Plant Biotechnol Rep (2011) 5:273–281
DOI 10.1007/s11816-011-0182-3

ORIGINAL ARTICLE

The epigenetic phenotypes in transgenic *Nicotiana benthamiana* for CaMV 35S-GFP are mediated by spontaneous transgene silencing

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Received: 17 April 2011 / Accepted: 3 May 2011 / Published online: 24 May 2011

Abstract Diverse epigenetic phenotypes are frequently found during research on transgenic plants. To understand the factors underlying such diversity, hundreds of independent 35S-GFP transgenic *N. benthamiana* plants were analyzed. The diverse GFP-expression phenotypes of the transgenic plants were classified into three major types based on the GFP expression patterns and their response to 35S-GFP agroinfiltration: steady-green, silenced and non-uniform phenotype. The non-uniform phenotype was further sub-divided into five minor phenotypes: variegated, red-dropped, on-silencing, partitioned and misty, according to the distribution of GFP expression on the leaves. Many of transgenic plants continuously generated diverse phenotypes over several generations despite the transgene identity. Such epigenetic GFP phenotyping was found to be the result of spontaneous transgene silencing mediated by either or both of post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). This finding was verified by the detection of 21- and 24-nt small interfering RNA (siRNA) molecules, and DNA methylation in the transgenic plants that showed repeated epigenetic variation. Agroinfiltration demonstrated that irregular distribution of GFP on a leaf was the result of erratic transgene silencing, and the technique also proved to be a rapid and effective method for selecting fully silenced plants within 3 days. Furthermore, two novel phenotypes described are potential materials for in-depth investigations into the genes and mechanisms responsible for spontaneous transgene silencing.

Keywords Spontaneous transgene silencing · Agroinfiltration · GFP-transgenic · *Nicotiana benthamiana* · CaMV 35S promoter · CMV 2b

Abbreviations

GFP	Green fluorescent protein
CaMV	Cauliflower mosaic virus
CMV	Cucumber mosaic virus
TGS	Transcriptional gene silencing
PTGS	Post-transcriptional gene silencing
siRNA	Small interfering RNA

Introduction

Agrobacterium-mediated plant transformation has been extensively applied for the introduction of useful genes with the aim of creating novel traits. However, transgenes are frequently incorporated into the genome in more than one copy, and transgene silencing is often facilitated by

Electronic supplementary material The online version of this article (doi:10.1007/s11816-011-0182-3) contains supplementary material, which is available to authorized users.

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ectopic expression of 35S promoter (Harper and Stewart 2000), multicopy or more than two T-DNA insertions in the same chromosome (Tang et al. 2007), and direct (DR) or inverted (IR) transgene repeats (Schubert et al. 2004). The initiation of gene silencing is recognized as involving the formation of complementary double-stranded RNA (dsRNA) and the downstream pathways of silencing have to a great extent been elucidated (Brodersen and Voinnet 2006). For instance, dsRNAs generated by IR transgenes are processed into short interfering RNA (siRNA) of 21 nt by a Dicer-like (DCL4) protein (Brodersen and Voinnet 2006; Dunoyer et al. 2005). The siRNAs are subsequently involved in the cleavage of target mRNA by RISCs containing AGO1 (Fagard et al. 2000; Morel et al. 2002). However, sense RNA silencing by ectopic expression (Palauqui and Vaucheret 1998; Schubert et al. 2004) and DR transgenes requires a different pathway, so that the concept of aberrant RNAs (Herr et al. 2006; Luo and Chen 2007), in which a polyA tail or 5′ cap structure is lacking, was introduced. This sense RNA silencing is believed to be initiated through the activation of RNA-dependent RNA polymerases (RDRs) (Wassenegger and Krczal 2006) that copy the aberrant RNA into complementary dsRNA, which subsequently leads to mRNA degradation by a pathway similar to that of IR-derived dsRNA. However, it is still not clear how aberrant RNAs are sensed inside the plant cell (reviewed in Kalantidis et al. 2008), and there are no reports on the mechanism of spontaneous initiation of endogene silencing. Reversions of silenced states were occasionally observed in petunia co-suppressed by CHS-A (Kanazawa et al. 2007) and in drug-resistant tobacco callus culture (Fojtova et al. 2003). Such destabilized silencing was speculated to be induced by epigenetic changes involving the methylation of transgenes (Kanazawa et al. 2007), and might be a limiting factor for useful transgene expression and use of RNA silencing as a tool for plant biotechnology. Undoubtedly, a sensitive method that allows detection of tissue-specific changes in gene expression is required to understand the mechanism(s) underlying the instability in spontaneous RNA silencing (reviewed in Kanazawa 2008). Thus, this study focused on the descriptive analysis of spontaneous transgene silencing resulting in diverse phenotypes produced in *N. benthamiana* following transformation with the GFP gene under the control of the 35S promoter. For this purpose, hundreds of transgenic plants were established and classified according to their GFP phenotype. To classify the phenotype more clearly, an additive agroinfiltration method was applied. It was based on the previous finding that an additively infiltrated transgene-homologous gene became silenced if the post-transcriptional gene silencing (PTGS) mechanism was already established (Matzke and Matzke 1995). To make the response clearer, a cucumber mosaic virus (CMV) 2b gene was co-infiltrated. The 2b gene is a viral suppressor of gene silencing, which prevents the initiation of PTGS (Lucy et al. 2002) by the directly binding to siRNAs (Goto et al. 2007). However, the 2b protein does not have any effect when PTGS has been previously established (Brigneti et al. 1998; Li et al. 1999). Nevertheless, viral suppressors can also be used to obtain a high level of gene expression in a transient expression system by preventing the onset of PTGS (Voinnet et al. 2003; Bucher et al. 2003; Choi et al. 2008). In this report, the phenomenon of diverse epigenetic phenotypes was investigated to determine the role of spontaneous transgene silencing. To associate epigenetic phenotyping with transgene silencing, representative plants of each classified phenotype were analyzed for their molecular properties, such as the presence of siRNAs and DNA methylation. In addition, the practical advantages of the agroinfiltration method and unique GFP phenotype plants for analyzing spontaneous transgene silencing are also described here.

