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Efficient transformation mediated by *Agrobacterium tumefaciens* with a ternary plasmid in *Pharbitis nil*

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Abstract *Pharbitis nil* Choisy has a large number of mutants and is well known in classical genetics. The mutants exhibit particularly rich and varied floral colors and patterns compared to other plants. *P. nil* is a typical short-day plant and has therefore been used as a model plant for the genetic analysis of floral colors and patterns and for the photoperiodic induction of flowering. In this paper, we describe an efficient transformation protocol mediated by *Agrobacterium tumefaciens* in *P. nil*. The binary vector pBI121, containing the *neomycin phosphotransferase II* (*NPT II*) gene, was used as a selectable marker, and the *luciferase* (*Luc*$^+$) gene was used as a reporter instead of *β-glucuronidase* (*GUS*). *Agrobacterium tumefaciens* strain GV3101 is carried in the binary vector, while that of LBA4404 is contained the binary and ternary vector, which expresses a constitutive *vir*G mutant gene (*vir*GN54D). We infected 393 somatic embryos with the *Agrobacterium* strain LBA4404/*vir*GN54D/pBI121-Luc$^+$. Fifty-seven kanamycin-resistant shoots were obtained after 2–3 months of culturing in a selection medium, and over 20% of the regenerated shoots were transgenic. Transformation was confirmed with PCR analysis, Southern hybridization, and Luc assay of the transgenic plants. All transgenic plants were morphologically normal and were fertile. The transformation efficiency in this study reached 3.1% of treated explants, which is high enough to produce transgenic *P. nil* with genes of interest.

Key words: *Pharbitis nil*, luciferase, somatic embryo, ternary vector, transformation.

Pharbitis nil Choisy (*Ipomoea nil* [L.] Roth), the Japanese morning glory, was first introduced into Japan from China during the Nara era (710–794) and has long been cultivated as a horticultural plant. Most of the existing mutants were isolated during the late Edo era (1603–1868). Japanese geneticists analyzed some of these mutants in 1910's (Imai 1930, 1938). A map consisting of ten linkage groups was constructed out of 15 groups based on the expected chromosome number (Hagiwara 1956; Imai 1929; Imai 1933; Imai and Tabuchi 1933; Yasui 1928).

These mutants would become very useful in genetic studies. *P. nil* has a large number of mutants and is well known in classical genetics. Mutants of this species exhibit particularly rich and varied floral colors and patterns compared to other model plants. Genetic analysis requires the ability to self-pollinate in order to isolate a recessive mutant, and *P. nil* can pollinate itself under natural conditions. In addition, when *P. nil* is grown under short-day conditions (10 h light: 14 h dark) and continuous temperature (28–30°C), it can be induced to flower within 1 month of sowing. The life cycle is comparatively short (2–3 months).

From the standpoint of evolutionary genetics, it is important that there are many related species of *P. nil* that can cross-breed with each other. Cross-breeding in this case is very easy to perform and observe, because the floral organ of *P. nil* is quite large. Furthermore, there is no differentiation of the genome except for insertion of the transposon and microsatellite region among various strains. Cloning of several genes can be effected by guiding the *Tpn1*-related transposable element, because most mutants of *P. nil* occur by *Tpn1* family transposon, which is similar to *En*/*Spm* in *Zea* mays.

Although *P. nil* is a typical short-day plant, the photoperiodic sensitivity of this species varies from genotype to genotype. In this study, we used one of the most sensitive genotypes, Violet. Seedlings of *P. nil* cv. Violet can be induced to flower by a single 16-h exposure to continuous darkness, whereas *P. nil* cv. Violet grown under continuous light cannot be induced to flower (Imamura 1967; Vince-Prue and Gressel 1985). Therefore *P. nil* cv. Violet has been used as a model plant for studies of photoperiodic induction of flowering.

Previously, we have described the isolation and characterization of several genes from *P. nil* cv. Violet (Sage-Ono et al. 1998a, b). In addition to using the technique of differential-hybridization screening,

Abbreviation: BA, benzylaminopurine; GUS, β-glucuronidase; IAA, indoleacetic acid; Luc$^+$, luciferase; NAA,α-naphthaleneacetic acid; NPT II, neomycin phosphotransferase II.

