Korean Journal of Life Science
Vol. 12. No. 1. 67~76, 2002

The increased GUS gene inactivation over generation in Arabidopsis transgenic lines

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Abstract

The effect of transgene inactivation in T2, T3 and F2 generations was analyzed in progeny seedlings which had been generated by *Agrobacterium* (LBA4404/pBI121)-mediated transformation in *Arabidopsis thaliana*. In a system which investigated in the expression of β-glucuronidase(GUS)gene in kanamycin-resistant (kan^R)seedlings, GUS inactivated seedlings were observed in 5 of 12 tested lines of T2 generation and the frequency of GUS inactivation was approximately 2.3%. Lines with multi-copies of T-DNA exhibited severe GUS gene inactivation with the frequency of 5.8% in T2 generation. In T3 generation lines exhibited GUS gene inactivation with the frequency of 1.3%. In contrast, inactivation increased dramatically up to 12.6% in multi-copy T-DNA line. A similar phenomenon was also found in F2 progeny from a transgenic line which had been crossed with wild-type Arabidopsis plant, WS-O (GUS gene inactivation frequency 9.9%). These results indicate that the foreign gene introduced into the plant was inactivated progressively in its transmission during subsequent generations and the transgenic line with multi-copies of T-DNA tended to show more increased inactivation.

Key words – *Arabidopsis thaliana*, T-DNA, Agrobacterium-mediated transformation, GUS inactivation, T2, T3 generation

Introduction

Many transgenic plants have been generated by *Agrobacterium*-mediated transformation or by a variety of direct gene transfer techniques. In general, transgenes introduced into receptive plant genome are inherited as simple, dominant Mendelian traits. However, variation in the expression of introduced foreign genes have frequently been observed in a number of transgenic plant systems[4,9,11]. Transgene inactivation has been studied extensively for a number of crop species including tobacco and petunia[13,21,23]. Most of the studies have focused on the elucidation of inactivation mechanism and factors, such as differences in copy number or integration sites affecting on transgene inactivation, and the relation between endogenous genes and transgenes [5,6,12,19]. In most cases this phenomenon, including the complete loss of transgene activity, is associated with increased methylation of the T-DNA[14,22]. These facts have been verified by a variety of experimental observations. For example, the demethylating agent, 5-azacytidine, treatment is used frequently to restore the activity of silenced genes[10,17]. Despite these numerous published reports, there is little detailed information on the effect of generation progression in transgene inactivation.

In this paper, the transgene inactivation in Arabidopsis transgenic lines was investigated in T2 and T3 generation, focusing on determining the relationship between the extent of transgene inactivation and generation progress.

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In order to do that, the differential inactivation system of GUS reporter gene and neomycin phosphotransferase-II (*nptII*) seletion marker was used as the approach of analyzing frequencies of transgene inactivation in T2 and T3 generation. This system is based on differential expression of the GUS and *nptII* gene in various transgenic lines and correlated with the degree of transgene methylation[1,16,20]. By investigating GUS gene inactivation from kanamycin resistant T2 and T3 progeny seedlings of transgenic Arabidopsis lines, the stability of transgene was evaluated over generation. These experimental results show strong evidence that the expression of transgene is decreased gradually when generation is proceed from T2 to T3 and that this inactivation is more severe in multi-copy T-DNA line than in single copy.

Materials and Methods

Bacterial strain and plant materials

Agrobacterium tumefaciens strain LBA4404, harbouring a binary vector (pBI121), was used to transform Arabidopsis. The binary vector carried GUS and *nptII* coding gene[8]. *Agrobacterium* were grown in YEP media, supplemented with antibiotics (streptomycin 100mg/L, kanamycin 50mg/L), with vigorous shaking (200rpm) at 28°C for 18 hours. The *Arabidopsis thaliana* L. Heynh, ecotype Wassilewskija (WS-O) was originally obtained from the ARS seed stock center (Koln, Germany) and used for transformation. Arabidopsis root explants were cultured on a germination medium (GM) containing MS macroelements, microelements, vitamins of Gamborg, 3% sucrose and 8g/L phytagar (Gibco). All media were buffered with 0.5g/L 2-(N-morpholino) ethanesufonic acid (MES) and adjusted to pH5.7. Growth regulators (6-benzylaminopurine, BAP; indole-3-acetic acid, IAA) were dissolved in dimethyl sulphoxide (DMSO), and antibiotics (kanamycin and vancomycin) were filter-sterilized and added to the medium. The following media were used for transformation and regeneration. GM, basal germination medium; Shoot-inducing medium (SIM1), GM supplemented with 2mg/L BAP and 0.2mg/L IAA; SIM2, SIM1 supplemented with 1000mg/L vancomycin; SIM3, SIM1 supplemented with 50mg/L kanamycin, 500mg/L vancomycin.

