Delayed activation of post-transcriptional gene silencing and de novo transgene methylation in plants with the coat protein gene of sweet potato feathery mottle potyvirus

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Abstract

The relationship between post-transcriptional gene silencing (PTGS) and DNA methylation was examined using Nicotiana benthamiana transformed with the coat protein gene including the 3’ non-translated region of sweet potato feathery mottle potyvirus. Line 4.28 showed a delayed activation of the transgene silencing in comparison with the other silenced lines, and showed complete resistance against the recombinant potato virus X engineered to contain the sequence homologous to the transgene when the silencing was activated. The transgene methylation in line 4.28 was less extensive in comparison with those of the other silenced lines before the silencing was activated. However, the extent of methylation increased in the course of plant development and became comparable with those in the other silenced lines. The activated silencing and the increased transgene methylation were reset after meiosis. However, the characters of delayed activation of the silencing and developmentally increased transgene methylation were meiotically transmitted to the next generation. These results suggest that transgene(s) itself has a potential to trigger and reset DNA methylation, which could determine a state of PTGS. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: DNA methylation; Gene silencing; Transgenic plants; Virus resistance

1. Introduction

Numerous attempts to confer virus resistance to plants using virus genes or sequences have been reported [1]. This form of resistance is known as pathogen-derived resistance (PDR) [2]. The first description of PDR was the introduction of the coat protein (CP) gene of tobacco mosaic virus into tobacco plants [3]. In this case, it is postulated that virus disassembly may be blocked by transgenic CP, thereby preventing different steps of the viral infection cycle including the replication and/or the cell-to-cell or long distance movement [4,5]. Other viral genes such as genes encoding replicase, movement protein, and other non-structural proteins have been shown to be effective for PDR [1]. A common characteristic of the viral protein-mediated resistance is that resistance usually correlates with high levels of expression of the transgene. Another mechanism of PDR to plant viruses is RNA-mediated and is typically associated with transgenic plants with low or undetectable level of transgene expression. This type of resistance was referred to as homology-dependent virus resistance (HDR) [6].

HDR has been postulated to operate in cytoplasm via a mechanism similar to cosuppression or post-transcriptional gene silencing (PTGS), because viral RNA replication is restricted to the cytoplasm [7–9]. HDR is highly specific for viruses having RNA sequences that are similar to the
virus sequences used for plant transformation [7–9]. Various factors have been suggested to play a role in activation of the silencing mechanism, e.g. exceeding an RNA threshold level [7,9], the production of aberrant RNA [10], and ectopic pairing of homologous DNA [11] and production of double-stranded RNA (ds RNA) [12]. These could be related to multiple copies of the transgene [13] or the state of methylation [10]. In the present study, the correlation between PTGS and de novo transgene methylation was examined using transgenic plants that show a delayed activation of PTGS.

2. Materials and methods

2.1. Virus and transgenic plants

The origin of the severe strain of sweet potato feathery mottle potyvirus (SPFMV-S) has been reported [14]. SPFMV-S does not infect Nicotiana benthamiana [14]. The transgenic N. benthamiana lines 4.28, 4.03, 7.22, 4.07, and 4.11 transformed with the CP coding sequence and the 3% non-translated region (NTR) of SPFMV-S have been previously described [15]. Lines 4.28, 4.03, and 7.22 (two loci of the transgene) showed PTGS, whereas lines 4.07 and 4.11 (one locus of the transgene) did not [15].

2.2. Virus constructs and in vitro transcription

The PVX vector, pP2C2S, was kindly provided by Dr D.C. Baulcombe, The Sainsbury Laboratory, Norwich, UK. For virus infection, the recombinant PVX, PVX.CPII, carrying the 5’ terminal 700 bp of the CP gene was employed [15]. To produce PVX.CPII, the plasmid pVC2, which contains the 3% half of the NIa gene, the NIb gene, the CP gene, and the 3’ NTR of SPFMV-S [16], was digested with EcoRI and EcoT22I (an isoschizomer of NsiI). The resulting fragment was endo-filled using the Klenow fragment of DNA polymerase I, gel purified, and ligated into the EcoRV site of pP2C2S. In vitro transcription reactions to produce infectious PVX and virus inoculation were described previously [15].

