Development of Visible Markers for Transgenic Plants and their Availability for Environmental Risk Assessment

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Monitoring of transgenic plants in the field is important, but risk assessment has entailed laborious use of invisible marker genes. Here, we assessed three easily visible marker transgenes – *green fluorescent protein* (*GFP*), *R*, and *Nicotiana tabacum* homeobox (*NTH*) *15* genes – for their potential use as marker genes for monitoring genetically modified plants. Transgenic *Arabidopsis thaliana* plants for each of these genes were visibly distinguished from wild-type plants. We determined the germination rate, 3-week fresh weight, time to first flowering, and seed weight of the transgenic plants to evaluate whether the expression of these marker genes affected the growth of the host. Introduction of *GFP* gene had no effect on the evaluated parameters, and we then used the *GFP* gene as a marker to assess the outcrossing frequency between transgenic and two *Arabidopsis* species. Our results showed that the hybridization frequency between transgenic plants and *Arabidopsis thaliana* was 0.24%, and between transformants and *Arabidopsis lyrata* it was 2.6% under experimental condition. Out-crossing frequency was decreased by extending the distance between two kinds of plants. Thus, the *GFP* gene is a useful marker for assessing the whereabouts of transgenes/transformants in the field. We also demonstrated that the *GFP* gene is possibly applicable as a selection marker in the process of generation of transgenic plants.

Key words: Green Fluorescent Protein, Risk Assessment, Transgenic Plant

Introduction

With the development of gene manipulation techniques, concerns have arisen about the potential consequences of the genetic modification of organisms, especially in agricultural crops (Raybould and Gray, 1994; Wolfenbarger and Phifer, 2000). Because predicting the impact, if any, novel transgenes will have on nature is difficult, risk assessment of genetically modified (GM) organisms typically is performed instead. These studies have focused on the toxicological or nutritional characteristics of transgenes and the proteins they encode (Richards *et al.*, 2003) and on the escape of GM plants into the environment through hybridization with their wild relatives (Raybould and Gray, 1993; Chevre *et al.*, 1997; Halfhill *et al.*, 2002; Rieger *et al.*, 2002; Wilkinson *et al.*, 2003; Zhu *et al.*, 2004). Hybridization has the potential for introgression of transgenes, allowing them to propagate in the environment (Raybould and Gray, 1993). Therefore, it is important to develop a widely applicable and suitable system for monitoring GM plants in the field.

In current monitoring methods, antibiotic- or herbicide-resistance genes are used as markers. To detect these genes to confirm the identity of GM plants is, however, hardly a simple task because it requires destruction of plant tissue and is time-consuming. An easier means of following marker genes to confirm the presence of GM plants in the field would be welcome.

Presented here are three candidate marker genes, the expression of which confers visible changes to plants and, therefore, could allow us to track GM plants macroscopically, even *in situ*.

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They are the *green fluorescent protein* (*GFP*), *R*, and *Nicotiana tabacum* homeobox (*NTH*) *15* genes. The *GFP* gene was first cloned from a jellyfish, *Aequorea victoria*, and its protein exhibits green fluorescence under blue or UV light without any additional substrates or co-factors (Prasher *et al*., 1992). Compared with the original jellyfish GFP sequence, the S65T mutation in the chromophore (sGFP) has yielded ~100-fold brighter fluorescent signals in plants and mammalian cells (Chiu *et al*., 1996; Haas *et al*., 1996). The *R* gene is a transcriptional activator that regulates the synthetic pathway of anthocyanin, and its overexpression turns plant organs red (Lloyd *et al.*, 1992). The *NTH15* gene is a *knotted*-type homeobox gene associated with leaf formation in plants. Tamaoki *et al*. (1997) reported that overexpression of *NTH15* led to morphological changes in tobacco leaves, and they proposed its use as a marker for assessment of GM plants in the field (Tamaoki *et al.,* 2003).

We investigated the possible use of *GFP*, *R*, and *NTH15* as marker genes. For utility as visible markers, the genes must express only their visual traits without any other side effects on host plants. To confirm this, we first introduced *GFP*, *R*, or *NTH15* into *Arabidopsis thaliana* and evaluated their effects on germination rate, seedling weight, time to first flowering, and seed weight in the host plants. We then assessed the frequency of outcrossing in wild-type *A. thaliana* (WT) and its close relative *A. lyrata* from transgenic GFP *A. thaliana* under experimental conditions.