Materials and methods

Plant transformation

Leaf-discs of wild-type *N. benthamiana* were transformed with *A. tumefaciens* harboring pGreen0229-35S:GFP (35S-GFP) (Choi et al. 2008; Haseloff et al. 1997) via leaf-disc transformation (Robert et al. 1988). The binary vector possessed the bar gene as a selection marker for plant transformation and regeneration. After 2 days of co-culture, leaf-discs were transferred for shoot induction (MS medium supplemented with 0.1 mg l^{-1} NAA, 2.5 mg l^{-1} BA), shoot elongation (0.1 mg l^{-1} BA) and subsequently for root induction (0.1 mg l^{-1} NAA). Kmoxilin (500 mg l^{-1}; Chong Kun Dang, Korea) and glufosinate-ammonium (5 mg l^{-1}; Riedel-de haën, Germany) were also supplemented to eliminate *Agrobacterium* and to select the putative transgenic plantlets, respectively. Finally, the fully regenerated plantlets were grown in the greenhouse for further growth and their seeds were harvested.

Agroinfiltration

To screen true transgenic plants and GFP-phenotyping, agroinfiltration was conducted with *Agrobacterium* harboring 35S-GFP in the absence or presence of pGreen0229-35S:2b (35S-2b) (Choi et al. 2008), respectively into the left or right-side on the same leaf of all the transgenic plants at T_1, T_2 and T_3 generations. The plants were grown for 6–7 weeks at an ambient temperature of between 25 and 30°C in the greenhouse. The detailed agroinfiltration conditions were basically followed as previously described

(Choi et al. 2008). The *Agrobacterium* carrying 35S-GFP or 35S-2b was commonly grown in LB medium containing 100 mg l^{-1} ampicillin, 50 mg l^{-1} kanamycin and 5 mg l^{-1} tetracycline for 2 days at 28°C. Each *Agrobacterium* culture was resuspended in the infiltration solution containing 10 mM $MgCl_2$ and 0.1 mM acetosyringone, and the concentration was adjusted to 0.2 at the optical density (OD) of 600 nm. Then, *Agrobacterium* solution was pushed into the leaf to transform mesophyll cells through a disposable syringe. The GFP phenotypes were photographed with an Olympus C-5050Z digital camera under the UV illumination (BIB-150;, Spectronics, USA).

Southern and northern blot analysis

To measure GFP transgene copy number and the steady-state level of its RNA transcripts, Southern and northern blot analysis was respectively performed by the procedures as previously described (Choi et al. 2008). To determine transgene copy number, genomic DNA was digested with *Hin*dIII which cleaves at a single site localized between 35S promoter and GFP gene in the T-DNA region. The transgene numbers were estimated based on the segregation ratios of resultant phenotypes verified by agroinfiltration, and confirmed with Southern blot analysis. To analyze transgene methylation, approximately 10 μg of genomic DNA was fully digested with the methylation-sensitive restriction enzyme *Sau*96I, which was used previously to detect methylation in the 35S-GFP cassette (Jones et al. 1999; Vaistij et al. 2002). The analysis by *Sau*96I-digestion was simple and provided an indicator of DNA methylation. The digested DNA was then electrophoresed on a 1.5% (w/v) agarose gel. For all the hybridization and visualization of bands, a DNA fragment ($\sim$700 bp) of P^{32}-labeled GFP gene was used as the probe.