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Felsheim and Das (1992), Krishna et al. (1992) and O'Neill et al. (1994) generated cDNAs of cotyledonous mRNAs, the levels of which were altered by photoperiodic treatment. The steady-state levels of some of these mRNAs showed circadian oscillations, which suggested participation in the photoperiodic induction of flowering (O'Neill et al. 1994; Liu et al. 2001). A reverse genetic approach would be highly useful to elucidate the function and roles of these genes. However, as no efficient and reliable *P. nil* transformation system is available, the functions of most of these genes have not been defined.

P. nil has been considered to be a recalcitrant species for plant regeneration *in vitro* (Messerschmidt 1974; Sangwan and Norreel 1975; Bapat and Rao 1977). However, Yoneda and Nakamura (1987) successfully regenerated *Pharbitis* plantlets by using immature embryos (Matsubara and Nakahira 1966). *Pharbitis* is susceptible to infection by *Agrobacterium*, and the transformation and production of kanamycin-resistant calli has previously been reported (Araki et al. 1989). We have reported a method for the generation of transgenic *P. nil* plants (Ono et al. 2000). However, the frequency of transformation and regeneration is very low using this method.

Fits et al. (2000) reported a "ternary transformation system" for plant cells. An *Agrobacterium tumefaciens* strain LBA4404, supplemented with a constitutive *vir*G mutant gene (*vir*GN54D) on a compatible plasmid, is capable of highly efficient T-DNA transfer to a diverse range of plant species. *Vir* gene expression is controlled by a two-component system formed by *vir*A and *vir*G. Activated *vir*G induces the expression of other *vir* genes. Extra copies of *vir*G (Liu et al. 1992; Hansen et al. 1994) or *vir*G-containing *vir*-region segments (Hiei et al. 1994; Ishida et al. 1996; Wenck et al. 1999) have been reported to increase the efficiency of *Agrobacterium*-mediated transformation of certain plant species. The activity of a constitutive *vir*G mutant carrying an Asn-54 to Asp amino acid substitution (*vir*GN54D) is independent of *vir*A, the sensor of the two-component system (Pazour et al. 1992).

In this paper, we describe a highly efficient transformation protocol mediated by *Agrobacterium tumefaciens* containing *vir*GN54D and plant regeneration in *P. nil* Choisy cv. Violet.

Materials and methods

Bacterial strain and plasmid

Agrobacterium tumefaciens strains LBA4404 and GV3101 have previously been described (Hoekema et al. 1983; Koncz and Schell, 1986). The binary vector was a modified pBI121 vector, in which the *GUS* gene was replaced by the *Luc*$^+$ gene (Promega, Madison, WI, USA). The constitutive N54D mutant *vir*G gene (*vir*GN54D) is cloned as a *Sac*I/*Hind*III fragment from pRAL6308 (Scheeren-Groot et al. 1994) into the plasmid pBBR1MCS ("ternary plasmid"; Kovach et al. 1994). Both pBI121-Luc$^+$ and the ternary plasmid were introduced into LBA4404, whereas GV3101 was included in the binary vector by a freezing methods (Holster et al. 1978; Zahm et al. 1984).

Plant material

Pharbitis nil Choisy cv. Violet (Marutane Co., Kyoto, Japan) was used throughout the experiments. Plants had been grown under short-day conditions (8 h light : 16 h dark) at 25°C after growing for 1 month under long-day conditions (16 h light : 8 h dark) at 25°C (fluorescent light at approximately 20 W/m^2).

For the isolation of immature embryos, immature fruits were harvested from plants about 2 weeks after flowering had begun. The immature fruits were surface-sterilized with 70% ethanol for 1 min and then with a 2% solution of sodium hypochlorite for 15 min. They were washed four times in a large volume of sterilized water and dissected for the isolation of immature embryos, which were 2 to 8 mm in length. The immature embryos were cultured on an embryoid induction (EI) medium [MS medium containing 3 mg l^{-1} α-naphthaleneacetic acid (NAA), 6% sucrose and 0.2% gelrite; Figure 1a; Jia and Chua 1992].