Furthermore, we also investigated the potential of the *GFP* gene as a selectable marker to generate transgenic plants for the following reason. The majority of the GM plants tested in field releases or commercialized contains selectable markers such as bacterial antibiotic-resistance genes (reviewed by Miki and McHugh, 2004). The hypothetical acquisition of plant-harbored antibiotic-resistance genes by bacterial communities from transgenic plants is often discussed as an undesired effect of large-scale applications of GM plants, due to well-known problems caused by antibiotic-resistant bacteria (Tschäpe, 1994). Actually, horizontal gene transfer from debris of transgenic plants carrying the *npt*II gene to bacteria was demonstrated, and in some cases, bacteria acquired kanamycine-resistance (Gebhard and Smalla, 1999). Thus, it is desired to develop a secure selectable marker for generating GM plants. Therefore, suitability of the *GFP* gene as a selection marker for generating transgenic plants was also studied.

Materials and Methods

Plant materials and growth conditions

A. thaliana (L.) Heyhn. accession Columbia (Col-0; The Arabidopsis Biological Resource Center, Columbus, OH, USA) was used as the WT plant. *A. lyrata* subsp. *lyrata* Pn2 was used for estimating natural hybridization frequency. GFP transgenic lines expressing modified jellyfish *green fluorescent protein* (*sGFP*) in a Col-0 background (lines nA5-2 and nA4-3; Niwa *et al*., 1999) were used in this experiment, and the nA5-2 line was designated as 35S::GFP. Transgenic *Arabidopsis* overexpressing the *R* gene and *NTH15* was created by introducing the 35S promoter of *Cauliflower mosaic virus* (*CaMV35S*)::*R* (pAL69) and *CaMV35S::NTH15* genes, respectively. These chimeric genes constructed in previous works (Lloyd *et al*., 1992; Tamaoki *et al*., 1997) were transformed independently into the T0 generation of *Arabidopsis* by the *Agrobacterium*-mediated vacuum infiltration method (Bechtold and Pelletier, 1998), and screened on medium containing 25 mg/L kanamycin. Transgenic *Arabidopsis* containing *CaMV35S::R* and *CaMV35S::NTH15* were designated as 35S::R and 35S::NTH, respectively. All the transgenic *Arabidopsis* used in this study were homozygous. Plants used in this study were grown in a growth chamber at 25 °C with a relative humidity of 50% to 60% under a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} in 14 h light/10 h dark cycles. Plants were watered with liquid fertilizer (Hyponex 5-10-5; Hyponex Japan, Osaka, Japan) diluted 2000-fold.

Measurement of seed germination rates, seedling fresh weight, time of first flowering, and seed weights

To determine seed germination rates, more than 100 seeds were placed on moist filter paper in Petri dishes and allowed to germinate. The number of emerged seedlings was counted 5 to 7 d later. A seed was considered to have germinated when both cotyledons emerged. The filter papers were kept moist by spraying with tap water during the experiment. Effects of the transgenes on the vegetative growth of *Arabidopsis* were determined by measuring the fresh weights of 3-week-old

seedlings. We sowed 5 to 10 seeds in each Jiffy-7 peat pellet (Jiffy Products International, Kristiansand, Norway) and maintained them in an Arasystem (Betatech bvba, Gent, Belgium). After 7 d, seedlings were thinned to one per pot. Exactly 3 weeks after sowing, the above-ground parts of the seedlings were harvested and weighed individually. To evaluate the effects of the transgenes on flowering and seed weight of *Arabidopsis*, we noted the time to first flowering and measured seed weights. When the plants had completed their life cycles and were well dried, the seeds of each plant were collected. Sets of seeds for each transgenic plant were chosen randomly, and 1000 seeds were weighed.

Investigation of out-crossing frequency between transgenic and non-transgenic plants