2.3. Northern blot analysis

Total RNA was extracted 2–8 weeks post-germination (wpg) as described by Sonoda et al. [15]. For RNA extraction of samples at 2–3 and 4 wpg, an individual plant seedling and the first to fourth systemic leaves of an individual plant were used, respectively. Newly emerged leaves were used for samples at 6 and 8 wpg. Seven micrograms of total RNA was size-fractionated on 1.2% agarose gel containing 0.66 M formaldehyde and transferred to Hybond-N + membrane (Amersham, Buckinghamshire, UK).

The plasmid pVC2 already described was digested with EcoRI and the resulting 1.2 kb fragment, which includes almost the entire CP gene and the 3’ NTR of SPFMV-S, was used as a probe (Fig. 3). The blot was hybridized with a random primed [32P]-labeled probe.

3. Results

3.1. The CP gene expression of T2 progeny in line 4.28

Some features of PTGS in N. benthamiana transformed with the CP gene including the 3’ NTR of SPFMV-S have been shown [15]. In total, seven lines that exhibited HDR against PVX engineered to contain the sequence homologous to the transgene were selected as those showing PTGS [15]. Among them, line 4.28 showed a high level of transgene expression by Northern blot analysis [15]. This contrasted with the other silenced lines, which showed low levels of transgene expression. To accurately analyze the expression of the transgene in line 4.28, we monitored steady-state mRNA accumulation in T2 transgenic plants at various stages of development.

At 3 wpg, readily detectable levels of the transgene mRNA were observed, suggesting that silencing had not been activated at this early time point (Fig. 1). The mRNA accumulation was reduced in one plant at 4 wpg (Fig. 1) and, by 6 wpg, a high proportion of plants showed degraded patterns of-
the mRNA, but some plants still accumulated mRNA at high levels (Fig. 1). Most of the plants showed degraded patterns of transgene mRNA by 8 wpg (Fig. 1). On the other hand, PTGS in the other silenced lines tested, lines 4.03 and 7.22 [15], was already activated at 2 wpg (data for line 4.03 shown in Fig. 1). The activated state was maintained at 4, 6, and 8 wpg. Lines 4.07 and 4.11, which do not show PTGS [15], always expressed detectable amounts of the mRNA (data for line 4.07 at 2 and 8 wpg shown in Fig. 1). These results suggest that the silencing in line 4.28 was activated spontaneously in the course of development but was delayed compared with the other silenced lines.

3.2. Response to virus inoculation of T2 progeny in line 4.28

It has been shown that a small number of plants in line 4.28 were resistant to the infection of the recombinant PVX, PVX.CPI, engineered to contain nearly the entire SPFMV-S CP gene, including the 3’ NTR (Table 1 in Sonoda et al. [15]). In contrast, the other silenced lines, lines 4.03, 4.09, 7.11, 7.12, and 7.22, always showed that a high proportion of plants were completely resistant to PVX.CPI (Table 1 in Sonoda et al. [15]). Therefore, line 4.28 has been regarded as a line showing a poor virus resistance. Since line 4.28 shows a delayed activation of the transgene silencing (Fig. 1), the effectiveness of its resistance after activation was examined. Responses of line 4.28 to recombinant PVX were analyzed using T2 plants at 4 and 8 wpg. As shown in Fig. 1, the silencing in line 4.28 was not activated in a high proportion of the plants at 4 wpg. On the other hand, at 8 wpg, the silencing was activated in most of the plants (Fig. 1). For virus inoculation, PVX.CPII, an infectious clone of PVX carrying the 5’ terminal 700 bp of SPFMV-S CP gene, was employed [15]. Eleven out of 15 plants analyzed at 4 wpg were susceptible to PVX.CPII. On the other hand, all ten 8-week-old plants showed complete resistance to PVX.CPII. We did not observe a recovery phenotype, i.e. plants infected with virus initially show symptoms but subsequently develop new leaves that have no virus symptom and have virus resistance [7,13]. Recombinant PVX is known to produce wild-type PVX resulting from the deletion of the inserted sequence as a result of homologous recombination among the duplicated CP promoters [17], so that in our system, plants that showed a recovery phenotype might be masked by infection with the wild-type PVX. We showed that the transgene expression was silenced in most of the plants at 8 wpg; however, one plant still expressed the mRNA at a high level (Fig. 1). This discrepancy between the transgene expression and the virus resistance might attribute to a phenomenon called ‘virus-induced gene silencing (VIGS)’, in which the transgene may become silenced after virus infection [18]. PTGS of the plant in which the transgene was not silenced at 8 wpg might be activated by infection of the recombinant PVX. All plants in the other silenced lines, lines 4.03, 7.11, and 7.22, at 4–5 wpg showed complete resistance to PVX.CPII [15]. The control N. benthamiana plants transformed with GUS-deleted pBI121 (Clontech, Palo Alto, CA) at 4 and 8 wpg were completely susceptible to PVX.CPII. These results suggest that after onset of PTGS, the degree of virus resistance in line 4.28 was comparable with that of the other silenced lines.

