

Abbreviations: AC, Ailsa Craig; CaMV, cauliflower mosaic virus; *Nr*, Never-ripe; PG, polygalacturonase; PGS, PG-silenced; PTGS, post-transcriptional gene silencing; RdRP, RNA-dependent RNA polymerase; *rin*, ripening-inhibitor; RNAi, RNA interference; siRNA, short interfering RNA

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en by a less active promoter did not [25]. Transgenic plants hemizygous for a β-1,3-glucanase *gn1* transgene did not show silencing, whereas plants homozygous for the transgene did [26]. These observations suggest a correlation between high transcript abundance and silencing. Features of transgene-induced virus resistance can also be reconciled with this model. In a study of transgene-mediated resistance to tobacco etch virus, it was found that three or more transgenes were necessary to establish a highly resistant state [27]. One or two transgene copies resulted in inducible resistance (or recovery), in which accumulation of homologous RNAs from the transgene and the virus may be over a threshold and trigger gene silencing [27,28]. However, not much has been learnt about the possible role of homologous endogenous mRNA in silencing.

Here we take advantage of the tomato PTGS system, with the developmental switch-on of the endogenous PG target gene and mutants expressing the endogenous PG gene at different levels, to test the effect of endogenous mRNA level on gene silencing. We introduced the silencing PG transgenes by crossing from the tomato PG-silenced line (PGS) [2,22] to low endogenous PG RNA tomato mutants *Never-ripe* (*Nr*, ~10% endogenous PG mRNA of that in wild type), *ripening-inhibitor* (*rin*, ~1% PG mRNA) [29] and wild type (Ailsa Craig) as a control. Northern analysis of small RNAs and endogenous and transgene PG mRNA in the F2 generation of these three crossing groups showed that abundant siRNAs are accumulated in most of the analysed lines containing PG transgenes, yet co-suppression or extensive silencing of the transgenes occurs only in lines with high and low PG background but not in very low PG background, suggesting that the level of endogenous PG mRNA does have an important role in PTGS.

2. Materials and methods

2.1. Plant materials

The PGS transgenic tomato line was derived from the progeny of the transgenic plant line T130 described by Smith et al. [2] and further analysed by Han and Grierson [22]. The transgene sequence corresponds to the 5′ end (730 bp) of the fruit-ripening-specific PG cDNA from tomato and is driven by the CaMV 35S promoter. The transgenes were transferred into *Nr*, *rin* and wild type by crossings, using the transgenic plant as the pollen parent. The progenies were selected on kanamycin medium and screened for *Nr* and *rin* phenotypes. *Nr* fruit ripen to an orange colour and remain firm compared to Ailsa Craig [30]. Mutant *rin* fruit are characterised by an enlarged corolla and calyx from a fairly early developmental stage [31] and the fruits turn yellow but do not ripen. The definition of the different stages of the fruit development was as described by Barry et al. [32].

2.2. Southern analysis

Genomic DNA was extracted from young leaves using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma). The DNA was digested with *Xba*I and *Hin*dIII overnight at 37°C and separated in agarose gels. Equal loading of the samples was checked by ethidium bromide staining. The DNA was transferred to Genescreen hybridisation membrane (Perkin Elmer Life Science). Prehybridisation was carried out at 65°C for 1 h in 5×SSPE/5×Denhardt's solution/1% sodium dodecyl sulphate (SDS)/100 μg/ml sheared and denatured salmon sperm DNA. The DNA probe used was made from the NPTII gene using the Rediprime II system (Amersham Pharmacia Biotech). Hybridisation was carried out at 65°C overnight, followed by washes with 2×SSC/0.1% SDS and 1×SSC/0.1% SDS at 65°C.

2.3. Northern analysis

RNA was extracted from fruits and leaves using methods described by Han and Grierson [17,22]. RNA (10 μg) was separated on a 25 mM sodium phosphate (pH 6.5)/3.7% formaldehyde/1.0% agarose gel and blotted to Genescreen hybridisation membrane. The filters were prehybridised in 50% formamide/1% SDS/1 M NaCl/10% dextran sulphate/100 μg/ml sheared and denatured salmon sperm DNA and hybridised with ^{32}P-random prime-labelled DNA probes at 42°C. The filters were washed in 0.1×SSC/0.1% SDS at 65°C and exposed to Kodak X-omat film between two intensifying screens at −70°C.