Estimation of out-crossing frequency between transgenic and non-transgenic plants was carried out in a greenhouse, separated from the outside, because the use of 35S::GFP *Arabidopsis* at field condition is not given approval by Japanese government as affairs now stand. The frequency of out-crossing between transgenic and WT or *A. lyrata* plants was investigated with the use of 35S::GFP as the pollen donor and WT or *A. lyrata* as the pollen acceptor. Seeds were sown on Jiffy-7 (Jiffy Products International) and maintained in the Arasystem (Betatech bvba) in the arrangements described in Results (see Fig. 4). 7 d after sowing, the seedlings were thinned to one per pot. When all flowering had finished, 35S::GFP plants were removed, and each of the remaining pollen acceptor plants was covered with a tube (height 40 cm) of the Arasystem. The WT or *A. lyrata* plants were maintained until they withered. After the plants had finished their life cycles and had dried, the seeds were collected from each individual plant. Each seed set was weighed to estimate the total number of seeds, which was calculated afterwards from the 1000-seed weight of a randomly selected plant from the experiment. All the collected seeds then were placed on moist filter paper in separate Petri dishes for each plant and allowed to germinate. 7 d after sowing, the emerged seedlings were screened under the excitation light (wavelength 488 nm) of a handheld non-UV lamp (Dark Reader Hand Lamp; Clare Chemical Research Inc., Dolores, CO, USA) for fluorescence in the dark, to estimate the frequency of GFP out-crossing. In the process, the emission light from GFP (wavelength 507 nm) was observed using Dark Reader Glasses (Model AG16; Clare Chemical Research Inc.) that block the blue excitation light generated by the Dark Reader Hand Lamp.

To confirm whether plants that showed GFP phenotype in out-crossing experiments were F1 hybrid between WT and 35S::GFP or not, the segregation of the GFP phenotype in the F2 generation was examined. Forty-seven lines of the GFP-fluorescent F1 seedlings were randomly selected, and their self-pollinating seeds were collected. Then, 40 seeds from each line were placed on moist filter paper in Petri dishes and allowed to germinate, and the number of seedlings was counted with GFP fluorescence of the seminal roots. Finally, we evaluated whether they displayed Mendelian segregation with the proportion of one non-GPF plant to three GFP plants or not.

Isolation of transgenic Arabidopsis with GFP fluorescence as a selection marker

Transgenic *Arabidopsis* overexpressing the *GFP* gene were created by introducing *CaMV35Somega::sGFP*. The chimeric gene was constructed in previous work (Niwa *et al.*, 1999) and transformed into the T0 generation of *Arabidopsis* as described above. The T1 seeds (about 20,000 seeds) were divided into halves, and one of that was germinated on medium containing 40 mg/L glufosinate (Basta; Bayer CropScience, Monheim am Rhein, Germany) to select transgenic plants (1st screening). Subsequently, glufosinate-resistant plants were checked for the presence of GFP fluorescence (2nd screening). The remaining seeds were sown on moist filter paper in Petri dishes, allowed to germinate, and 7-day-old seedlings with GFP fluorescence of the seminal roots were selected (1st screening). After that, plants were transferred on medium containing 40 mg/L glufosinate for 7 d to determine whether plants showed glufosinate-resistance or not (2nd screening).

Results

Generation of transgenic Arabidopsis and phenotypes of plants

Introducing the *CaMV35S::R* and *CaMV35S::NTH15* genes into *Arabidopsis* resulted in five lines of homozygous 35S::R and six lines of homozygous 35S::NTH. Two of the five 35S::R trans-

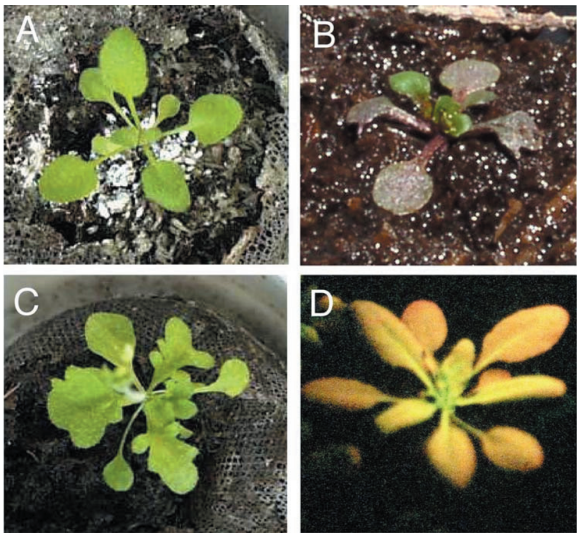

Fig. 1. Phenotypes of transgenic plants. (A) Col-0: Wild-type *Arabidopsis* used as control. (B) 35S::R: Transgenic *Arabidopsis* carrying the *CaMV35S::R* chimeric gene. The plant formed red leaves. (C) 35S::NTH15: Transgenic *Arabidopsis* carrying the *CaMV35S::NTH15* chimeric gene. The leaves of plants exhibit lobed morphologies. (D) 35S::GFP: Transgenic *Arabidopsis* carrying the *CaMV35S::GFP* chimeric gene. Yellowish-green fluorescence in leaves was observed as described in Materials and Methods.

genic plants showed extreme variation in plant color, whereas the other three lines were indistinguishable in appearance from WT. The transgenic 35S::R *Arabidopsis* with the severe phenotype formed leaves containing red pigment (Fig. 1B) and other organs, such as the stem, cauline leaves, flower petals, and siliques, also showed red pigmentation (data not shown). Because the red phenotype produced a high amount of anthocyanin, this line was used for further experiments.