Detection of siRNAs

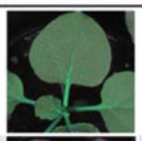
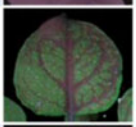
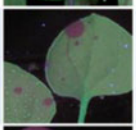
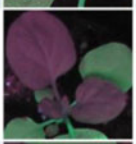
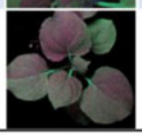
For detection of siRNAs, low molecular weight (LMW) RNA was isolated from transgenic plants using RNAzol®RT (Molecular Research Center, USA). Then, 50 μg of RNA was electrophoresed on 20% (w/v) polyacrylamide gels containing 7 M urea at 250 V, and then transferred onto Hybond™ N^+ nylon membranes using a Semi-Dry Transfer Cell (Bio-rad, USA). Hybridization with a P^{32}-dCTP GFP-specific probe and visualization of siRNAs were as described by Goto et al. (2003). To confirm the sizes of siRNAs, microRNA marker (N2102S; New England BioLabs, UK), which consisted of three synthetic single-stranded RNA oligonucleotides of 17, 21 and 25 nt in length, was used as a standard.

Results

The classification of diverse GFP-phenotypes

The GFP-phenotypes of *N. benthamiana* transgenic plants were so diverse that they were finally classified into three major phenotypes: the steady-green, silenced and non-uniform. The non-uniform phenotype was further sub-divided into variegated, red-dropped, on-silencing, partitioned, and misty. The classification of each GFP phenotype was based on the spatial distribution and expression timing of GFP on the leaves (Table 1). To verify the GFP phenotyping, *Agrobacteria* with 35S-GFP alone or in the presence of 35S-2b were infiltrated into the leaves of each transgenic plant as shown in Fig. 1a. Generally, GFP expression reached a peak at 3 days post-infiltration (dpi) and the presence of 2b clearly increased the expression of co-infiltrated GFP (wild-type in Fig. 1a). The common findings were that the infiltrated-GFP was expressed only in the GFP-expressing regions additively, and never expressed in red-colored regions of the silenced, variegated, red-dropped and partitioned phenotypes even when the suppressor 2b was co-introduced (Fig. 1c–g). This indicates that such red-colored regions have already entered a GFP silencing state, and that this silencing state is not reversed by the presence of 2b. By exploiting this finding, fully GFP silenced plants were successfully identified from the population of wild-type-like transgenic plants. That is, fully silenced plants did not express the infiltrated GFP even with the presence of 2b (Fig. 1c), while non-transgenic plants clearly expressed the infiltrated GFP (Fig. 1a). Thus, the additive GFP expression by agroinfiltration enabled us to screen a large population of the transgenic plants within a short period of 3 days, and was also effective in verifying the regionalized silencing in a plant. To approximately determine the rate of occurrence of each phenotype, a total of 1,420 T_1 progeny plants, which were composed of 10 progeny generated from 142 independent T_0 transgenic plants, were classified with the help of the agroinfiltration method. The approximate frequency of each phenotype was as follows: the steady-green and silenced phenotypes comprised 36 and 19% T_1 plants, respectively. The remaining 45% non-uniform phenotypes were composed of the variegated (25%), red-dropped (4%), on-silencing (6%), partitioned (2%), and misty (8%) (Table 1), in which the detailed definition of each phenotype is also given. It should be noted that the frequency of occurrence of the phenotypes can be changed if the successive segregation of transgenes continues over several generations. However, the data in Table 1 can be utilized to estimate the probability of phenotypic diversity in transgenic plant research.

Table 1 Classification, feature and approximate frequency of diverse GFP-phenotypes

GFP-phenotype	Sub-type	Typical pattern	Feature	Frequency (%)
Steady-green			Uniformed and constitutive GFP-expression in a whole plant	36
Silenced			No detectable of GFP-expression in a whole plant	19
Non-uniform	Varigated		Specifically GFP-silencing mainly in vein region on leaves	25
	Red-dropped		GFP-silencing drops on leaves of steady-green plant	4
	On-silencing		Abrupt transition from GFP-expression to fully-silencing in the subsequent emerging leaves	6
	Partitioned[a]		Sharply partitioned GFP-expression and silencing region within a leave	2
	Misty		Too weak and misty GFP-expression in a whole plant	8
Total				100

The non-uniform was further divided into five sub-phenotypes such as the variegated, red-dropped, on-silencing, partitioned and misty. The approximate frequency of each phenotype was converted into a percentage based on the observation of 1,420 T_1 progeny plants generated from 142 independent T_0 plants

[a] The silencing region is gradually enlarging in the subsequently emerging leaves