2.4. Extraction and detection of small RNAs

Small RNAs were extracted and transferred to Hybond-NX membrane (Amersham Pharmacia Biotech) as described previously [20]. After the first ethanol precipitation in a total RNA preparation [17,22], the pellet was re-dissolved in 2 ml water. High molecular weight nucleic acids were removed by precipitation in 10% PEG 8000/0.5 M NaCl and small RNAs were enriched using a Qiagen-tip 20 (Qiagen). Small RNAs were separated through 15% polyacrylamide/7 M urea gels, transferred onto Hybond NX filters by electrophoretic transfer at 250 mA for 30 min and cross-linked by UV using a Stratalinker® (Stratagene). Prehybridisation was performed in 40% formamide, 7% SDS, 0.3 M NaCl, 0.05 M Na_2HPO_4–NaH_2PO_4 (pH 7), 1×Denhardt's solution, 100 μg/ml sheared and denatured salmon sperm DNA for 30 min at 30°C. Hybridisation was in the same solution for 16 h at 30°C and the filters were washed with 2×SSC/0.2% SDS at 50°C for 3×10 min.

2.5. Hybridisation probes

The random prime-labelled DNA probes used for the detection of PG were made from the 5′ half of the PG cDNA using the Rediprime II system (Amersham Pharmacia Biotech). An antisense-specific riboprobe corresponding to the PG transgene was generated using an in vitro transcription system (Promega) as described previously [33]. Riboprobes were treated by RNase-free DNase (Promega) to remove the DNA template and hydrolysed to an average size of 50 nt by mixing them with 200 μl of alkaline buffer (120 mM Na_2CO_3, 80 mM $NaHCO_3$) and incubating at 60°C for 2–3 h depending on the length of the riboprobes.

3. Results

3.1. Strong co-suppression is correlated with high levels of siRNAs

Strong co-suppression of the endogenous and PG transgene was observed in the PGS line, bred from the progeny of T130 containing truncated sense PG transgenes driven by the CaMV 35S promoter [2]. This line was shown to contain PG transgene inserts at two loci, corresponding to 4 kb and 9 kb *Xba*I/*Hin*dIII fragments (Fig. 1A). Transferring the PGS transgenes into wild type Ailsa Craig (AC++) by crossing PGS pollen to AC++ and then selfing resulted in AC×PGS lines containing different copies of the transgene and rearrangement of the transgenes in the genome. Lines 16 and 29 inherited the 4 kb locus from PGS and the transgene was homozygous (Fig. 1A). Line 22 contained transgenes in both loci as in PGS but it was hemizygous at the 9 kb locus. In addition to the two loci of the PGS parent, line 31 had an extra transgene rearranged in a different locus and at all three loci the transgenes were hemizygous. Line 33 lacked the 9 kb locus compared with line 31. The variation of transgene number and hemi-/homozygous status of the transgene locus among these lines presented an advantage for analysing the dosage effect of the transgene on co-suppression. Northern analysis of the RNA from ripening fruits (5 days post-breaker or B+5, breaker stage refers to fruits that are beginning to change colour) of these lines using a probe made from the PG transgene indicated that higher gene dosage of the transgenes or the presence of the 9 kb transgene in lines 22 and 31 might be responsible for the stronger co-suppression in these lines compared to those without the 9 kb insert and showing fewer copies of the transgenes (lines 16, 29 and 33) (Fig. 1A,B).