Transformation procedure of Arabidopsis root explants

For transformation of Arabidopsis root explants, root segments were spread on SIM1 agar plate and incubated for 2 days for conditioning. The conditioned root segments were transferred to 10ml liquid SIM1 into which 1ml of Agrobacterium overnight culture was added, and were incubated for 5 minutes with gentle shaking. Root segments were then transferred to SIM1 plates and incubated in a growth chamber. After 2-3 days of cocultivation, overgrowth of *Agrobacteria* around the root segments was evident. The cocultivated root segments were then transferred to 10ml of SIM2 and shaken vigorously to wash off the *Agrobacteria*. The washed root segments were then incubated on a SIM3 plate. After approximately two or three weeks, formation of green calli was observed. After 2-3 additional weeks, the small regenerating shoots (approximately 0.5cm in height) were transferred to GM plates to set seed.

In-planta transformation

The growth of plant materials, transformation procedure and selection of transformed plants were performed as described before[2].

Kanamycin resistance test and GUS assay

T2 seeds from transgenic plants were tested for kanamycin resistance and GUS expression. The T2 seeds were surface-sterilized and placed on a MS basal medium plate containing 50mg/L kanamycin. Following cold treatment for 3 days at 4°C to improve germination, the plates were transferred to the growth room. The T2 plants were grown at 22°C for 1 week under constant

fluorescent light (3000 Lux). The T2 seeds from the transformed plants were germinated and kan^R seedlings developed green cotyledons on a MS medium plate containing kanamycin. For kanamycin sensitive (kan^S) seedlings, the cotyledons began to bleach and never developed primary leaves or roots. For *in situ* staining to detect GUS activity, plant tissues were incubated for 6 hrs in a staining buffer containing 1mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-gluc, Research Organics Co.) as a substrate[7]. The T2, T3 and F2 kan^R seedlings from each line were assayed *in situ* for GUS activity with X-gluc.

PCR amplification of GUS and *nptII* gene fragment

The gene-specific primers for amplifying the GUS gene fragment (primer P1 and P2), and *NPTII* (primer P3 and P4) were designed and used for PCR. The sequence of primers are as follows (P1, ACGAACTGAACTGGCA-GACTATCCCC; P2, CGTACCT CGCATTACCCTTACGCT; P3, ACTCGGGAGCGGCGATACTGTA; P4, GAGGCTAT-TCGGCTATGACTG). The reaction was carried out in a total volume of 100μl that contained 0.2mM of each deoxynucleotide, 0.5μg of each primer, 2 unit of Taq polymerase and approximate 100 ng of Arabidopsis DNA, and was overlaid with 50μl of mineral oil. Plant DNA for PCR was extracted from leaves according to the method of Dellaporta[3]. Samples were heated to 94°C for 5 min and then amplified in a PCR machine. Conditions for the amplification were as follows: denaturation for 2 min at 94°C; annealing for 2 min at 60°C; polymerization for 2.5 min at 72°C. The cycle was repeated 30 times. Each PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

Results

Transformation of *Arabidopsis thaliana*

Two methods of the Agrobacterium-mediated transformation of Arabidopsis were developed and used for this study. One is the root-explants transformation method which employs a direct shooting media containing BA and IAA[18]. The other is *in-planta* transformation as a non-tissue culture approach, which involves simple *in planta* inoculation of Agrobacterium on wounded plant [2]. The *Agrobacterium tumefaciens* strain used for transformation harbors a binary vector pBI121 containing *nptII* genes which confer kanamycin resistance in the T-DNA region, allowing selection of transformed plant cells. Also, pBI121 carries the GUS gene, allowing confirmation of transformation by GUS gene expression[8]. With the root-explants transformation, the first kanamycin resistant shoots, 3-4 mm in size, were obtained only 2 weeks after cocultivation and numerous shoots were formed thereafter. The transformation frequency (the number of shoots to treated root segments) was 33%. In these results, the plants that regenerated from primary transformed shoots are designed T1 and the seeds from T1 plants are T2 seeds. Subsequent generations derived from T1 plants and T2 seeds are T2 plants, T3 seeds, T3 plants and T4 seeds.