All six 35S::NTH lines showed varying degrees of phenotypic changes in leaf development; we used the line that displayed the most prominent morphological change for further studies. In WT plants, the first two leaves produced by the juvenile vegetative meristem are small, round, and entire, whereas later adult leaves are larger, spatulate, and serrate (Fig. 1A). In the 35S::NTH line that we used, the first two leaves were normal in shape, whereas all subsequent rosette leaves were lobulated (Fig. 1C).

We confirmed that the 35S::GFP plants (line nA5-2) showed the expected green fluorescence. Yellowish-green fluorescence was observed over their entire leaves as described previously (Fig. 1D, Niwa *et al.*, 1999), whereas only a background of red chlorophyll fluorescence was detected in WT leaves (data not shown). The marked difference in fluorescence colors confirmed that the 35S::GFP would be useful for further experiments.

Effects of transgenes on plant growth

The germination rates, fresh weights of seedlings, times to first flowering and seed weights of the host plants were measured in WT and transgenic plants, and these parameters were compared to determine whether the transgenes conferred any undesirable effects on the host plants. The germination rate of vernalized seeds [mean ± standard error (SE); 3 independent experiments] was

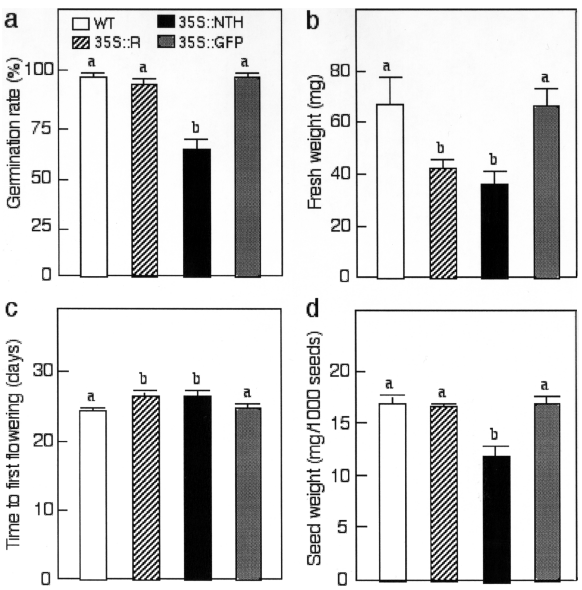

Fig. 2. Various developmental parameters of wild-type (WT) and transgenic plants. (a) Germination rate in WT and transgenic plants. More than 100 seeds were germinated on a filter paper, and the germinated seeds were counted after 7 days. The values shown are the averages of three independent experiments ± standard error (SE). (b) Fresh weight of seedlings in WT and transgenic plants. The fresh weight of 3-week-old plants was measured. The values shown are average plant fresh weights ± SE (n = 23 to 40). (c) Time to first flowering in WT and transgenic plants. The period from sowing to the appearance of the first flower was investigated. The values shown are average times to first flowering ± SE (n = 23 to 40). (d) Total seed weight in WT and transgenic plants. The values shown are the average 1000-seed weights ± SE (n = 10). Mean values with the same letter are not significantly different ($p < 0.05$) from WT.

(96.4 ± 1.8)% (n = 111) for WT, (96.5 ± 1.5)% (n = 144) for 35S::GFP, (93.3 ± 2.0)% (n = 150) for 35S::R, and (61.9 ± 4.3)% (n = 126) for 35S::NTH (Fig. 2a). Only the germination rate of 35S::NTH was significantly ($p < 0.05$) different from that of WT.

The fresh weight (mean ± SE) of 3-week-old seedlings was (65.0 ± 9.4) mg (n = 23) for WT, (64.7 ± 5.5) mg (n = 28) for 35S::GFP, (41.1 ± 1.7) mg (n = 40) for 35S::R, and (35.1 ± 3.7) mg (n = 23) for 35S::NTH (Fig. 2b). The average weights of both 35S::R and 35S::NTH seedlings were significantly less than that of WT seedlings ($p < 0.05$), whereas the difference between the average weights of 35S::GFP and WT seedlings was not significant.