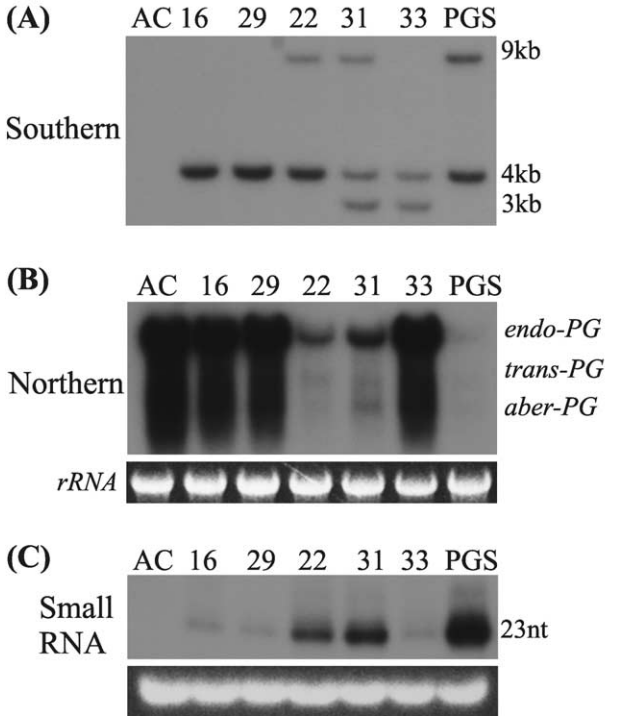

Fig. 1. Strong co-suppression in a high endogenous PG mRNA background. PG transgenes were transferred by crossing from the PGS line into wild type (AC) resulting in AC×PGS lines. A: The copy number of the transgenes analysed by Southern hybridisation. The genomic DNA was digested with *Xba*I/*Hin*dIII and probed with the transformation selection marker gene NPTII. B: The expression of the endogenous PG gene (endo-PG), the PG transgene (trans-PG) and aberrant PG RNA (aber-RNA) in ripening fruits (B+5, 5 days after the fruit starts to turn red). C: Accumulation of small RNAs. The probes used for Northern and Southern hybridisation were made from the 5′ half of the PG cDNA and NPTII (the selection marker) gene respectively. The riboprobe used for the detection of small siRNAs was made from the in vitro transcribed 5′ half of the PG gene and hydrolysed as described by Han and Grierson [22]. The positions of the related mRNAs and sizes of the genomic DNA fragment are shown on the right. Equal loading of genomic DNA was confirmed before Southern blotting (data not shown) and equal loading of RNA samples for Northern analysis of total RNA and small RNAs was shown by ethidium bromide staining of rRNA and the predominant RNA species respectively.

Hybridisation of small RNA species extracted from leaves of AC×PGS lines with a hydrolysed antisense-specific PG transgene riboprobe showed that siRNAs about 23 nt in size were present in all these lines and the PGS parent (Fig. 1C). Lines 22, 31 and PGS containing more transgene copies than the other lines and the 9 kb transgene locus accumulated more siRNAs (Fig. 1C) than the other lines and showed co-suppression (Fig. 1B). There appeared to be a correlation between higher copy number of the transgenes and strong silencing. However, it cannot be excluded that a particular transgene insert (9 kb locus) may be linked with the abundant siRNAs and strong co-suppression as indicated below (Figs. 2 and 3). Analysis of siRNAs in immature green (IMG) fruits in these lines showed the same pattern as in the leaves (data not shown).

3.2. Endogenous PG gene is preferentially suppressed relative to the PG transgene in a low PG mRNA background

PG transgenes were transferred into the *Nr* background by crossing with the PGS line. The F1 population was screened for the PG transgene using polymerase chain reaction detection, selfed and the resulting F2 population was screened for plants homozygous for the *Nr* phenotype and analysed by Southern hybridisation. *Nr* fruit ripen to an orange colour and remain firm compared to wild type Ailsa Craig. Line 10 inherited the transgene at the 4 kb locus and lines 11, 27 and 31 inherited the transgene at the 9 kb locus (Fig. 2A). The transgenes at these loci were all homozygous. However, lines 11 and 27 showed a hemizygous *Nr* phenotype. From Northern hybridisation of the RNA from ripening fruits (B+5) in these lines (Fig. 2B), it was observed that the *Nr* homozygous lines 10 and 31 showed slight and strong suppression of the endogenous PG gene respectively compared to PG expression