Fig. 1. SPFMV-S CP mRNA level in transgenic N. benthamiana lines 4.28, 4.03, and 4.07. RNA was extracted at the indicated weeks post-germination (wpg) from T2 progeny, and 7 μg RNA were subjected to denaturing gel electrophoresis, transferred to nylon membrane and hybridized with a [32P]-labeled CP-specific probe. In RNA samples at 4–8 wpg in line 4.28, each lane corresponds to the identical plant. The photographs of the ethidium bromide-stained RNA gels before transfer are also shown.
Fig. 2. Southern blot analysis of the transcribed region in lines 4.28, 4.03, and 4.07 using methylation-sensitive restriction enzymes, *Hpa*II, *Hae*III, and *Sau*3AI, in combination with *Xba*I and a probe specific for the CP sequence. The *Xba*I site of both transgene copies was unmethylated and readily digested (data not shown). *Hpa*II/*Xba*I digestion of DNA extracted from 2-week-old plants produced a 1.8 kb fragment and traces of smaller fragments, 0.4, 0.9, and 1.3 kb, indicating that *Hpa*II site 1 was partially methylated (Figs. 2 and 3). On the other hand, this treatment of 8-week-old plants gave 1.8 and a trace of 1.3 kb fragments, but not 0.4 and 0.9 kb fragments (Fig. 2). This indicated that *Hpa*II site 1 was fully methylated (Fig. 3). *Hae*III/*Xba*I digestion of DNA extracted from 2-week-old plants produced 0.35 and 0.85 kb fragments (Fig. 2), indicating that *Hae*III sites 1 and 2 were not methylated (Fig. 3). On the other hand, additional fragments of 1.2 and a trace of 1.5 kb were detected in samples from 8-week-old plants (Fig. 2). This indicated that *Hae*III sites 1 and 2 were partially methylated (Fig. 3). *Sau*3AI/*Xba*I digestion of DNA extracted from 2-week-old plants showed fragments of 0.35, 0.7, and a trace of 1.05 kb (Fig. 2). The expected 0.1 kb fragment was probably not detected in our system because it was too small. The fragment sizes suggest that the *Sau*3AI site 2 was largely unmethylated, and that *Sau*3AI sites 1 and 3 were partially methylated (Fig. 3). Samples from 8-week-old plants gave 0.35, 0.7, 1.05, and 1.4 kb fragments, and the 1.05 kb fragment was the most prominent band (Fig. 2). This suggests that *Sau*3AI sites 2 and 3 were partially methylated and that *Sau*3AI site 1 was not (Fig. 3). Thus, methylation of *Hpa*II, *Hae*III, and *Sau*3AI sites in the transgene was obviously increased when PTGS was activated. No remarkable variation in the transgene methylation was observed among T2 plants 8 weeks old, in which PTGS was activated (data not shown). These results reveal a correlation between the activation of the silencing and the developmentally increased transgene methylation in line 4.28. The other silenced lines 4.03 and 7.22 showed similar methylation patterns in the CP gene and the 3′ NTR for samples extracted at 2 and 8 wpg with all
the three endonuclease digestions (blot for line 4.03 at 2 wpg shown in Fig. 2, and data for lines 4.03 and 7.22 summarized in Fig. 3). Non-silenced lines 4.07 and 4.11 had identical hybridization patterns of less methylated in the CP gene and the 3’ NTR at 2 and 8 wpg (blot for line 4.07 at 8 wpg shown in Fig. 2). *Hpa*II/*Xba*I digestion of DNA extracted from lines 4.07 and 4.11 produced a 1.3 kb fragment, indicating that *Hpa*II site 1 was partially methylated. Transgene methylation in a non-silenced line was reported in our previous study [15].