The time to first flowering (mean ± SE) was (26.1 ± 0.32) d (n = 24) for WT, (26.6 ± 0.41) d (n = 27) for 35S::GFP, (28.4 ± 0.62) d (n = 40) for 35S::R, and (28.3 ± 0.67) d (n = 23) for 35S::NTH (Fig. 2c). The difference between the average flowering times of WT and 35S::GFP was not significant, whereas both 35S::R and 35S::NTH came into flower significantly later than WT did ($p < 0.05$).

The 1000-seed weight (mean ± SE; 10 independent experiments) was (19.3 ± 0.51) mg for WT, (19.3 ± 0.42) mg for 35S::GFP, (18.7 ± 0.32) mg for 35S::R, and (13.7 ± 0.71) mg for 35S::NTH (Fig. 2d). There was no significant difference between WT and 35S::GFP, nor between WT and 35S::R. By contrast, the average 1000-seed weight of 35S::NTH was significantly ($p < 0.05$) lower than that of WT.

Out-crossing frequency between 35S::GFP and Arabidopsis species

As described in the preceding section, only 35S::GFP showed similar growth to WT, indicating that introduction of the *CaMV35S::GFP* gene had no apparent deleterious side effects on the growth of host plants. We concluded that *GFP* could be used as a marker gene for monitoring transgene movements. Indeed, the 35S::GFP plants could easily be distinguished from the WT plants by their yellowish-green fluorescence from leaves (Fig. 3), so we used 35S::GFP plants in further experiments to assess out-crossing frequency.

We first investigated whether heterozygotic hybrids would show GFP fluorescence. Seedlings of artificially generated F1 hybrid WT/35S::GFP or

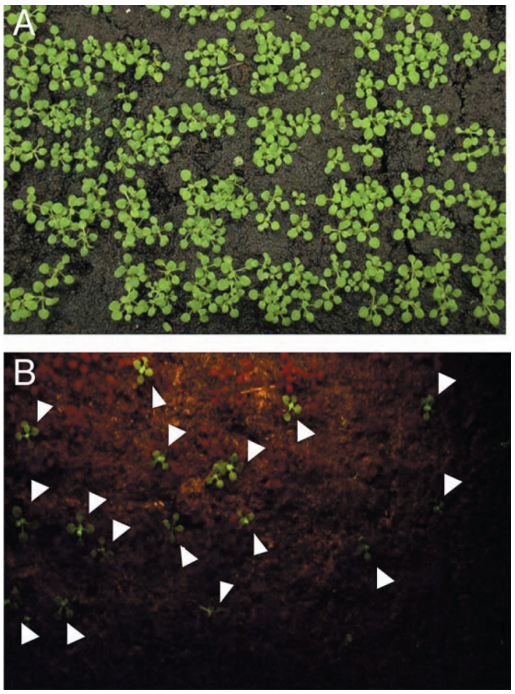

Fig. 3. Identification of 35S::GFP plants from a field overgrown with many *Arabidopsis* plants. (A) An *Arabidopsis* community observed under white light. (B) GFP fluorescence of the same field as in (A). Some transgenic plants carrying the *CaMV35S::GFP* transgene (yellowish-green plants) were visually identified among many WT plants (red plants). White arrows indicate 35S::GFP plants.

A. lyrata/35S::GFP F1 hybrids showed the red fluorescence of chlorophyll in their cotyledons but green fluorescence in their seminal roots, whereas WT seedlings showed only the red fluorescence of chlorophyll in their cotyledons (data not shown). Therefore, heterozygous WT/35S::GFP and/or *A. lyrata*/35S::GFP F1 hybrids can show green fluorescence in their seminal roots, and we used this parameter in further studies to indicate the presence of the *GFP* gene.