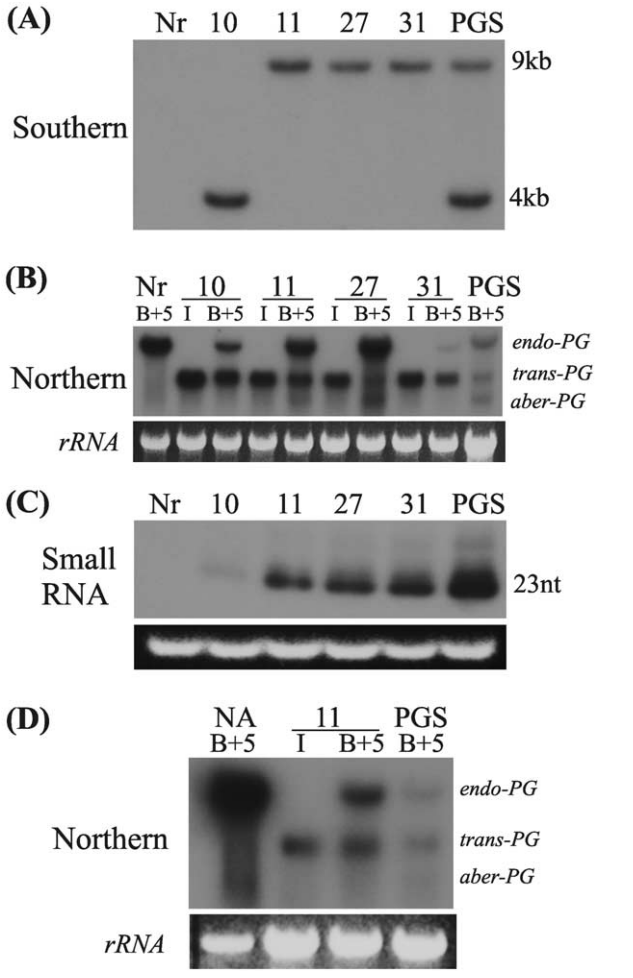

Fig. 2. Preferential silencing of the endogenous PG gene compared to the truncated PG transgenes in a low endogenous PG mRNA background. PG transgenes were transferred by crossing from the PGS line into a ripening mutant 'Never-ripe' (*Nr*), resulting in *Nr*×PGS lines. A: The copy number of the transgenes analysed by Southern hybridisation as described in Fig. 1. B: The expression of the endogenous PG gene (endo-PG), the PG transgene (trans-PG) and aberrant PG RNA (aber-RNA) in ripening fruits (B+5, 5 days after the fruit starts to change colour). Transgene mRNA in immature green fruits (referred to as 'I' for immature) was also analysed as a control to test the silencing of the transgene during ripening. C: Accumulation of small RNAs. D: The endogenous PG gene in *Nr*×PGS line 11 (*Nr* hemizygous) is silenced in comparison to that in NA (non-transgenic *Nr* hemizygous line generated by crossing *Nr* to wild type AC). Preparation of probes and loading controls were as described in Fig. 1.

in *Nr* during ripening (B+5). The transgene expression, however, decreased only slightly in ripening fruits compared to that in immature green fruits. For each line, the difference in hybridisation signals of the endogenous PG mRNA between *Nr* and line 10 or 31 was greater than that of the transgene between the immature green stage and ripening stage. This means that the suppression of the endogenous PG gene is much stronger than the suppression of the transgene. This is also true with line 11 (Fig. 2D). From the Northern results in Fig. 2B, it appeared at first sight as though there was no silencing of the endogenous PG gene in lines 11 and 27. However, it should be stressed that the background PG expression level of these transgenic lines is higher than that of *Nr*, as these lines are hemizygous at the *Nr* locus. To investigate this, we generated a new *Nr* hemizygous line NA by crossing *Nr* and AC as a control for these two *Nr* hemizygous lines. The endogenous PG mRNA level in line 11 was compared with that in NA and the results showed that the endogenous PG gene is suppressed in line 11 (Fig. 2D).

Probing for small PG RNA species in the leaves of the four *Nr*×PGS lines and *Nr* showed that 23 nt PG siRNAs were present in the *Nr*×PGS lines, not in the *Nr* mutant (Fig. 2C). The level of these 23 nt siRNAs was much higher in lines 11, 27 and 31 than line 10, and the high level of siRNAs in these lines was, therefore, linked with the 9 kb transgene locus. There seemed to be a correlation between the level of 23 nt siRNAs and the degree of silencing. Line 10, containing the 4 kb insert only, accumulated less siRNA and showed weaker silencing whereas line 31, containing the 9 kb insert, accumulated more siRNA and showed stronger silencing (Fig. 2B).