The independent 110 transformants in T1 generation were generated using both transformation methods. These T2 seeds collected from T1 plants were screened for kanamycin resistance encoded by *nptII* gene. 28% of the lines showed either kanamycin sensitivity or no clear segregation, but most of the remaining lines showed a Mendelian segregation ratio on kanamycin resistance. The 12 T2 lines showing genetic segregation of 3:1 or 15:1 were chosen and studied in this research. Most of these 12 lines showed ratios compatible with the segregation of single or multiple ($\geq$2 copies) T-DNA inserts (Table 1).

After 1 week of germination on selective media containing kanamycin (50 mg/L), the kan^R seedlings were assayed with X-gluc for GUS gene expression. Among the kan^R seedlings, we found the phenomenon of GUS gene inactivation which kan^R seedlings had no detectable GUS activity. In order to verify the presence of T-DNA in genomic DNA from kan^R seedlings with no detectable

Table 1. GUS inactivation in transgenic lines of *Arabidopsis thaliana* in T2 generation

Transformants[a]		Segregation		GUS inactivation	Frequency (%)[b]
		kan^R	kan^S	GUS(-) / kan^R	
Single copy lines	R11	17	6	0 / 17	-
	R17	141	26	0 / 36	-
	R45*	43	18	2 / 41	4.9
	R53	117	26	0 / 36	-
	R65	74	16	0 / 67	-
	R67	34	25	0 / 29	-
	R68	37	13	0 / 33	-
	R77	16	5	0 / 16	-
Multi-copy lines	R10*	22	1	1 / 23	4.3
	R36*	67	2	3 / 24	12.5
	R37*	287	16	1 / 43	2.3
	R63*	34	1	2 / 31	6.5

[a]R17, R53, R68 from single copy T-DNA lines and R37 from multi-copy lines were selected for further investigation in T3 generation.
[b]GUS inactivation frequencies were determined as proportion of kanamycin resistant-GUS negative seedlings to total seedlings.
*GUS inactivation line

GUS activity, PCR analysis was conducted. In all PCR reactions using four specific primers; two (P1 and P2) for recognizing coding sequence of GUS gene, and two (P3 and P4) for *NPTII* gene, the results indicated that kan^R seedlings contained GUS and *NPTII* gene in their genome, even if they did not have GUS activity (data not shown).

GUS gene inactivation of kan^R seedlings in T2 generation

We used the GUS gene inactivation as the indicator in order to investigate transgene stability in T2 and T3 generation. The results of the GUS assay in T2 generatin are shown in table 1. In five (R10, R36, R37, R45 and R63) of all 12 tested lines, the GUS inactivation of kan^R seedlings were observed with a total inactivation frequency of 2.3% (No. GUS inactive seedlings/No. tested kan^R seedlings = 9/396).

In respect of the effect of T-DNA insert number on GUS gene inactivation, GUS(-)kan^R seedlings were found in all four lines (R10, R36, R37 and R63) which showed a segregation ratio of 15:1 or more for kanamycin resistance, hence containing two or more copies of T-DNA in their genome. However GUS gene inactivated seedlings were found in one of eight lines which contained single copy T-DNA. In conclusion, GUS gene inactivation was much higher in multi-copy T-DNA lines (GUS inactivation frequency 7/121 = 5.8%) than in single T-DNA containing lines (2/275 = 0.7%).