Phenotypic variation over generations

In attempt to fix each phenotype genetically and phenotypically, successive selection for a targeted phenotype was conducted up to the T_3 generation by using the agroinfiltration method, and their transgene numbers were determined by Southern blotting. For the steady-green phenotype, a successive selection of fully green plants from T_0 transgenic plant 266 led to the phenotypic fixation at the T_3 generation (Fig. 2). The selected T_2 plant (266-3-1) and its progeny (266-3-1-2, 5, 8, 11) was confirmed to have a single transgene copy. In this process, the agroinfiltration method was not applied because the criterion of selection was fully green plant. For the silenced phenotype, plants showing the fully silencing response (as determined by agroinfiltration) were successively selected. The T_1 plant 018-1 repeatedly produced silenced and non-transgenic plants with the ratio of 3:1 at the subsequent T_2 and T_3 generations (Fig. 2). This phenotypic segregation ratio was the result of a single dominant gene, and the 1st and 2nd selected plants must be heterozygous caused by coincidental selections of heterozygous plants repetitively at T_1 and T_2 generations. However, it was confirmed by Southern blot analysis that two transgenes were incorporated in all the silenced plants. Therefore, it can be explained that two transgenes must be located on the same chromosome and behave like a single gene phenotypically. The partitioned plant of T_0 plant 029, unexpectedly and repeatedly, produced diverse phenotypes, and the 2nd selected partitioned plant (029-5-6) also produced the steady-green (029-5-6-2, 3), silenced (029-5-6-5), and partitioned (5-6-9) plants at the T_3 generation (Fig. 2). However, the T_2 plant

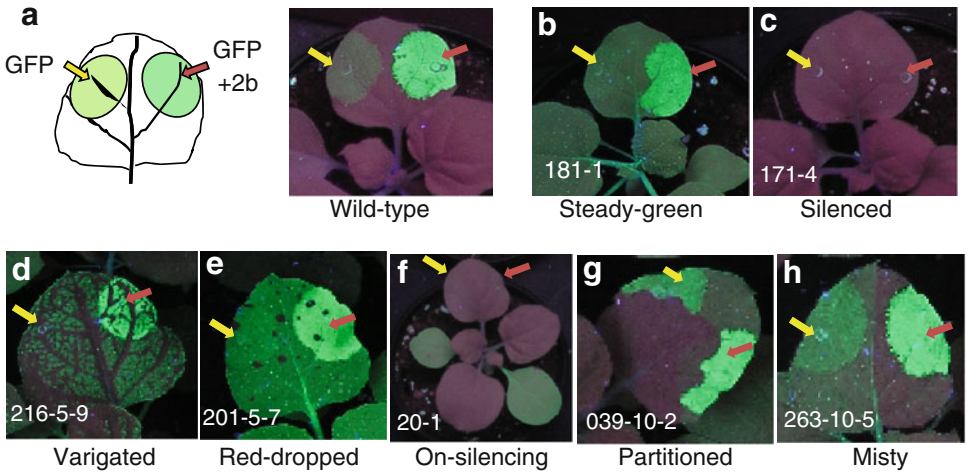


Fig. 1 Responses of the agroinfiltration with 35S-GFP in the absence and presence of 35S-2b on diverse phenotypes at 3 dpi. The typical GFP-expression in wild-type (**a**) resulted from the agroinfiltration with 35S-GFP alone (*GFP*) or the presence of the 35S-2b (*GFP + 2b*). The responses of the agroinfiltration are shown in the steady-green (**b**), silenced (**c**), variegated (**d**), red-dropped (**e**), on-silencing (**f**), partitioned (**g**) and misty (**h**), respectively. The *yellow* and *red-colored arrows* indicate the responses of the infiltrated GFP alone and GFP + 2b, respectively. The *numbers* on the *left* in the photos indicate the serial pedigree numbers (T_0-T_1-T_2) of each transgenic plant

