Developmentally Regulated Transgene Silencing in *Torenia*

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Summary

We demonstrated developmental transgene silencing and resetting in *torenia* (*Torenia fournieri* Lind.). The experiments used transformants carrying the neomycin phosphotransferase II gene, the β -glucuronidase (GUS) gene, and the hygromycin phosphotransferase gene. GUS activity was measured at various developmental stages in GUS-homozygous and GUS-hemizygous plants derived from a primary transformant, R₀-1. Rapid silencing of the GUS gene occurred 12 to 19 days after germination in the homozygous plants, while it occurred at a later developmental stage, 54 days after germination, in the hemizygous plants. The silenced state seemed to be stable through vegetative propagation in both the homozygotes and the hemizygotes. Northern blot analysis showed that mRNA reduction correlated with a reduction in GUS activity. A single GUS gene locus was sufficient to trigger the silencing, and initial levels of GUS activity correlated with the timing of silencing. These results suggested that the GUS gene silencing observed in *torenia* is not dependent on allelic interaction of the GUS genes, but is correlated with a dose effect. This type of silencing can be explained by the RNA threshold model.

Key Words : transgene silencing, *Torenia fournieri*, RNA threshold model, transformation, β -glucuronidase gene.

Introduction

Transgene silencing has been reported for various genes in several plant species, and several models for silencing mechanisms have been proposed (Matzke and Matzke 1995; Meyer and Saedler 1996). The broad range of silencing might result from several different mechanisms. Silencing of transgenes might be occurred when they integrated into a chromosomal region of highly repetitive DNA (Prols and Meyer 1992) or when they integrated as an inverted repeats (Jones *et al.* 1987). Silencing loci carrying transgenes could inactivate other genes that are controlled by the homologous promoter (Park *et al.* 1996). These kinds of silencing phenomena

are often associated with methylation of transgene, and might be resulted from a gene inactivation at the transcriptional level.

De Carvalho *et al.* (1992) reported that suppression of the β -1,3-glucanase transgene in tobacco occurred only in homozygous plants. They found that the silencing phenomenon was not dependent on allelic interaction between T-DNA copies present at the same locus, but rather is correlated with the transgene dose in the plant genome. Elmayan and Vaucheret (1996) reported that tobacco plants carrying the β -glucuronidase (GUS) gene at a single locus showed silencing in each generation, but the timing of the silencing was different among the transformants. They concluded that post-transcriptional silencing occurred through a dose effect and not through DNA-DNA interaction. Kunz *et al.* (1996) reported developmentally regulated chitinase-transgene silencing in *Nicotiana glauca*. The chitinase-gene silencing depended on transgene dose rather than on the interaction of alleles at the same transgene locus. Pang *et al.* (1996) reported that, in homozygous lettuce plants, silencing of the nucleocapsid (N) protein gene of the tomato spotted wilt virus was activated at an earlier developmental stage than in hemizygous lettuce plants. These kinds of silencing phenomena resulted from a gene inactivation at the post-transcriptional level, and seem to be regulated by the same mechanism, possibly explained by the RNA threshold model (Smith *et al.* 1994).

Our initial observation of *torenia* (*Torenia fournieri* Lind.) transformants, examined 4 to 5 weeks after germination, was that the plants homozygous on the GUS gene locus (GUS gene was regulated by the cauliflower mosaic virus 35S (CaMV35S) promoter) showed lower levels of GUS activity than the hemizygous plants. Further experiments showed that the activity in the homozygotes was rapidly reduced at an earlier developmental stage than in the hemizygotes. Here we report, developmentally regulated transgene silencing and resetting observed in *torenia* transformants that might also be explained by the RNA threshold model.

Materials and Methods

Plant materials

The primary transformant, R₀-1 (previously referred to as transformant-1), which was transformed with a binary vector plasmid pIG121-Hm (Hiei *et al.* 1994) was obtained from a previous experiment (Aida and Shibata 1995). The R₀-1 is carrying the neomycin phosphotransferase II (NPT II) gene, the β -glucuronidase (GUS) gene, and the hygromycin phosphotransferase (HPT) gene. Progeny of the R₀-1 were used for materials. All plant materials were grown *in vitro*.