GUS gene inactivation of kan^R seedlings in T3 generation

The inactivation of GUS gene of kan^R seedlings containing single copy and multi-copy T-DNA was investigated in T3 generation. Single copy T-DNA lines used in this experiment were 3 lines (R17, R53, R68) which were randomly selected. These lines had a single copy of T-DNA which was determined by segregation ratio of kan resistance, and did not show GUS inactivation in their T2 generation (table 1). In two (R53 and R68) of the three tested lines, the GUS inactivated kan^R seedlings were observed with a frequency of 2.1%(4/144) and 2.2% (4/180), one line(R17) had no GUS inactivated seedlings in their progenies at the T3 generation (0/276). The inactivation frequency of three single copy lines in T3

generation was approximately 1.3% (8/600). Table 2 shows the results of kanamycin selection and GUS expression from T3 progeny seedlings in R68 line.

Among multi-copy T-DNA lines, the R37 was chosen for the analysis in T3 generation, since this line exhibited weak GUS inactivation with the frequency of 2.3% in T2 generation (table 1). The selfed 54 progeny lines in T3 generation were generated and tested for kanamycin resistance. Subsequently, the kan^R seedlings were assayed for GUS gene expression. Table 3 gives data on the kanamycin selection and GUS expression in the progeny lines. The copy number and genotype of the integrated T-DNA were determined by segregation pattern of T3 progeny seedlings on selective media. 22 of the 51 tested lines were shown to have GUS inactivated seedlings. The frequency of GUS inactivation (77/612 = 12.6%) was strikingly higher, up to 10 times, in comparison to those observed in transgenic T2 lines. In some T3 progeny lines (R37-9, R37-10, R37-34 and R37-51), the severe inactivation of GUS gene was detected with frequencies of more than 50%. Eleven of the fifteen lines which contained 2 copies of T-DNA with the heterozygous genotype, had GUS inactivated seedlings among their selfed T3 progenies. In contrast to this result, inactivation was observed in four of fifteen progeny lines with heterozygous, single T-DNA copy. However the frequency of inactivation observed in T3 generation did not show the relation with genotypes of T-DNA (homozy-

Table 2. GUS gene inactivation in progeny seedlings of single T-DNA copy line (R68) in T3 generation

Line No.	Segregation		Genotype[a] of T-DNA	GUS inactivation	Frequency
	kan^R	kan^S		GUS(-)/kan^R [b]	
R68-1	149	0	T-DNA(+/+)	0 / 12	3 / 60 (5.0%)
R68-2	147	0		0 / 12	
R68-3*	143	0		1 / 12	
R68-4*	126	0		2 / 12	
R68-5	111	0		0 / 12	
R68-6	119	26	T-DNA(+/-)	0 / 12	1 / 120 (0.8%)
R68-7	156	57		0 / 12	
R68-8	180	65		0 / 12	
R68-9	150	41		0 / 12	
R68-10	146	50		0 / 12	
R68-11*	76	17		1 / 12	
R68-12	186	42		0 / 12	
R68-13	165	51		0 / 12	
R68-14	103	53		0 / 12	
R68-15	148	51		0 / 12	
R68-16	0	114	T-DNA(-/-)	-	
R68-17	0	86		-	
R68-18	0	140		-	
R68-19	0	76		-	
R68-20	0	128		-	
R68-21	0	132		-	

[a]Genotype of T-DNA was determined by the segregation ratios of kanamycin resistance.
[b]12 kan^R seedlings were random-selected and assayed for GUS activity by *in situ* staining with X-Gluc.
*GUS inactivation line

Table 3. GUS inactivation of T-DNA in progenies of multi T-DNA copy line (R37[a]) in T3 generation