3.4. The CP gene mRNA expression and transgene methylation status in *T*<sub>3</sub> progeny in line 4.28

To assess meiotic inheritance of the activation of PTGS in *T*<sub>2</sub> transgenic plants, *T*<sub>2</sub> plants were self-fertilized and the resulting *T*<sub>3</sub> plants were analyzed for the expression of the transgene by Northern blot hybridization. At 3 wpg, *T*<sub>3</sub> plants showed detectable levels of the transgene mRNA (Fig. 4), suggesting that the silenced state was not inherited to the next generation. Then, the steady-state mRNA accumulation was reduced at 4 wpg (Fig. 4). By 8 wpg, a high proportion of the plants showed patterns of degraded mRNA (Fig. 4). The proportion of *T*<sub>3</sub> plants in which the transgene expression was silenced at 8 wpg was slightly lower than that observed in *T*<sub>2</sub> plants at 8 wpg (Figs. 1 and 4). However, in our several repeated analyses, there was substantially no difference in the proportion of the silenced plants between *T*<sub>2</sub> and *T*<sub>3</sub> plants at 8 wpg (data not shown). Genomic DNA extracted from *T*<sub>3</sub> plants at 2 and 10 wpg were subjected to methylation analysis in the same as the *T*<sub>2</sub> plants. The methylation pattern of the 2- and 10-week-old *T*<sub>3</sub> plants closely resembled those of the 2- and 8-week-old *T*<sub>2</sub> plants, respectively (Fig. 5). This suggests that the same re-setting and development of transgene methylation is occurring in each new generation.

4. Discussion

Line 4.28 accumulated a high level of SPFMV-S CP mRNA during the first 3 weeks of development, but this level was dramatically reduced in the course of plant development. The reduction in transgene mRNA correlated with the development of complete resistance against a recombinant PVX engineered to express the sequence homologous to the transgene. These observations suggest that PTGS, resulting in virus resistance, is being developmentally induced in the plants. This is in contrast to lines 4.03 and 7.22, in which PTGS was

![Fig. 3. Methylation status of the transgenes in the silenced lines. Restriction map of the transgene in the silenced lines was shown for *Hpa*II, *Hae*III, and *Sau*3AI. Restriction site for *Xba*I and DNA fragment used as a probe are also shown. The methylation status of the transgene for *Hpa*II, *Hae*III, and *Sau*3AI sites from Southern blots are indicated: open circle, unmethylated; partially filled circle, partially methylated; filled circle, fully methylated. Data for the transgene in lines 4.03 and 7.22 was reported in Sonoda et al. [15]. LB, T-DNA left border; nos, nopaline synthase 3’ end; CP and 3’ NTR, the CP coding sequence and the 3’ NTR of SPFMV-S; 35S, cauliflower mosaic virus 35S promoter.](image-url)
already activated at 2 wpg. In line 4.28, the delayed activation of PTGS was reset after meiosis and showed the same developmental activation in the next generation. This developmental activation of PTGS seems similar to that in transgenic petunia with a chalcone synthase gene [20]. The endogenous chalcone synthase gene in flowers was developmentally expressed in the presence of the highly expressed transgene. The gene silencing in some plants occurred only when there were elevated levels of total amount of chalcone synthase mRNA accumulated. This coincides with the threshold model [7] and may not be related to de novo methylation of the transgene DNA. However, our results suggest some relationship between PTGS induction and the increased level of de novo methylation of the transgene DNA during development.