Natural out-crossing frequencies between WT and 35S::GFP plants were examined for the arrangements described in Fig. 4. The experiment with the "Zigzag array" (Fig. 4A) was conducted with three trays, and 93,348, 93,142, and 89,775 seeds were obtained from each tray. When the seeds were all allowed to germinate, 258, 202, and 205 of the resulting seedlings, respectively, exhibited green fluorescence in seminal roots. Therefore, the out-crossing frequencies were (0.28 ±

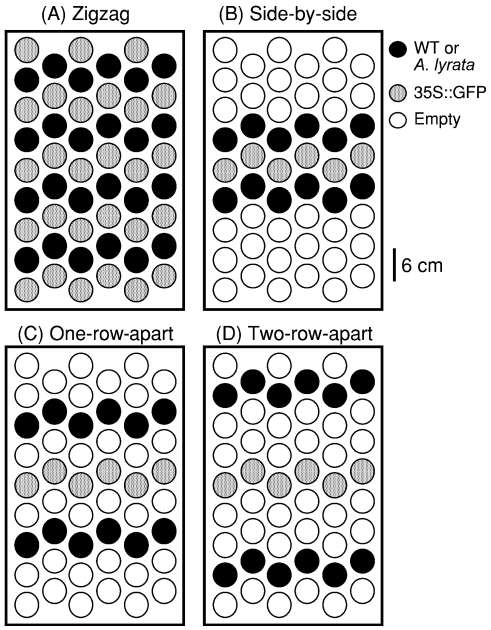

Fig. 4. Arrangements used to investigate the hybridization rate between WT or *A. lyrata* and 35S::GFP plants. Four arrays of pots were designed for measurement of hybridization frequency. (A) Zigzag array: WT or *A. lyrata* and 35S::GFP were set in parallel. (B) Side-by-side array: A single 35S::GFP array was sandwiched between WT arrays. (C) One-row-apart array: A single WT array was set on both sides, parallel to 35S::GFP arrays with a distance of 12 cm. (D) Two-row-apart array: A single WT array was set on both sides, parallel to 35S::GFP arrays with a distance.

0.021)%, (0.22 ± 0.015)%, and (0.23 ± 0.022)%, respectively, for the individual trays and (0.24 ± 0.02)% overall (Table I).

Whether these GFP-fluorescent seedlings were the products of hybridization between WT and 35S::GFP was answered by investigating the segregation of the GFP phenotype in the F2 generation. We allowed 47 lines of the F1 GFP-fluorescent seedlings to self-pollinate and collected the seeds. We then placed 40 seeds from each line on moist filter paper in Petri dishes, allowed them to germinate, and counted the number of seedlings with fluorescence of the seminal roots. F2 progenies of the GFP hybrids displayed Mendelian segregation with the proportion of one non-GPF plant to three GFP plants ($p > 0.05$) when 1381 seedlings from the 1823 seeds [rate of fluorescence (75.8 ± 1.0)%] exhibited fluorescence. This result shows that the F1 hybrids were heterozygous and inherited a single dominant *CaMV35S::GFP* gene, confirming that the F1-fluorescent seedlings were in fact hybrids between WT and 35S::GFP plants.

Estimation of natural out-crossing frequencies between *A. lyrata* and 35S::GFP plants was also carried out under the same conditions as above except for the sowing day. In our growth conditions, the first flower in 35S::GFP plants appeared after (26.6 ± 0.41) d (Fig. 2C) whereas the average of the first flower blooming in *A. lyrata* was (95.3 ± 2.51) d (data not shown). Thus, we sowed *A. lyrata* seeds two month before sowing seeds of 35S::GFP to adjust the flowering period. Accordingly, 4569, 5236, and 3989 seeds were obtained from each tray. When the seeds were all allowed to germinate, 103, 127, and 119 of the resulting seedlings, respectively, showed green fluorescence in seminal roots. Therefore, the out-crossing frequencies were (2.3 ± 0.4)%, (2.4 ± 0.3)%, and (3.0 ± 0.6%), respectively, for the individual trays and (2.6 ± 0.4)% overall (Table I). The segregation of the GFP phenotype at F2 generation of F1 hybrid between *A. lyrata* and 35S::GFP could not be determined because the plants were pollen sterile and could not self-pollinate (data not shown), as shown previously (Nasrallah *et al.* 2000).

Array type	Total number of seeds per experiment	Number of fluorescent seedlings	Out-crossing rate (%)
Zigzag (WT X 35S::GFP)	92,088 ± 1158	222 ± 44	0.24 ± 0.02
Side-by-side	11,767 ± 346	8 ± 0.9	0.068 ± 0.016
One-row-apart	14,923 ± 640	2 ± 0.7	0.016 ± 0.006
Two-row-apart	12,056 ± 971	0	0
Zigzag (*A. lyrata* X 35S::GFP)	4598 ± 624	116± 12	2.6 ± 0.4

Table I. Out-crossing frequencies between WT or *A. lyrata* and 35S::GFP in various arrays. All seeds of acceptor plants were allowed to germinate, and the fluorescent seedlings were counted. Each experiment was performed three times, and the data are represented as means ± standard error. Array types are indicated in Fig. 4.