Plant transformation and regeneration

Agrobacterium tumefaciens for transformation were grown overnight at 28°C on an LB medium containing antibiotics. The cells were washed and suspended with the secondary embryoid formation (SEF) medium (MS medium containing 0.5 mg l^{-1} NAA, 6% sucrose and 0.2% gelrite; Ono et al. 2000) that had been prepared without Gelrite^{TM} (Monsanto Co., St. Louis, MO, USA). Somatic embryos induced from immature embryo culture (Figure 1b) were inoculated with the *Agrobacterium* suspension for 5 min and transferred to plates of an SEF medium and supplemented with 10 mg l^{-1} acetosyringone. After 2 days of co-cultivation, the somatic embryos were transferred to an SEF selection medium containing 25 mg l^{-1} kanamycin and Augmentin (125 mg l^{-1} Potassium Clavulanate and 250 mg l^{-1} Amoxicillin; Glaxo SmithKline K.K., Uxbridge, UK). One month after selection (Figure 1c), they were transferred to an embryoid maturation and germination (EMG) medium [MS medium containing 0.2 mg l^{-1} indoleacetic acid (IAA), 2 mg l^{-1} benzylaminopurine (BA), 3% sucrose and 1.2% agar; Ono et al. 2000] that contained 50 mg l^{-1} kanamycin and Augmentin. When the shoots were regenerated (Figure 1d), they were transferred to a hormone-free 1/2MS medium with 25 mg l^{-1} kanamycin and Augmentin

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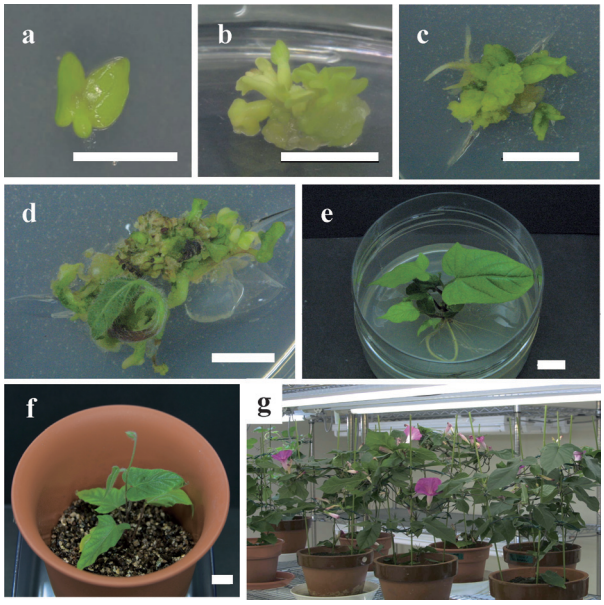

Figure 1. Transformation and regeneration of *Pharbitis nil*. (a) an immature zygotic embryo culture of *P. nil*, (b) a somatic embryo used for transformation, (c) secondary embryos during selection, (d) regeneration shoot, (e) root formation, (f) plantlet transplanted to vermiculite for acclimatization, (g) transgenic plant 2 months after acclimatization. Scale bar: (a) 0.5 cm, (b)–(f) 1.0 cm.

(Figure 1e). When the roots were induced on the regenerated shoots, plantlets were transplanted to moist vermiculite (Tekunon Co. Ltd. Gunma, Japan) for acclimatization (Figure 1f). Once the shoots had begun to grow, plantlets were transplanted to moist soil (Figure 1g).

Southern hybridization analysis

Total DNA was isolated from leaves using the CTAB extraction method (Murray et al. 1980; Wagner et al. 1987). The DNA digested with *Eco*R I was fractionated by electrophoresis on a 1% agarose gel. The separated DNA was blotted onto a nylon membrane filter (Biodyne B; Nihon Pall, Ltd., Tokyo, Japan). The membrane was hybridized with a ^{32}P-labeled 1328-bp *Luc*$^+$ gene fragment, amplified by PCR from a *Luc*$^+$ coding region. The washing of the membrane was performed in 2×SSC plus 0.1% SDS for 10 min at 65°C and then with 0.1×SSPE plus 0.1% SDS for 10 min at 65°C. Hybridization signals were detected using a BAS-5000 image analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

PCR analysis

Genomic DNA was extracted from leaf tissues according to the CTAB method. We used the following primer sets to amplify the *NPTII* and *Luc*$^+$ sequences: *NPTII* forward primer, 5′-GAGGCTATTCGGCTATGACT-3′; *NPTII* reverse primer, 5′-TCCCGCTCAGAAGAACTCGT-3′; *Luc*$^+$ forward primer, 5′-GGAAGACGCCAAAAACATAA-3′; and *Luc*$^+$ reverse primer, 5′-AGCCACCTGATAGCCTTTGT-3′. Reactions were carried out on a thermal cycler, starting denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. Amplified DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and observed under UV illumination.