For analyzing the evolution of GUS activity in developing seedlings of GUS-homozygote and GUS-hemizygote, two GUS-homozygous plants of the R₁ progeny (No. 20 and No. 25) were either self-fertilized or back-crossed with wild-type plants to obtain GUS-homozygote or GUS-hemizygote, respectively. Seeds were sown *in vitro*, and GUS activity of the seedlings was measured in the whole plant (day 5 only) or the newest developed leaf from the top of 3 or 6 randomly chosen plants until day 82. Apical shoots were excised and transplanted to new medium 65 days after germination, and GUS activity of the first leaf from the top of the plants was measured 103 and 138 days after germination. Each plant was used for only one measurement to avoid the putative bias induced by the cutting stress.

GUS assay

GUS activity was examined following the procedure reported by Jefferson *et al.* (1987). Substrates used were 4-methylumbelliferyl- β -D-glucuronide for quantitative assay, and 5-bromo-4-chloro-3-indolyl glucuronide for histochemical assay. The GUS assay buffer used in this experiment contained 20% methyl alcohol to eliminate the endogenous GUS activity reported by Kosugi *et al.* (1990). For quantitative analysis, GUS activity was expressed as nanomoles of 4-methylumbelliferon (4-MU) produced in 30 min at 37 °C per mg protein (nmol 4-MU/mg protein/30min). GUS background activity in the wild type control was $\leq$ 10 nmol 4-MU/mg protein/30min.

Leaf tests

Kanamycin resistance and hygromycin resistance of transformants were determined by leaf tests. Leaf segments were cultured on Murashige and Skoog solid medium (Murashige and Skoog 1962) containing 1 mg/l benzyladenine(BA), 300 mg/l carbenicillin and 300 mg/l kanamycin to determine kanamycin resistance, or a medium containing 1 mg/l BA, 300 mg/l carbenicillin and 10 mg/l hygromycin to determine hygromycin resistance. Throughout the determination of GUS activity evolution, the leaf tests were performed at the same time as GUS determination with the same plant (3 or 6 plants were used at each developmental stages). From day 19, the same leaves were used, but on day 12, different

cotyledons of the same plant were used since they were too small for both the leaf test and GUS determination.

Southern blot analysis and Northern blot analysis

Total DNA was isolated for Southern blot analysis as follows: Leaf tissue was homogenized with homogenization buffer containing 15% sucrose, 50 mM EDTA, 250 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 0.1% 2-mercaptoethanol. After centrifugation, DNA was extracted from the pellet with ISOPLANT (Nippon Gene, Toyama, Japan) as suggested by the manufacturer. Total RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan) as suggested by the manufacturer for Northern blot analysis. Southern and Northern blotting was performed using DIG-High Prime and DIG Luminescent Detection Kit for nucleic acid (Boehringer, Mannheim, Germany).

Results and Discussion

GUS activity level among the progeny of a primary transformant

We previously reported the Mendelian inheritance of the introduced GUS gene from primary transformants to their progenies (Aida and Shibata 1995). We measured GUS activity in the progenies 4 to 5 weeks after germination. Some of the self fertilized progeny (R₁) of the primary transformant R₀-1 (having a single GUS gene locus) showed GUS activity levels of greater than 10 and less than or equal to 500 nmol 4-MU/mg protein/30min (Fig. 1b). These lower levels were rarely shown among progeny of the R₀-1 $\times$ wild type (Fig. 1a). We hypothesized that levels of GUS activity would be lower in GUS-homozygous plants than in GUS-hemizygous ones. We randomly selected two GUS-strong and two GUS-weak plants from the R₁ progeny to determine whether they were homozygous or hemizygous. Table 1 shows the segregation of GUS activity in the progeny of R₁ $\times$ wild type or R₂. All the progeny of the two GUS-weak R₁ plants showed GUS activity, suggesting that the plants showing lower activity were homozygous on the GUS gene. On the other hand, the progeny of the two GUS-strong plants fell into the ratio of about 1 : 1 (GUS+ : GUS-) when crossed with the wild type, and 3 : 1 (GUS+ : GUS-) when self fertilized, suggesting that the plants showing higher activity were hemizygous on the GUS gene. Distribution of GUS activity in the progeny of R₁ $\times$ wild type and R₂ is shown in Fig. 2. The levels in the progeny of the GUS-weak R₁-20 (homozygous) $\times$ wild type, all of which were considered hemizygous, were higher (Fig. 2a) than in the R₁-20 self fertilized progeny, all of which were considered homozygous (Fig. 2b). Some of the self fertilized progeny of the GUS-strong R₁-16 (hemizygous) plants, that might segregate into the ratio of about 1 : 2 : 1 for GUS-homozygous : GUS-hemizygous : no gene, showed a lower GUS activity level (Fig. 2d). Such levels were rarely shown among the

Fig. 1. GUS activity levels among offspring of the primary transformant, R_0-1 , which carry a single GUS gene locus. GUS activity of the first leaf from the top was measured 4 to 5 weeks after germination.