Table II. Efficiency of *GFP* gene as a selection marker for generation of transgenic plants. About twenty thousand of T1 seeds that had transformed *CaMV35Somega::sGFP* chimeric gene were divided into halves, and 1st screening with glufosinate or GFP was carried out, independently. Then, glufosinate-resistant plants were checked for GFP fluorescence (2nd screening). Plants that showed GFP fluorescence at 1st screening were transplanted on medium containing glufosinate to check glufosinate-resistance (2nd screening). Transformation efficiency was calculated with dividing number of plants isolated at 2nd screening by the number of T1 seeds.

No. of T1 seeds	Selection marker of 1st screening	Result of 1st screening	Result of 2nd screening (%)[a]	Transformation efficiency (%)
11,294	glufosinate	49[b]	39[c] (80)	0.35
12,587	GFP	42[c]	42[b] (100)	0.33

[a] Values show correctness of 1st screening and are calculated with dividing number of plants isolated at 2nd screening by number of plants isolated at 1st screening.
[b] Screening with glufosinate resistance.
[c] Screening with GFP fluorescence.

Distance effect on out-crossing frequency

We assessed the effect of distance on out-crossing frequency by using three arrangements of array. The "side-by-side" array had a row of WT plants on either side of a row of 35S::GFP plants (Fig. 4B), with a distance of 6 cm between WT and 35S::GFP rows. The "one-row-apart" array followed the same pattern as the side-by-side array, but the rows were 12 cm apart (Fig. 4C). The out-crossing rate (mean ± SE) for the side-by-side array was (0.068 ± 0.016)%, whereas that for the one-row-apart array was (0.016 ± 0.006)% ($p < 0.05$) (Table I). No hybridization was observed when WT plants were set at least 18 cm apart from 35S::GFP plants (Fig. 4D, Table I).

Isolation of transgenic Arabidopsis with GFP fluorescence as a selection marker

To assess whether GPF fluorescence could be applicable for a selection marker to generate transgenic plants or not, we carried out two ways for screening of transgenic plants using T1 seeds derived from one transformation experiment. The one was resistant to glufosinate, the other was GFP-fluorescent. 49 plants showed glufosinate-resistance screening 11,249 T1 seeds (1st screening), and 39 of that showed GFP fluorescence both rosette leaves and roots (2nd screening, Table II). Thus, transformation efficiency using glufosinate-resistance is calculated as 0.35%. When 12,587 T1 seeds were screened with GFP fluorescence as indicator (1st screening), 42 seedlings showed GFP fluorescence in seminal roots (Table II). Surprisingly, all isolated seedlings showed glufosinate-resistance (2nd screening), suggesting that application of GFP as a selection marker results in isolation of few non-transgenic plants. The transformation efficiency using GFP fluorescence is calculated as 0.33%, that is similar to the result of screening with glufosinate-resistance.

Discussion

We developed three different transgenic *Arabidopsis* strains – 35S::GFP, 35S::R, and 35S::NTH. Among these plants, 35S::R and 35S::NTH plants could easily be distinguished from WT plants by the leaf color or morphology, respectively (Figs. 1A, B, C). The 35S::GFP plants also were clearly distinguishable from their host plants (Fig. 1D), but they have two minor disadvantages compared with 35S::R and 35S::NTH. The first is that detection of GFP fluorescence requires some equipment such as a blue- or UV-light illuminator and cut-off filter. The second is that GFP fluorescence can be observed only in the dark. Although the use of 35S::GFP plants has some drawbacks, they were clearly distinguishable from WT plants, and these findings suggest that the three genes used here are suitable as markers for monitoring transgenic plants.

In principle, marker genes should express their traits without any other effects on host plants. We therefore compared the germination rate, seedling weight, flowering time, and seed weight of *Arabidopsis* carrying each transgene with those of WT. Compared with WT, 35S::R did not show significant differences in germination rate or seed weight (Figs. 2A, D), but the 3-week fresh weight was lower (Fig. 2B), and opening of the first flowers was delayed (Fig. 2C). The last two findings indicate that introduction of the *CaMV35S::R* gene affected plant growth. Inhibition of growth and late

flowering was also observed in other transgenic lines (data not shown). In addition, progenies of 35S::R lacked red organs despite being screened beforehand by kanamycin resistance (data not shown); there seemed to be insufficient expression of the *R* gene for the organs to appear red. As a result, 35S::R differed from WT in some of the tissues of concern, therefore it doesn't appear beneficial to use *R* as a marker in *Arabidopsis*.