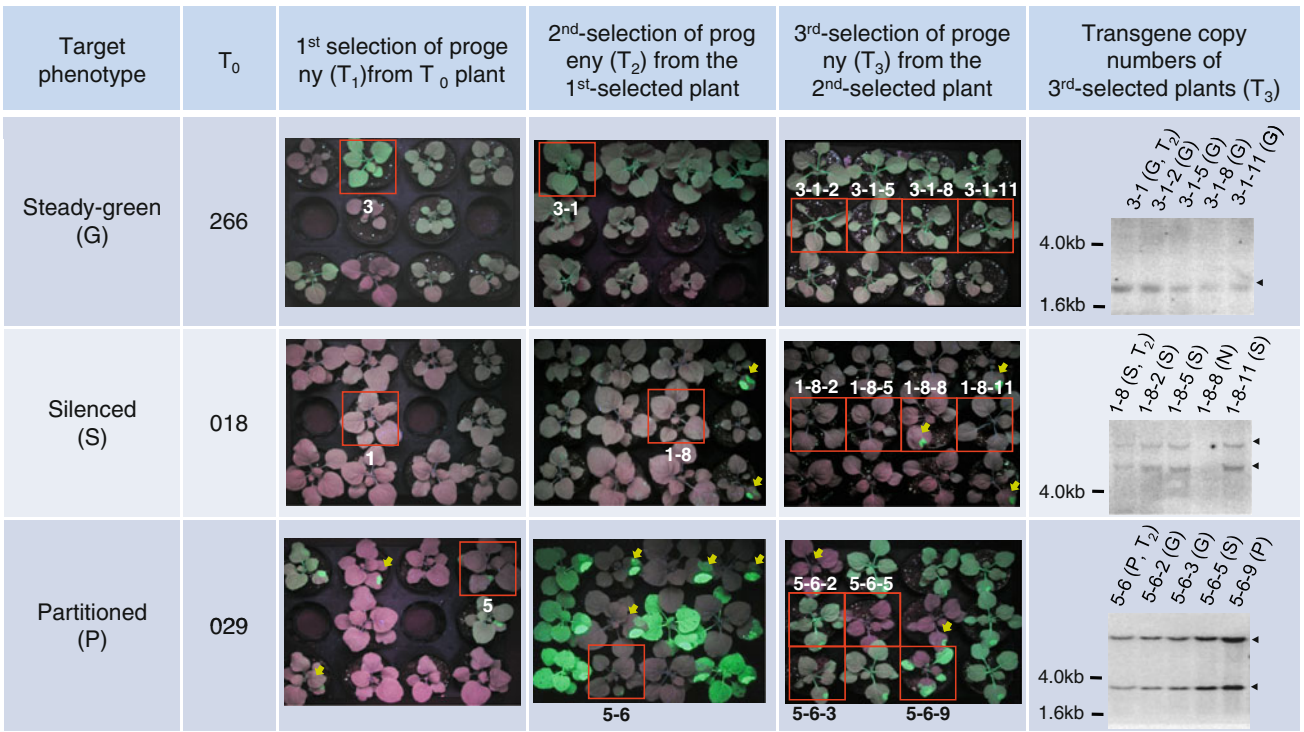


Fig. 2 The targeted-selection of specific phenotypes by generation advancement up to T_3 and transgene copy numbers of the selected plants. The plants in *red-lined squares* at T_1 and T_2 generation were selected for the fixation of specific phenotypes. The selected plants in *red-lined squares* at T_2 and T_3 were analyzed to determine the transgene number. The *numbers* on the *upper* or *lower* part of the *squares* indicate the serial pedigree numbers from T_0 plants. For the silenced (018) and non-uniform (029) plants, the agroinfiltration with GFP and GFP + 2b was applied to all the progenies to distinguish the non-transgenic plants. The plants indicated by *yellow-colored arrows* in the silenced and partitioned were confirmed as the non-transgenic plants. *G*, *S*, and *P* indicate the steady-green, silenced and partitioned, respectively, and the *black arrowheads* at the right-side of the Southern blot indicate the locations of incorporated GFP-transgenes

and its progeny were revealed to harbor two transgenes which appeared to be located at the same chromosome based on the segregation ratio of 3:1 as revealed by agro-infiltration at the T_1 and T_2 generations. Taking into account the diverse phenotyped plants originating from an identical genotype, this phenotypic diversity appeared to be induced by epigenetic factors.

Additional epigenetic variation

More epigenetic variations were observed in other transgenic plants (Fig. S1). For instance, the steady-green plant (003-1-5), which harbored two transgenes at T_2, generated three different phenotypes at the next generation (T_3): the steady-green, silenced and on-silencing. Although single-copy plants (003-1-5-5, 8), produced by the resultant segregation of two transgenes, showed the steady-green phenotypes, the other two-copied plants exhibited the silenced (003-1-5-4, 9) or on-silencing (003-1-5-3, 10) phenotypes which were different phenotypes from that of their progenitor. Other partitioned (039-10-8) and variegated (216-5-9) plants showed epigenetic variations at a subsequent generation (T_3). Likewise, they also produced diverse phenotypes, such as the steady-green, silenced, variegated and misty (Fig. S1). The common finding is that four transgenic plants (029-5-6, 003-1-5, 039-10-8, 216-5-9) exhibiting epigenetic variations also contain two transgenes. This suggested that the presence of two transgenes might facilitate epigenetic diversity. To understand such variations, the molecular mechanisms underlying epigenetic variation should be identified. Although obtaining a desired transgenic plant involves selecting the single-copy plant for practical use, it will be worth identifying the endogenous factors of plant origin that spontaneously initiate or regulate the epigenetic variation.