A: GUS activity levels among offspring of the $R_0-1 \times \text{wild type}$. It is supposed that about half are GUS-hemizygous, and the remainder do not carry the GUS gene locus.

B: GUS activity levels among offspring of the $R_0-1 \text{ self}$. It is supposed that the offspring fall into the ratio of 1 : 2 : 1 for GUS-homozygous : GUS-hemizygous : no gene. Some of the progeny show lower levels of GUS activity rarely seen in $R_0-1 \times \text{wild type}$ plants. It is thought that the GUS-homozygous plants might show lower levels of GUS activity than GUS-hemizygous plants.

Table 1. Segregation of GUS activity in $R_1 \times \text{wt}^{1)}$ or R_2 generation of a primary transformant R_0-1

R_0-1 offspring	GUS+ : GUS-	Expected ratio	χ^2	Expected zygosity Probability on the parental R_1 plant
R_1-20 (GUS weak) $\times \text{wt}^{1)}$	36 : 0	1 : 0	0	1 homozygous
R_1-20 (GUS weak) self	38 : 0	1 : 0	0	1 homozygous
R_1-25 (GUS weak) $\times \text{wt}^{1)}$	41 : 0	1 : 0	0	1 homozygous
R_1-25 (GUS weak) self	40 : 0	1 : 0	0	1 homozygous
R_1-16 (GUS strong) $\times \text{wt}^{1)}$	26 : 17	1 : 1	1.884	0.1 - 0.2 hemizygous
R_1-16 (GUS strong) self	27 : 16	3 : 1	3.419	0.05 - 0.1 hemizygous
R_1-22 (GUS strong) $\times \text{wt}^{1)}$	25 : 21	1 : 1	0.348	0.5 - 0.7 hemizygous
R_1-22 (GUS strong) self	9 : 4	3 : 1	0.231	0.5 - 0.7 hemizygous

¹⁾wt : wild type

progeny of the $R_1-16 \times \text{wild type}$ (Fig. 2c), that might segregate into the ratio of 1 : 1 for GUS-hemizygous : no gene. These results suggested that the GUS-homozygous plants showed lower activity levels than the GUS-hemizygous plants 4 to 5 weeks after germination.

Evolution of GUS activity in developing seedlings of GUS-homozygote and GUS-hemizygote

Two GUS-homozygous plants of the R_1 progeny (No. 20 and No. 25) were either self-fertilized or back-crossed with wild-type plants to obtain homozygotes or hemizygotes on the GUS gene for determining GUS-activity evolution in developing seedlings. We confirmed the inheritance of the GUS gene locus by Southern blot analysis (Fig. 3). GUS activity in plants of two homozygote and two hemizygote groups derived from R_0-1 was measured at various developmental stages (Table 2). Each plant was used for only one measurement to avoid the putative bias induced by the cutting stress. The level of GUS activity was high in all

the plants at day 5 (5 days after germination) and at day 12. The level in the homozygote was about double that in the hemizygotes. This result is reasonable because the homozygotes have 2 GUS gene locus in their genome, while the hemizygotes have only 1. At 19, the activity level in the homozygotes decreased suddenly compared to that of day 12 and never returned to a high activity level. This result suggests that the silencing of the GUS gene occurred rapidly and synchronously between day 12 and day 19 in the homozygous plants. However, most of the hemizygotes maintained a high activity level until day 54, with most of them showing a much reduced activity level by day 68. Variation in activity was observed among individual hemizygotes at day 54 and day 68. These results suggest that the silencing also occurred in the hemizygotes, but at a later stage.

Distribution of GUS activity in a homozygous and a hemizygous seedling was analyzed by histochemical assay at day 40 (Fig. 4). High activity was observed throughout the whole plant in the hemizygote, but in the

Fig. 2. GUS activity levels among $R_1 \times$ wild type and the R_2 generation of R_0-1 .