Line No.	Segregation		Genotype of T-DNAs	GUS inactivation	Frequency
	kan^R	kan^S		$GUS(-)/kan^R$	
R37-1	63	0		0 / 12	
R37-2	73	0		0 / 12	
R37-3*	60	0		2 / 12	
R37-4	60	0		0 / 12	
R37-5	78	0		0 / 12	
R37-6	68	0		0 / 12	
R37-7*	50	0		3 / 12	
R37-8	55	0		0 / 12	
R37-9**	72	0		6 / 12	
R37-10**	57	0	T-DNA(+/+)(+/+)	12 / 12	30 / 252 (11.9%)
R37-11*	43	0	T-DNA(+/+)(+/-)	1 / 12	
R37-12	61	0	T-DNA(+/+)(-/-)	0 / 12	
R37-13	68	0		0 / 12	
R37-14	55	0		0 / 12	
R37-15**	67	0		5 / 12	
R37-16	95	0		0 / 12	
R37-17	46	0		0 / 12	
R37-18	20	0		0 / 12	
R37-19	94	0		0 / 12	
R37-20*	54	0		1 / 12	
R37-21	58	0		0 / 12	
R37-22*	63	2		1 / 12	
R37-23*	44	4		1 / 12	
R37-24*	82	2		2 / 12	
R37-25	71	3		0 / 12	
R37-26	94	2		0 / 12	
R37-27	107	3		0 / 12	
R37-28*	115	7		2 / 12	
R37-29*	71	2	T-DNA(+/-)(+/-)	2 / 12	30 / 180 (16.6%)
R37-30*	69	4		2 / 12	
R37-31*	53	2		4 / 12	
R37-32*	72	4		1 / 12	
R37-33	80	5		0 / 12	
R37-34**	59	3		12 / 12	
R37-35*	72	6		1 / 12	
R37-36*	70	4		2 / 12	
R37-37	60	19		0 / 12	
R37-38	32	11		0 / 12	
R37-39	57	14		0 / 12	
R37-40*	24	5		1 / 12	
R37-41	66	17		0 / 12	
R37-42	63	14		0 / 12	
R37-43	34	20		0 / 12	
R37-44	35	10	T-DNA(+/-)(-/-)	0 / 12	17 / 180 (9.4%)
R37-45	31	11		0 / 12	
R37-46	50	27		0 / 12	
R37-47	43	6		0 / 12	
R37-48*	38	5		1 / 12	
R37-49	60	22		0 / 12	
R37-50*	86	38		3 / 12	
R37-51**	71	23		12 / 12	
R37-52	0	36		-	
R37-53	0	117	T-DNA(-/-)(-/-)	-	
R37-54	0	55		-	

[a]R37 line was identified as transgenic plant with 2 copies of intact T-DNA by Southern DNA blot analysis (data not shown) and showed a GUS inactivation frequency of 2.3% in T2 generation as described in Table 1.

*GUS inactivation line.

**Strong GUS inactivation line with higher frequency.

gote or heterozygote).

Similar inactivation patterns were also observed in F2 progenies from transgenic line crossed with wild-type (GUS inactivation frequency 40/403 = 9.9%; Table 4). Some F2 lines (F2-10 and F2-14) showed extremely high GUS inactivation frequencies of more than 80%.

Discdussion

The experimental results are in accordance with those of Ottaviani et al.[16] who reported the non-expression of introduced reporter gene in transgenic plants following selection for a linked selectable marker gene. They showed that the inactivation of the GUS gene occurred frequently in transgenic potato plants and that this phenomenon is induced mainly by specific methylation of the GUS gene. The fact that only one of the co-introduced genes was repressed selectively was also reported in transgenic tobacco[1]. Ottaviani's data indicated that the cytosine residues in most of the analyzed sites within the CaMV 35S promoter and GUS coding region were methylated, but the sites of the coding region of the *nptII* gene were suprisingly not methylated in GUS negative shoot lines. Linn et al. also demonstrated that inactivation of the introduced foreign gene in transgenic petunia plants was associated with increased methylation of sites within the 35S promoter preferentially[15]. It may thus be concluded that the GUS gene containing CaMV 35S promoter is probably more sensitive to methylation than the *nptII* gene containing *nos* promoter in lines transformed with pBI121. However the mechanism of differential methylation remains unrevealed. Since the binary vector pBI121, identical to Ottaviani's, was used for the transformation of Ara-

Table 4. GUS gene inactivation in F2 progeny lines from transgenic line[a] X WS-O wildtype

F2 Line	Segregation		GUS inactivation	Frequency of GUS inactivation (%)
	kan^R	kan^S	GUS(-) / kan^R	
F2-1	57	11	0 / 30	-
F2-2*	41	15	1 / 30	3.3
F2-3	159	18	0 / 29	-
F2-4*	146	16	1 / 28	3.6
F2-5	158	55	0 / 47	-
F2-6*	99	34	2 / 11	18.1
F2-7	69	13	0 / 25	-
F2-8	12	1	0 / 10	-
F2-9	69	16	0 / 30	-
F2-10**	44	11	15 / 15	100.0
F2-11*	25	2	1 / 20	5.0
F2-12	40	1	0 / 28	-
F2-13	31	5	0 / 23	-
F2-14**	31	8	20 / 23	87.0
F2-15	15	2	0 / 10	-
F2-16	40	14	0 / 25	-
F2-17	40	11	0 / 19	

[a]The transgenic plant used was T2 plants of R37 line. After selfing F1 plants, the 17 F2 lines from each of the F1 plants were collected and used in this experiment. It was confirmed that T2 plant used in the cross exhibited the GUS activity.