Association of epigenetic variation with spontaneous transgene silencing

Eleven plants, which comprised the five different phenotypes, were investigated at the molecular level to address the possible link between epigenetic variations and transgene silencing. This attempt stemmed from a finding that red-colored regions of the silenced and non-uniform phenotype might be the result of the localized silencing as observed using the agroinfiltration method (Fig. 1c–g). The GFP RNA transcripts were readily detected in all the plants which expressed GFP fully or even locally as follows: the steady-green (193-7-4, 266-3-1), variegated (216-5-9), partitioned (039-10-8) and misty (190-7-2) phenotypes (1–5 in Fig. 3b). In contrast, GFP RNA transcripts were barely detected in any of the silenced plants (6–11 in Fig. 3b). For the siRNAs and DNA methylation, the siRNA of 21 nt in length was clearly detected in most of the plants of non-uniform and silenced phenotypes (3, 4, 6–11) except the misty phenotype (5 in Fig. 3c), and the 24nt-siRNA were also faintly detected in some of the non-uniform and silenced plants (3, 7, 8, 11 in Fig. 3c). Methylation of genomic DNAs was analyzed based on the patterns of *Sau*96I-digestion on the 35S-GFP cassette (Fig. 3e), larger-sized DNA fragments ranging from 0.67 to 1.43 kb were produced (SD in Fig. 3d) when the 35S promoter and GFP gene were methylated. The fragments of 0.67 and 0.76 kb indicate methylation on the GFP gene, and the 1.43 kb-fragment indicates that 35S promoter and GFP gene were concurrently methylated. The digested patterns of most of the non-uniform and silenced plants resulted in larger-sized fragments over 0.5 kb and their intensities were strong (3–9, 11 in Fig. 3d). However, only smeared fragments around 0.5 kb were visualized in two steady-green plants (1, 2 in Fig. 3d). This demonstrates that there are significantly higher extents of methylation of 35-GFP cassette in most of the non-uniform and silenced plants. As an exceptional case, the methylation was lacking in the silenced plant (060-1-5, 10 in Fig. 3d). The detection of siRNAs and DNA methylation was recognized as evidence for PTGS and TGS, so that most of the plants must have been in the status of active transgene silencing mediated by both PTGS and TGS pathway. However, a misty (5 in Fig. 3c, d) or silenced (10 in Fig. 3c, d) plant appeared to have a single pathway of either TGS or PTGS based on the detection of DNA methylation or siRNA alone, respectively. The overall relationships of the molecular analysis to the specific GFP-phenotypes are illustrated in Table 2. As shown in previous reports (Schubert et al. 2004; Tang et al. 2007), more than two copies of a transgene facilitated transgene silencing. Most of the silenced and non-uniform plants possessed the silencing mechanism in combination with PTGS and TGS. In this study, two interesting phenotypes were obtained. The first was the silenced (010-1-3 in Table 2), which showed an unusual response to the agroinfiltration, unlike that of other silenced plants, and might have a somewhat different silencing pathway. That is, the presence of the 2b appeared to induce the reversion of GFP silencing to expression, although its response was slow and weak. This unusual phenotype was named as the mid-silenced, and it would be useful to investigate the interfering pathway of silencing peculiar to the silenced plant (010-1-3) by the 2b. The second is the partitioned phenotype (039-10-2, 8 in Fig. 1; Fig. S1; Table 2), which is a unique plant with a phenotype so far unreported. That is, transgene silencing took place spontaneously, without external factors being involved, resulting in a leaf showing a sharp partition of GFP-expressing and silencing regions, so that a tight mechanism manipulating such partition must be activated. It could be regarded as an outstandingly