- A: GUS activity levels among offspring of the GUS-weak R_1-20 (GUS-homozygous) $\times$ wild type. All the offspring might be GUS-hemizygous.
- B: GUS activity levels among offspring of R_1-20 (GUS-homozygous) self. All the offspring might be GUS-homozygous, with levels lower than the GUS-hemizygous progeny.
- C: GUS activity levels among offspring of the GUS-strong R_1-16 (GUS-hemizygous) $\times$ wild type. It is supposed that about half are GUS-hemizygous and the remainder do not carry the GUS gene locus.
- D: GUS activity level among offspring of R_1-16 (GUS-hemizygous) self. It is supposed that the offspring fell into the ratio of 1 : 2 : 1 for GUS-homozygous : GUS-hemizygous : no gene. Some of the progeny shows lower levels of GUS activity which are rarely seen in $R_1-16 \times$ wild type.

Fig. 3. Southern blot analysis on the GUS gene.

Total DNA digested with *Hind* III was electrophoresed and transferred to a nylon membrane. The coding region of the GUS gene was used as a probe. Blots were finally washed with $0.5 \times$ SSC, 0.1% SDS at 68°C . The primary transformant, R_0-1 , shows a single band. The homozygotes and the hemizygotes on the GUS gene locus, which were progeny of R_0-1 , were used for analyzing the evolution of GUS activity. They also show a single band at the same place as the parent, which suggests they have the GUS gene(s) at the same locus as the parent.

homozygote, high activity was detected only around the cotyledons with the upper parts showing faint activity. It was optically demonstrated that the GUS gene silencing in early developmental stages occurred only in the

homozygote and not in the hemizygote. The silenced state was stable through vegetative propagation both in the homozygotes and in the hemizygotes. Vegetatively propagated plants derived from apical shoots of the

Table 2. Evolution of GUS activity (nmoles 4-MU/mg protein/30min) in developing torenia seedlings and vegetatively propagated plants

Days after germination	5	12	19	26	40	54	68	82	103	138
R ₁ -20 self	8259	5998	87	35	49	47	19	5	124	46
homozygote	11324	5547	116	200	118	18	6	4	58	16
	4628	6707	80	4	84	8	14	5	71	12
	8871	8540	90	27			16	5	10	65
	9361	8866	73	44			12	6	23	14
	11837	8948	79	47			11	4	101	34
R ₁ -20 × wild	6794	4527	2121	3189	4912	6610	12	27	37	306
type	6963	3987	3115	2242	4242	2722	30	221	15	102
hemizygote	3551	3706	1608	4889	3347	2049	31	268	26	113
	4682	4129	1881	3543			626	115	41	87
	5579	4730	1603	2884			89	192	44	32
	6751	3941	1855	3420			21	110	28	12
R ₁ -25 self	9122	7066	522	314	35	6	7	13	16	6
homozygote	10298	7298	115	128	30	6	2	4	7	5
	10724	7415	166	1281	50	2	6	18	10	15
									239	19
									114	8
									40	15
R ₁ -25 × wild	4007	4387	1790	3570	3725	69	11	50	225	250
type	5319	4968	1912	4401	1368	2657	50	516	295	70
hemizygote	4169	4470	1949	4732	3432	2394	996	100	465	103
	4732	4684	2029	4098			182	102	463	65
	5579	5456	3025	307			724	5	754	57
	6536	3678	3227	4609			1040	5	445	422

Fig. 4. Distribution of GUS activity 40 days after germination.

Left: hemizygote, right: homozygote

GUS activity was examined with 5-bromo-4-chloro-3-indolyl glucuronide as a substrate. High activity is observed in the whole plant in the hemizygote, but in the homozygote, high activity is detected only around cotyledons with upper parts showing only faint activity. GUS gene silencing at early developmental stages is shown only in the GUS-homozygous plant.

Fig. 5. Northern blot analysis on GUS gene.

Total RNA was extracted from the homozygotes and the hemizygotes derived from R₁-20. The RNA was extracted from entire plants (day 12), lower part including cotyledons (day 26 lower part), upper part excluding cotyledons (day 26 upper part), or top leaves (day 138). The RNA was electrophoresed and transferred to a nylon membrane. The coding region of the GUS gene was used as a probe. Blots were finally washed with 0.1 × SSC, 0.1% SDS at 68 °C. At a day 26, the mRNA level is reduced only in the homozygotes, especially in the upper part. The mRNA is hardly detected in either the homozygotes or in the hemizygotes at day 138. It seems that the mRNA reduction correlates with the reduction of GUS activity.

primary seedlings maintained lower activity levels than the levels at earlier stages (Table 2, days 103 and 138). Northern blot analysis showed that mRNA levels of the GUS gene at day 26 were reduced only in the homozygotes (Fig. 5). The mRNA was hardly detected in either the homozygotes or the hemizygotes at day 138. It seemed that the mRNA reduction was correlated with the reduction of the activity.