Table 1. Effect of different type of antibiotics in the medium on shoot regeneration.

No. of experiments	Component of medium	No. of somatic embryos	No. of regeneration shoots
EX1	Claforan[a]	72	0
	Augmentin[b]	162	10
EX2	Claforan	91	1
	Augmentin	48	0
EX3	Claforan	81	1
	Augmentin	249	14
Total	Claforan	244	2 (0.8%)
	Augmentin	459	24 (5.2%)

[a] Cefotaxime Sodium 200 mg l^{-1}. [b] Potassium Clavulanate 125 mg l^{-1}, Amoxicillin 250 mg l^{-1}.

Luciferase assay

Luminescence assays were performed with the whole petal of the T1 generation and with leaves that were hollowed out during the T2 generation. The petals were sprayed with 1 mM Luciferin (Beetle Luciferin, Potassium Salt; Promega, Tokyo, Japan) contained in 0.01% Triton X-100, and the leaves were dipped. The photon counting was performed using an ALGUS-20 (Hamamatu Photonics Co., Japan) for 1 h.

Results

Confirmation of transformation efficiency on previously reported Agrobacterium-mediated transformation

The efficiency of the transformation system that was previously reported by Ono et al. (2000), in which immature embryos were cultured on an EI medium, was confirmed. After 3 to 4 weeks, somatic embryos were observed from immature embryos. These 244 somatic embryos were infected and co-cultured with *Agrobacterium* in an SEF co-cultivation medium. After 2 days, they were transferred to plates of an SEF selection medium for 4 weeks. Two of the 244 somatic embryos were regenerated after transfer to an EMG medium, (Table 1). However, no transgenic *P. nil* were obtained from the selected somatic embryos, indicating that the transformation efficiency of the previous protocol is quite low.

Effect of Augmentin on regeneration

To improve the efficiency of regeneration, we compared Claforan to Augmentin. Augmentin was present in the

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Table 2. Effect of ternary plasmid *vir*GN54D in *Agrobacterium* on transformation frequency.

No. of experiments	Strain*1	No. of somatic embryos	No. of regeneration shoots	No. of transgenic shoots
EX1	GV3103*2	162	10	2
	LBA4404*3, *virG*N54D*4	150	26	5
EX2	GV3103	48	0	0
	LBA4404, *virG*N54D	33	3	1
EX3	GV3103	249	14	0
	LBA4404, *virG*N54D	210	28	6
Total	GV3103	459	24 (5.2%)	2 (0.4%)
	LBA4404, *virG*N54D	393	57 (14.5%)	12 (3.1%)

*1 Binary vector: pBI121-Luc$^+$.
*2 GV3101 (pMP90).
*3 LBA4404 (pAL4404).
*4 pBBR*vir*GN54D.

SEF selection medium, the EMG medium and the 1/2 MS medium, in place of Claforan. In each transformation experiment, 459 of the somatic embryos were cultured in these media containing Augmentin after infection and co-cultivation with *Agrobacterium*. The frequency of somatic embryos that generated kanamycin-resistant shoots was 5.2% in these media containing Augmentin but only 0.8% in these media containing Claforan (Table 1). These results suggest that Augmentin is useful for the *in vitro* regeneration of *P. nil* shoots.

Effects of VirGN54D on the efficiency of transformation in P. nil

To improve the efficiency of transformation, we examined the effects of *vir*GN54D (ternary plasmids) on shoot formation from somatic embryos. We infected 459 or 393 somatic embryos with *Agrobacterium* strain GV3101 (pMP90) or LBA4404 (pAL4404) with a copy of *vir*GN54D. All of these were transferred to plates with media containing Augmentin. The frequency of regenerated shoots from somatic embryos that were infected with LBA4404 with a ternary vector was higher. Twelve transformants were obtained from somatic embryos infected with the *Agrobacterium* strain LBA4404 after 3 months in culture. In this experiment, the transformation efficiency was at least 3.1% (Table 2), a ratio of about 7.8-fold of GV3101. These results indicate that *vir*GN54D affects the efficiency of transformation in *P. nil*.