3.3. *Transgenes are not silenced in a very low PG mRNA background*

PGS transgenes were transferred by crossing into *rin*, which produces approximately 1% PG mRNA, and the F2 population (*rin*×PGS) was obtained by the same approach as for *Nr*×PGS. Southern analysis showed that lines 5 and 25 inherited the homozygous transgene at the 9 kb locus (Fig. 3A). Transgenes in line 26 were located at both loci as in the PGS parent, with the 9 kb locus being hemizygous. Line 31 contained only a hemizygous transgene at the 4 kb locus. Since the level of endogenous PG transcript is so low that it is barely detectable by Northern hybridisation [29], we compared the levels of transgene expression in fruits between the immature green stage and 7 weeks post-anthesis (when fruits start to turn yellowish, a stage equivalent to B+5 in the Ailsa Craig and *Nr* controls) (Fig. 3B). None of the four lines tested showed any suppression of the transgenes at 7 weeks post-anthesis. The transgenes in these lines were also expressed at a higher level than in the PGS line (Fig. 3B). However, the accumulation of small RNAs complementary to the PG transgene transcripts in *rin*×PGS lines 5, 25 and 26, which contained the 9 kb transgene insert, is similar to that in the PGS line, and much higher than in line 31 which is hemizygous for the transgene at the 4 kb locus (Fig. 3C).

It appeared that higher accumulation of siRNAs was associated with the 9 kb locus in the plants resulting from the three crosses, since lines that inherited the 9 kb locus (AC×PGS lines 22 and 31, *Nr*×PGS lines 11, 22 and 31, *rin*×PGS lines 5, 15 and 26) showed much higher accumulation of 23 nt siRNAs than lines that just inherited the 4 kb locus.

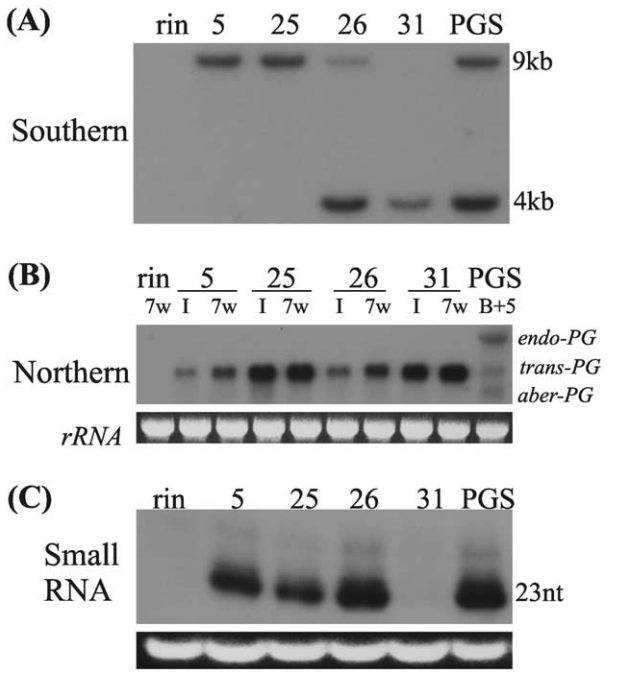

Fig. 3. Trangenes are not silenced in a very low PG mRNA background. PG transgenes were transferred by crossing from the PGS line into a ripening mutant 'ripening-inhibitor' (*rin*), which contains less than 1% endogenous PG mRNA, resulting in *rin*×PGS lines. A: The copy number of the transgenes was analysed by Southern hybridisation as described in Fig. 1. B: The expression of the PG transgene (trans-PG) in fruits at 7 weeks after anthesis (equivalent to the ripening stage B+5 of PGS fruits). Transgene mRNA in immature green fruits (referred to as 'I' for immature) was also analysed as a control to test the silencing of the transgene during ripening. C: Accumulation of small RNAs. Preparation of probes and loading controls are as described in Fig. 1.