Rapid silencing of the GUS gene was observed in both the homozygotes and the hemizygotes, but it occurred at an earlier developmental stage in the homozygotes. It seemed that a single GUS gene locus was enough to initiate the silencing, and initial levels of GUS activity correlated with the timing of silencing. This suggests that the GUS gene silencing observed in *torenia* was not dependent on allelic interaction of the GUS gene loci, but was correlated with dose effect. The plants used for evaluation of GUS activity evolution were daughters of the GUS-homozygous R_1 plants that showed lower GUS activity. In the daughters, the higher GUS activity observed at early developmental stages suggested that the lower activity condition of the parental plants had changed to a higher activity condition during reproduction. This kind of reactivation of the transgene during reproduction has been reported by several researchers (Hart *et al.* 1992; Elmayan and Vaucheret 1996; Kunz *et al.* 1996). Park *et al.* (1996) reported that post-transcriptional gene silencing is meiotically reversible, but transcriptional silencing, which is associated with methylation, is meiotically heritable. The GUS gene silencing in *torenia* seems to be correlated with initial GUS activity level, and the silent state can be reset during reproduction. This type of silencing can be explained by the RNA threshold model (Smith *et al.* 1994) rather than other possibilities that are associated with methylation of transgene such as integration into a chromosomal region of a highly repetitive DNA, integration as an inverted repeats organization, or interaction of homologous promoters. The GUS-homozygous *torenia* plants, whose transcriptional rates might be higher than that of the GUS-hemizygous plants, may exceed a threshold level at an earlier stage, activating the elimination system for the mRNA which then results in low steady-state levels of the mRNA.

Expression of transgenes linked to the GUS gene

The primary transformant, R_0 -1, was transformed with a binary vector plasmid pIG121-Hm (Hiei *et al.* 1994) carrying the NPT II gene, the GUS gene, and the HPT gene. The GUS-homozygotes and the GUS-hemizygotes, progeny of R_0 -1, took over the three transgenes and showed resistance to both kanamycin and hygromycin, forming shoots during the leaf test. Non-transformed controls survived neither on the kanamycin medium nor on the hygromycin medium.

We examined both kanamycin and hygromycin resistance in the seedlings of the homozygotes and the hemizygotes at various developmental stages. The leaf

tests were performed at the same time as GUS determination with the same plant. All the homozygotes and the hemizygotes showed resistance to both kanamycin and hygromycin throughout the examined developmental stages—even in the completely GUS-silenced plants (data not shown). It seemed that only the GUS gene was silenced throughout the plants' development—the linked NPT II gene, which precedes the GUS gene, and the HPT gene, which follows the GUS gene, are never silenced.

Expression levels of different linked transgenes have been found to have no correlation with each other in transgenic tobacco calli (An 1986; Peach and Velten 1991) or plants (Mlynarova *et al.* 1995). We previously reported GUS gene silencing in *Kalanchoe blossfeldiana* that were also transformed with pIG121-Hm (Aida and Shibata 1996). Some GUS-silenced *kalanchoe* transformants showed resistance to both kanamycin and hygromycin. The independent expression levels of linked transgenes could be explained by the silencing phenomenon. The tendency of gene silencing might be different for each transgene, which makes their expression levels different. The RNA threshold model demonstrates that high expression of a gene leads to its silencing. The CaMV 35S promoter, which regulates the GUS and HPT genes, promotes higher expression than the promoter of nopaline synthase which regulates the NPT II gene (Odell *et al.* 1985). Although the GUS gene and the HPT gene are both regulated by the CaMV35S promoter, our results suggested that the GUS gene might be more easily silenced than the HPT gene. But it is not clear that the HPT gene might be developmentally silenced, since we only analyzed the hygromycin resistance until the day 138. Nuclear run-on assay should be performed to analyze the actual expression levels of both genes in the future to discern the reason for these differences.

Conclusion

We demonstrated developmental transgene silencing and resetting in *torenia*. Transgene silencing has been studied mainly in tobacco; however, the study of the silencing phenomenon in *torenia* will also be useful due to its transformation efficiency and ability to yield seeds under *in vitro* condition (Aida and Shibata, in press). Analysis of transgene silencing would contribute to developing a stable transgene expression system for plants.

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