Molecular analysis and Luciferase assay of transgenic plants

The integration of the transgene in the genomes of T1 transgenic plants was confirmed by PCR analysis and Southern hybridization. *NPTII* and *Luc*$^+$ were amplified in transgenic plants by specific primers. These bands were not amplified in non-transformed plants (data not shown). PCR-positive plants were selected as putatively transformed plants. Five of 12 plants were checked for

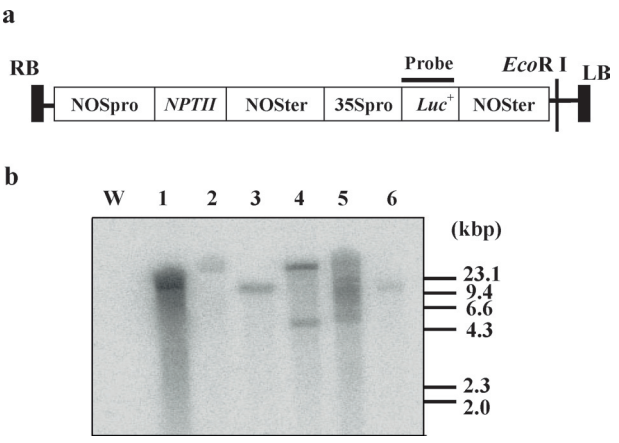

Figure 2. Southern hybridization analysis of transgenic *Pharbitis nil*. (a) The structure of the construct (pBI121-Luc$^+$) used for transformation. (b) Analysis of genomic DNA from six Luc-positive plants (1–6) and non-transgenic control plant (W). DNA (20 μg) was digested with *Eco*R I, and the transgene was detected with a ^{32}P-labeled DNA fragment of the Luc gene (indicated in a). 1: the transformant from somatic embryos infected with *Agrobacterium* carried pBI121-Luc$^+$. 2–6: the transformants from somatic embryos infected with *Agrobacterium* carried pBI121-Luc$^+$ with *vir*GN54D. NOS-pro, promoter of the gene for nopaline synthase; *NPTII*, gene for neomycin phosphotransferaseII; NOS-ter, terminator of the gene for nopaline synthase; 35S-pro, 35S promoter of cauliflower mosaic virus; *Luc*$^+$, the gene for Luciferase; RB, right border of T-DNA; LB, left border of T-DNA.

the copy number of the transgene by Southern hybridization. There is only one *Eco*R I restriction site within the T-DNA region (Figure 2a). We used a DNA fragment containing the *Luc*$^+$-coding region as a probe. Of the lines tested, lines 2, 3, and 6 had a single copy of T-DNA in their genomes. Line 4 had two copies, and line 5 had multiple copies of T-DNA in their genomes. In addition, T1 line 1, which was infected with *Agrobacterium* without *vir*GN54D, had multiple copies of the transgene (Figure 2b). To confirm the expression of the transgene for *Luc*$^+$, we attempted a Luciferase assay of the petals of transformed plants. No signal was detected from the non-transformed control plant (Figure

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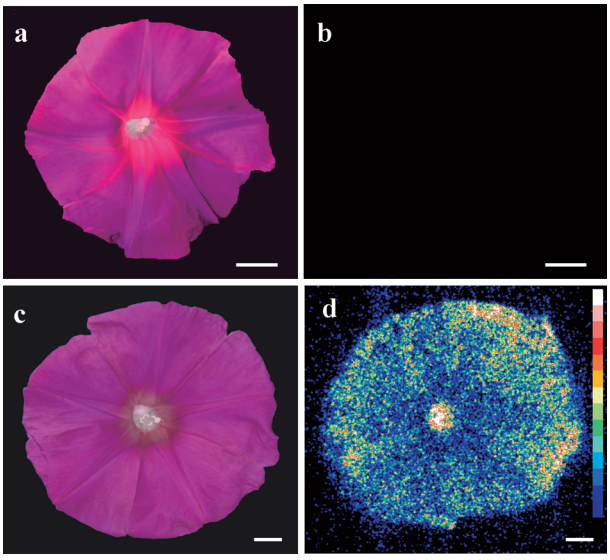

Figure 3. Bioluminescence image of a petal of transgenic T1 *Pharbitis nil*. Panels on (a) and (c) show photographs. (b) and (d) show bioluminescence images. (a), (b) non-transgenic control plant. (c), (d) transgenic T1 plant of line 3. The color scale shows the intensity of luminescence. Scale bar: 1 cm.