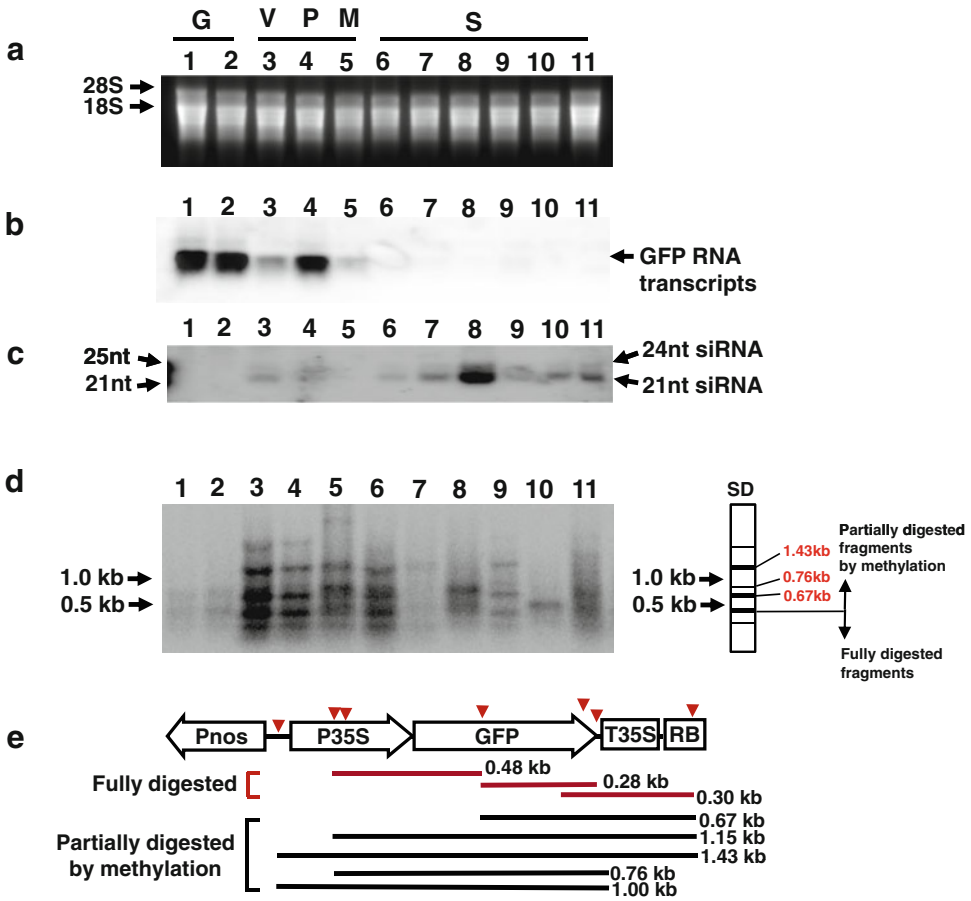


Fig. 3 The steady-state levels of RNA transcripts, and detections of siRNAs and DNA methylations of 35-GFP transgene. The phenotypes analyzed comprised of two steady-green (*1* 193-7-4, *2* 266-3-1), variegated (*3* 216-5-9), partitioned (*4* 039-10-8), misty (*5* 190-7-2) and six silenced (*6* 010-1-3, *7* 018-1-8, *8* 029-5-6, *9* 040-3-5, *10* 060-1-5, *11* 067-3-2). The steady-state level of GFP RNA transcripts (**b**) were visually detected from total RNAs (**a**). The 24- and 21-nt small RNAs (**c**) and DNA methylation (**d**) were detected. The SD shows the sizes of DNA fragments produced by the digestion with *Sau*96I. The expected DNA fragments of the methylated 35S-GFP cassette were estimated based on the nucleotide sequences (**e**). The *red-colored arrowheads* indicate the digestion sites by *Sau*96I. The *red* or *black lines* are fully or partially digested DNA fragments due to DNA methylation, respectively. *G*, *V*, *P*, *M* and *S* indicate the steady-green, varigated, partitioned, misty and silenced phenotypes, respectively

useful plant for cloning a large number of endogenous silence-specific genes and dissecting the regulation mechanism of such tight transgene silencing.

Discussion

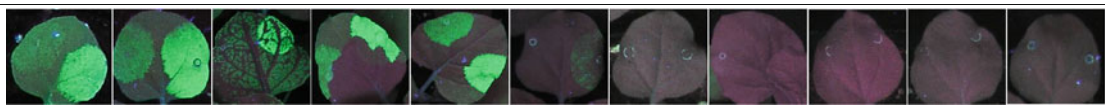
Efficacy of additive agroinfiltration

This study has revealed that the agroinfiltration was an effective technique to select transgene silenced plants. In fact, Bastar® spray was not applied due to concerns that the bar gene silenced plant might be lost by the indiscriminate spray, so that the agroinfiltration technique was developed as an alternative selection in this study. Fortunately, it proved to be a powerful technique and also had practical advantages: for instance, the selection could be achieved within the short timeframe of 3 days, a large population of transgenic plants could be handled simply by injecting *Agrobacterium* suspensions, and finally the technique was simpler than other techniques, such as PCR and Southern analysis. This technique can be applied to invisible and hard-to-confirm genes by fusing a partial sequence of GFP to them at the process of binary vector construction. Furthermore, the agroinfiltration method is also useful to identify the transgene silenced regions in the epigenetic phenotyped plants.