*GUS inactivation line.

**Strong GUS inactivation line with higher frequency.

bidopsis, it seems that the GUS inactivation in kanamycin resistant transgenic lines is due to the increased methylation in the GUS region of T-DNA.

We also observed a similar phenomenon on *nptII* gene, indicating the suppression of that gene in the T3 or T4 generations. On kanamycin-containing medium, a few lines showed a yellowish or a green and white mottled appearance of seedlings. This is apparently an unstable expression of *nptII* gene, since these lines showed all kanamycin resistance in their former generation. Some kanamycin sensitive seedlings showed a strong GUS expression. Although it has not been possible to determine the precise frequency of *nptII* gene suppression, this must be less low (1%>) when compared to that of GUS inactivation. From the above it is proposed that *nptII* gene including *nos* promoters may be methylated at low frequency. The cause of these results that *nptII* gene is less sensitive to methylation, appears to be the effect of selection pressure when the seedlings are maintained on selection medium. The experimental result indicated that GUS gene inactivation occurred frequently in plants with multicopy integration of the transgene. It has become obvious from published reports that copy number of the introduced T-DNA affect on gene expression negatively and multiple insertions tended to have increased methylation of the integrated T-DNA [5,15,21].

In summary, these experimental results indicate that (a) GUS gene inactivation increases progressively over generation and (b) transgenic plants with multiple t-DNA insertions tend to have more increased inactivation of GUS genein subsequent generation. The results presented here have important implications for plant breeding programs, in which useful transgenic plants are produced and applied for agricultural improvement. For the long term use of transgenic plants, the introduced transgene must be inherited in a predictable manner and maintain a stable expression over many generations. The above data strongly indicates that a system of selection for transgenic plants which contain a single intact copy, may be particularly useful in improving the stability of transgene. And many studies on the stability of promoters have to be done extensively. Also the fact that transgene inactivation increases dramatically over generations have to be considered essentially to evaluate the uniformity of valuable agronomical traits in transgenic crop plants which may be in T3 or T4 generation in the farmer's field.

Acknowledgements

I thank Kee Yeun Kim and Yong Hwi Yoon for the analysis of experimental data and maintaining Arabidopsis transgenic lines.

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(Received January 23, 2002; Accepted February 18, 2002)

초록 : 애기장대 형질전환 식물체의 세대경과에 따른 GUS유전자의 비활성화에 관한 연구

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Agrobacterium(LBA4404/pBI121)을 이용하여 형질전환된 애기장대 (*Arabidopsis thaliana*)를 대상으로 T2, T3, F3 세대에서의 도입된 외래 유전자의 비활성화 현상을 조사하였다. Kanamaycin 저항성 개체들의 GUS 유전자 발현을 분석한 결과, T2세대에서 조사된 12계통 중 5계통에서 GUS 비활성 개체가 관찰되었다 (GUS 유전자 비활성율 2.3%). Multi copy T-DNA 계통을 조사한 결과, GUS 비활성 정도가 더욱 심해짐이 관찰되었다 (5.8%). T3 세대에서 single copy T-DNA 계통들은 1.3%의 GUS 비활성율을 보인 반면, multi-copy T-DNA 계통에서의 비활성율은 12.6%로 급격히 증가하였다. 유사한 현상이 형질전환 식물체와 정상개체를 교배하여 생산된 F2 계통에서도 관찰되었다 (비활성율 9.9%). 본 실험으로 식물체에 도입된 외래 유전자가 후대에서의 전이과정동안 점진적으로 비활성화되고, 이 현상은 multi copy T-DNA 계통에서 훨씬 심각함이 밝혀졌다.