3a, b), whereas the expression in all transformants was detected as shown in Figure 3c, d.

Segregation analysis

To confirm of presence of the transgene from T1 transgenic plants in T2 transformants, we examined 14 seeds of line 3 by segregation analysis via Southern hybridization. Ten bands had the –9-kbp fragment, depending on the T-DNA region (Figure 4a). Fourteen plants of line 3 were further analyzed for the expression of the *Luc*$^+$ transgene by the Luc assay. All T2 plants that contained the transgene in the genome showed Luc activity, whereas the remaining T2 plants did not (Figure 4b, c). In addition, we investigated 14 seeds of each of the other two lines, 4 and 5, by Luc assay (Table 3) and PCR (data not shown). In T1 line 5, all 14 T2 transgenic plants were confirmed to have integrated both the *NPTII* and *Luc*$^+$ genes. However, seven of these transformants did not show Luc activity (data not shown). These results indicate that the transgene of multiple copies is not inherited stably, whereas that of single copy is inherited stably.

Discussion

To date, few attempts have been made to establish a transformation system in *P. nil*. In previous studies (e.g., Ono et al. 2000), we were confronted by two difficulties. The first was the lower frequency of transformation, and another was that of regeneration. In this study, we established an efficient protocol for plant regeneration and the *Agrobacterium*-mediated transformation of *P. nil*.

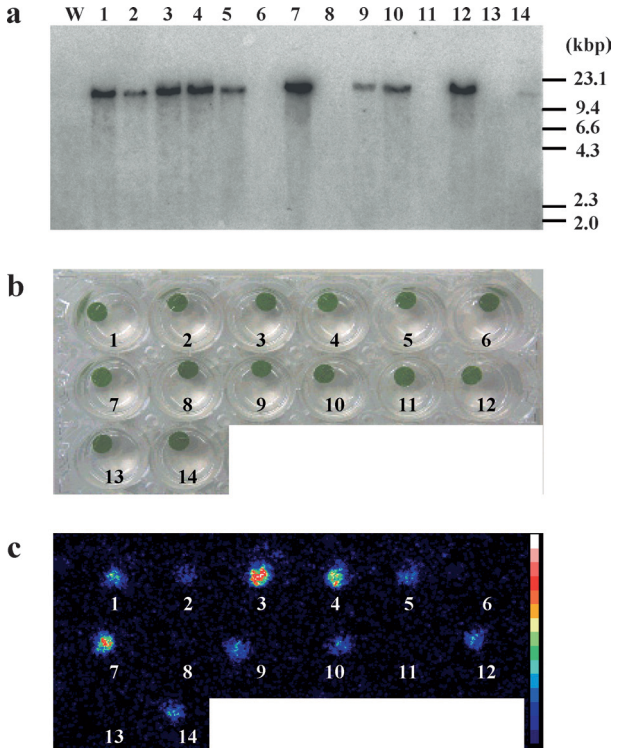

Figure 4. Southern hybridization of the transgene into the genome and bioluminescence image of *Pharbitis nil* leaf of line 3 T2 transgenic plants. (a) Analysis of genomic DNA from 14 T2 plants (1–14) and non-transgenic control plant (W). DNA (10 μg) was digested with *Eco*R I, and the transgene was detected with ^{32}P-labeled DNA fragment of the Luc$^+$ gene (indicated in Figure 2a). (b) Panel shows photographs. (c) bioluminescence image of the clipped leaves of T2 plants. The scale shows the intensity of luminescence.

Table 3. Segregation analysis of transgene in transgenic plants.

Line	No. of seeds	Luc assay		Ratio
		Positive	Negative	
3	14	10	4	2.5 : 1 (3 : 1 χ^2=0.095)
4	14	6	8	3 : 4
5	14	7	7	1 : 1

First, we investigated the effect of antibiotics on the regeneration of somatic embryos. The process of eliminating *Agrobacterium* with antibiotics is very important after infection and co-culturing with *Agrobacterium*. However, little attention has been given to whether there is a relationship between antibiotics and plant culture (Ogawa et al. 2002). Augmentin as a beta-lactam antibiotic has a lesser effect on cultured plantlets than Claforan (Dr. H. Ezura, University of Tsukuba, personal communication). Compared to Claforan, Augmentin induced many of the kanamycin-resistant shoots (Table 1). These results indicated that the first problem had been adequately addressed. However, we will not attempt to discuss the effects of Augmentin here, because we do not have definitive information on antibiotics.