4. Discussion

Introduction of truncated PG transgenes into tomato plants caused co-suppression of both the transgene and the endogenous ripening-specific PG gene [2]. We transferred the PG transgenes into low PG tomato ripening mutants *Nr* and *rin* and to wild type (as control) by crossing, producing *Nr*×PGS, *rin*×PGS and AC×PGS lines respectively. Lines that were homozygous and hemizygous at the transgene locus or *Nr* locus were also selected. Among AC×PGS and *Nr*×PGS lines, we observed that strong silencing was correlated with high levels of the siRNAs (Figs. 1A,B and 2A,B). A particular insert of the transgene (9 kb locus), but also the copy number of the transgene, appeared to be related to the high levels of siRNAs (Figs. 1B,C and 2B,C) and strong silencing (Fig. 1A,B). This last result is consistent with the dosage effect of transgenes on silencing observed previously [34–36].

We showed previously that siRNAs were accumulated in leaves and green fruits of the PGS plants even before the endogenous PG was transcribed [22], indicating that there is a potential for silencing. This appears to be correlated with the presence of strong silencing transgenes. When PGS transgenes were transferred into tomato plants with lower expression levels of the ripening-specific PG gene (*Nr* 10%, *rin* 1% of PG mRNA in wild type), however, the expression of the transgene was not inhibited much during ripening,

even though siRNAs as abundant as in the control lines (AC×PGS) were accumulated in some *Nr*×PGS and *rin*× PGS lines (Figs. 1C, 2C and 3C). In the *Nr* background, the expression of the transgene was not inhibited much after the endogenous PG was switched on, even though the endogenous PG was clearly suppressed (Fig. 2B); in the very low PG expression background (*rin*, 1% of wild type PG mRNA), the expression of the transgene did not decrease during the 'ripening' process in this mutant (Fig. 3B), when less than 1% of the normal PG mRNA is accumulated [29]. Therefore, the expression level of mRNA from the endogenous PG gene appeared to affect the initiation and degree of silencing of the transgenes. The relationship between transgene and endogenous gene has been investigated since the phenomenon of silencing was first discovered. It has been observed that high levels of transgene mRNA, due to either a strong promoter or multiple insertions of the transgene, were associated with silencing (as discussed in Section 1) [25–27]. High levels of transgene mRNA in the shoots to be used as scions are also required for grafting-transmitted gene silencing [37]. Similarly, association of high expression of homologous endogenous genes with co-suppression was also reported [2,38]. It was also shown that transgenes could interfere with the processing of homologous endogenous pre-mRNA, leading to much higher accumulation of unspliced transcript of the gene compared to that in the wild type [38,39]. Examples of the interaction between transgene and endogenous gene, and its influence on the levels of transgene and endogenous gene transcripts, have also been reported [39,40].

The silencing of the transgene in the background of high endogenous PG mRNA (AC and *Nr*), but not in the background of very low PG mRNA (*rin*), may be explained by the following model. Accumulation of siRNAs generated from the transgenes may inhibit the action of Dicer on the dsRNA produced from the transgene, as suggested by the in vitro experiments in *Drosophila* embryo lysates and wheat germ extract [41]. Thus, in the high endogenous PG mRNA background, many siRNAs are incorporated into RISC to target endogenous PG mRNA and may subsequently be degraded during the action. This may result in transient reduction of siRNAs, leading to activation of cleavage of transgene dsRNA and a decreased level of sense transgene mRNA as we observed. In the *rin* background, with very low PG mRNA, only small amounts of siRNAs are required to target the endogenous PG, therefore the inactivation of Dicer is not relieved and the transgene dsRNA is not cleaved. Active involvement of the endogenous PG gene in the silencing process may be another explanation for our results. Recently, it was demonstrated that endogenous genes play an active role in transgene-mediated co-suppression in tobacco [42]. Endogenous-specific siRNAs were detected in the co-suppressed plants, in greater abundance than those from transgenes, causing more efficient silencing of a virus containing a transgene sequence. However, this is not supported by our failure to detect any small RNAs generated from the 3′ end of the endogenous PG gene, but only from the truncated transgene [22].

In conclusion, we have demonstrated that siRNAs were generated in leaves and green fruits before co-suppression [22], the level of siRNAs is linked with a particular transgene insert and also possibly transgene copy number, and high levels of endogenous PG mRNA were important for the effective silencing of the endogenous PG and the transgene mRNAs.

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