Epigenetic variations resulted from spontaneous transgene silencing

One of the achievements obtained from the agroinfiltration method was to connect epigenetic variation with transgene silencing. Consequently, epigenetic diverse phenotypes

Table 2 The molecular characteristics and possible pathways of transgene silencing to each phenotype

Transgenic plant (GFP-phenotype)	193-7-4 (G)	266-3-1 (G)	216-5-9 (V)	039-10-8 (P)	190-7-2 (M)	010-1-3 (S)[a]	018-1-8 (S)	029-5-6 (S)	040-3-5 (S)	060-1-5 (S)	067-3-2 (S)
Response of GFP infiltration (at 3 dpi)											
Transgene number	1	1	2	2	3	3	2[b]	2[b]	3[b]	2	4
DNA methylation	ND	ND	Yes	Yes	Yes	Yes	Yes	Yes	Yes	ND	Yes
small RNAs (21 and 24 nt)	ND	ND	Yes	Yes	ND	Yes	Yes	Yes	Yes	Yes	Yes
Possible cause of transene-silencing			TGS+ PTGS	TGS+ PTGS	TGS	TGS+ PTGS	TGS+ PTGS	TGS+ PTGS	TGS+ PTGS	PTGS	TGS+ PTGS

Transgenic plants are T_2 plants and numbered by the serial numbers of pedigree from T_0 plants. *G*, *V*, *P*, *M* and *S* indicate the steady-green, varigated, partitioned, misty and silenced phenotypes, respectively. The GFP-agroinfiltration was conducted as described in Fig. 1a and photographed at 3 dpi. The transgene numbers were estimated by Southern blotting and agroinfiltration, and the possible pathways of transgene silencing were judged by the DNA methylation and presence of siRNAs

[a] Named as the mid-silenced plant in which slow and weak expression of the infiltrated-GFP was detected only in the presence of 2b

[b] Transgenes were identified to be inserted into a single chromosome based on the Southern blot and phenotypic segregation ratios at T_2 and T_3 generations

were shown to result from erratic transgene silencing. This finding was supported by the detection of both of siRNAs (21 and 24 nt) and DNA methylation in most of the non-uniform and silenced plants. To date, the formation of dsRNA is recognized as the common initiator of PTGS and TGS pathways. In PTGS, the 21-nt siRNAs released from the digestion by a DCL4 are incorporated into a RNA-induced silencing complex (RISC) to degrade the target mRNA (Dunoyer et al. 2005). In contrast, the 24-nt siRNAs are released by the digestion of dsRNA by a different dicer (DCL3) (Xie et al. 2004), and then incorporated into a different RISC with the involvement of additional components inducing DNA and histone modifications resulting in TGS (Vaistij et al. 2002; Eamens et al. 2008; Kanazawa 2008; Frizzi and Huang 2010). Thus, the 24-nt siRNAs are regarded as the indicator of TGS. The methylated DNA is thought to act as a template for the aberrant RNA transcription, which repeatedly forms dsRNA to create a self-perpetuating RNA silencing loop (Vaucheret 2005). Such methylated DNA (Stokes et al. 2002; Bender 2004) and histone methylation (Cheung and Lau 2005) were reported to meditate epigenetic gene regulation. However, the detection of both 21- and 24-nt siRNAs in most of the non-uniform and silenced plants suggested that both PTGS and TGS pathways were involved in fully or localized transgene silencing. Therefore, the epigenetic variation observed in this research must be the result of the combined action of PTGS and TGS. To suppress such spontaneous transgene instability, it will be necessary to discover the regulating genes involved in their initiation. If such genes could be deployed to control gene silencing, it would represent ground-breaking progress in agricultural biotechnology.

Novel materials for spontaneous transgene silencing research

Some of the transgenic plants, which have the potential to be of great value for elaborate tuning of spontaneous transgene silencing, were additionally obtained. Of particular interest is the unique partitioned phenotype. The sharp partitions occurred despite the identical conditions of genetic background, developmental stage and external environment. Thus, it could be excellent material for investigating the specific mechanisms of the initiation, regulation and maintenance of spontaneous transgene silencing. For instance, a differential screening of specific genes between two regions could open doors for discovering the genes involved in the tight regulation of localized transgene silencing in contrast to those involved in induced silencing such as plant virus-mediated gene silencing (Cakir and Tör 2010; Ruiz et al. 1998). The second plant is the mid-silenced plant. It was characterized by the restoration of GFP expression by presence of the 2b, although the GFP expression was not as strong as that of agro-infiltrated GFP into wild-type *N. benthamiana*. However, the mid-silenced plant could allow the verification of previous reports on the 2b, particularly its lack of efficacy on established PTGS (Brigneti et al. 1998; Li et al. 1999). Finally, another two silenced plants (190-7-2, 060-1-5) detected with the existence of either DNA methylation or siRNAs, respectively, could also be useful materials for separate in-depth investigation of TGS and PTGS pathways.

Acknowledgments This study was carried out cooperatively with the John Innes Centre (JIC), and was funded by the National Academy of Agricultural Science (NAAS, PJ006437) and additionally supported by the Biogreen21 program (BG21) of Rural Development

Administration (RDA), the Republic of Korea. JIC is grant-aided by the Biotechnology and Biological Sciences Research Council (BBSRC), UK.

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