The involvement of DNA methylation in PTGS has been examined in several studies [9,10,19]. However, the exact time course of PTGS and corresponding alteration in transgene methylation have not determined in all cases. We have investigated the alteration of transgene methylation in line 4.28 during plant development. When the PTGS was not activated (2 wpg), the transgene methylation in line 4.28 appeared to be less extensive compared with those in lines 4.03 and 7.22. However, the methylation status became to be comparable with those in lines 4.03 and 7.22 when the silencing was activated. At this time point, the degree of virus resistance in line 4.28 was comparable with that in the other silenced lines including lines 4.03 and 7.22. The transgene methylation increased from 2 to 8 wpg and was reset after meiosis. However, the character of a developmentally increased transgene methylation was transmitted to the next generation. These results suggest that the degree of PTGS and the level of transgene methylation increase in the course of plant development and reset after meiosis. In VIGS, virus-induced DNA methylation was associated with activation and re-setting of PTGS [21,22]. However, our results suggest that the transgene(s) itself has the potential to trigger and re-set DNA methylation and PTGS. DNA methylation was not detected in the 35S promoter in line 4.28 (data not shown), as has been reported in other studies [10,19].

Fig. 4. SPFMV-S CP mRNA level in transgenic N. benthamiana line 4.28. RNA was extracted at the indicated weeks post-germination (wpg) from T3 progeny, and 7 μg RNA were subjected to denaturing gel electrophoresis, transferred to nylon membrane and hybridized with a [32P]-labeled CP-specific probe. In RNA samples at 4–8 wpg, each lane corresponds to the identical plant. The photographs of the ethidium bromide-stained RNA gels before transfer are also shown.

Fig. 5. Southern blot analysis of the transcribed region in line 4.28 using methylation-sensitive restriction enzymes, HpaII, HaeIII, and Sau3AI, in combination with XbaI. Southern blot data for the transgene of T3 progeny at 2 and 10 wpg are shown. Total DNA (20 μg) digested with restriction enzymes was size-fractionated on a 1.5% agarose gel, transferred to nylon membrane and hybridized with a [32P]-labeled CP-specific probe. The leftmost numbers of the blots indicate the size of DNA fragments (kb).
In the present study, de novo DNA methylation appeared to be involved in the silencing in line 4.28. DNA methylation has been implicated in the production of aberrant RNA [10]. It has been postulated that methylated regions of DNA can lead to premature termination of transcription producing aberrant RNA, which can serve as templates for the plant-encoded RNA-dependent RNA polymerase (RdRp) to produce short antisense RNA. Alternatively, the methylation might result in locally altered chromatin structure and the formation of aberrant RNA preferentially identified by RdRp [22]. The antisense RNA may form ds RNA with the transgene mRNA, and the resulting ds RNA may be degraded by a host-encoded ds RNA-specific RNase [12].

The activation of PTGS in line 4.28 was delayed compared with that in the other silenced lines, including lines 4.03 and 7.22. In an early developmental stage, line 4.28 might produce little or no aberrant and/or antisense RNA to activate PTGS due to the less extensive transgene methylation. The transgene methylation observed at 2 wpg when the PTGS was not activated (Fig. 3, HpaII site 1 and Sau3AI site 2) might reflect the basal level of transgene methylation due to the presence of two copies of the transgene [15]. An additional level of de novo methylation in the course of further development, together with the basal one, might initiate aberrant and/or antisense RNA production, thereby PTGS and resulting complete resistance to PVX.CPII might have been displayed. On the other hand, in the other silenced lines 4.03 and 7.22, the basal level of the transgene methylation might be higher than that of line 4.28 and no additional level of de novo methylation might be required to activate PTGS, so that PTGS in those silenced lines could be activated without delay. The difference of the basal methylation between line 4.28 and the other silenced lines might be reflected in the degree of DNA–DNA interactions based on the transgene arrangements in the genome. Transgenes arranged as inverted repeats have supposed to provoke methylation of the transcribed region [23]. Analyses of the transgene arrangements in line 4.28 and the other silenced lines would be an intriguing future investigation. The mechanism that triggers the additional level of de novo methylation observed in this study might be attributed to RNA-directed DNA methylation reported in plants carrying transgenes derived from the potato spindle tuber viroid [24]. The transgene methylation still might be merely a consequence of PTGS. So far, molecular features of aberrant RNA have not been elucidated. However, both transgene methylation and aberrant RNA remain to be attractive factors that are associated with PTGS.

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