All of the characteristics of 35S::NTH that we examined were different from those of WT plants. The germination rate was lower, and the vegetative growth of 35S::NTH was poorer than that of WT (Figs. 2A, B). Floral development also was affected by introduction of the *NTH15* gene, which delayed blooming (Fig. 2C) and caused a decreased 1000-seed weight (Fig. 2D). In addition, inhibition of growth factors with transformation of *NTH* gene also appeared in other lines (data not shown). Considered together, these results suggest that the *CaMV35S::NTH15* gene is unsuitable as a marker gene in *Arabidopsis*. However, in a previous study, overexpression of *NTH15* in tobacco led to various leaf morphologies with little effect on the growth of the host tobacco plants (Tamaoki *et al*., 1997). Perhaps, *NTH15* could be applied to plants that form relatively big leaves, like tobacco.

By contrast to 35S::R and 35S::NTH plants, the germination rate, 3-week fresh weight, flowering time, and seed weight in 35S::GFP plants were not significantly different from those of WT plants (Fig. 2). No inhibition of growth factors with transformation of *GFP* gene was also observed in other lines (line nA4-3 ; Niwa *et al*. 1999, data not shown). In addition, the fluorescence trait was dominantly inherited by F1 hybrids between 35S::GFP and WT (Fig. 3) and remained stable without kanamycin selection until at least the T7 generation (data not shown). We therefore concluded that *GFP* fulfills the requirements of a marker gene, although its use requires equipment such as a blue- or UV-light illuminator or a polarized filter and a dark room to detect the fluorescence.

We also show that the *GFP* gene is suitable for a selection marker to generate transgenic plants. The gene is also used as *in vivo* marker to follow the processes of protoplast fusion, regeneration and selection of hybrid citrus (Olivares-Fuster *et al.,* 2002). Further, usage of *GFP* gene, as selection marker, is more effective than glufosinate-resistance because no non-transformants were screened using GFP fluorescence at 1st screening (Table II). Selectable markers conferring antibiotics or herbicide-resistance have widely been used to introduce valuable genes into crop plants, but there are two major problems: (i) selective reagents have sometime negative effects on plant diet; (ii) there is uncertainty regarding the environmental impact of many selectable marker genes such as horizontal gene transfer from plants to bacteria (Gebhard and Smalla, 1999). Application of *GFP* gene for selection marker overcomes these problems because introduction of *GFP* gene not affects plant growth (Fig. 2), and if the gene has incorporated to bacteria, no adverse effect is supposed to provide bacteria. Marker-free transformation system such as usage of MAT vector (Ebinuma *et al.,* 1997) will also overcome the above problems, but application of this method has disadvantages by following reasons: (i) The frequency of marker-free transgenic plants is relatively low because it requires recombination that is rarely occurred in plant cells; (ii) it requires long time to obtain transgenic plants (*e.g.* 6 months in tobacco and 8 months in aspen; Ebinuma *et al*., 1997). In addition, the use of *GFP* gene as selection marker is more advantageous than other methods. As described in this study, adult plants overexpressing *GFP* gene could be chased with GFP fluorescence (Fig. 3). Therefore, it is concluded that the *GFP* gene is a good marker for generating transgenic plants.

Researchers have been fascinated by the use of *GFP* genes as markers in many hosts (Oparka *et al*., 1997; Harper *et al*., 1999; Harper and Stewart, 2000; Hudson *et al*., 2001). Various transgenic *GFP* genes have been developed (Halfhill *et al.*, 2003), and one of them actually was used as a marker gene to detect GM plants in field experiments (Warwick *et al*., 2003). However, overexpression of *GFP* was reported to be toxic to plant growth and development (Haseloff *et al.*, 1997), which we did not find in our present study. This attribute may depend on the particular *GFP* (Harper *et al.*, 1999) and host plant used.

There is a tendency to focus on the main traits of transgenes of interest, but our present results indicate that attention should be paid to probable side effects that might accompany expression of the transgene. Transgenes are valuable tools indeed, but further studies addressing their potential effects on nature are needed if we are to use these tools well in the field.

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