To improve the efficiency of transformation, we tested

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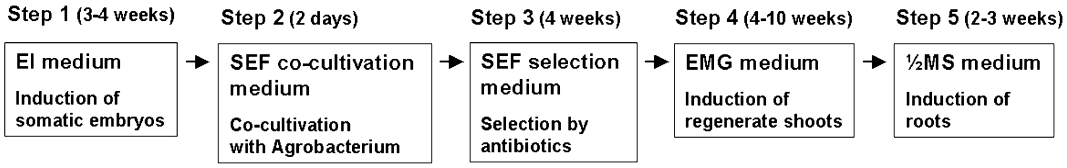

Figure 5. Schematic representation of *Agrobacterium*-mediated transformation of *Pharbitis nil*. The time schedule is indicated on the left.

the so-called ternary transformation system. Fits et al. (2000) reported that stable transformation frequencies were dramatically increased by using this system on a diverse range of plant species. Therefore, the same results were anticipated for *P. nil*. A 7.8-fold increase was observed when copies of *vir*GN54D were present in *A. tumefaciens* (Table 2). These results indicate that the presence of the ternary plasmid carrying *vir*GN54D increased T-DNA transfer to the *P. nil* cell. Therefore, the ternary transformation system improves the opportunity for the genetic engineering of *P. nil* that has been considered refractory to *Agrobacterium*-mediated transformation.

We examined 14 T2 seeds of each of the three lines for segregation analysis (Figure 4, Table 3). Southern hybridization, PCR analysis, and bioluminescence analysis of the expression of Luc^+ revealed that the Luc^+ transgene was heritable. T1 line 3 had a single copy of T-DNA (Figure 2b). The segregation ratios in the T2 generation of T1 line 3 was 2.5 : 1, similar to the ratio of 3 : 1 that could be expected from a single integration event (Figure 4a, Table 3). In addition to the results of the bioluminescence analysis, this result agreed with the results of Southern hybridization. All of the T2 non-luminescent plants, for example, lines 6, 8, 11, and 13, had no Luc^+ transgene (Figure 4a, c). The segregation ratio in the T2 generation was 3 : 4 and 1 : 1, as in T1 line 4 and 5 (Table 3). These plants putatively carried two or more copies of the transgene (Figure 2b). In T2 plants of line 5, seven of these plants did not have Luc activity, although these plants had the Luc^+ transgene (data not shown). These results suggest that a single copy of the transgene is inherited stably. However, in some plants that have multiple copies of the transgene, expression of the transgene may be down-regulated by co-suppression.

The method for *Agrobacterium*-mediated transformation of *P. nil* is summarized in Figure 5. Immature zygotic embryos, which were used as starting material, were cultured in an EI medium for the induction of somatic embryos. Somatic embryos were infected and co-cultivated with *Agrobacterium*, including *vir*GN54D (ternary plasmid) in an SEF co-cultivation medium for 2 days. These embryos were transferred to an SEF selection medium for the induction of kanamycin-resistant embryos. The secondary embryos were transfered to an EMG medium. Regenerated kanamycin-resistant shoots were excised and rooted with plates of half-strength MS medium. When roots had formed on the regenerated shoots, plantlets were transplanted to moist vermiculite for acclimatization. Once shoots had started to grow, plantlets were transplanted to moist soil. Using this protocol, transgenic *P. nil* is produced within 17 weeks after the commencement of cultures.

In conclusion, we have established a highly efficient protocol for the transformation of *P. nil* that allows the stable integration and expression of a Luc^+ transgene, as confirmed by Luciferase assay, molecular analysis, and the habitability of the transgene. Genes related to the photoperiodic induction of flowering and to floral morphogenesis, as well as genes in mobile genetic elements, have been isolated from *P. nil* (Parfitt et al. 2004; Lee et al. 2003; Nitasaka 2003; Suzuki et al. 2003; Yoshida et al. 2002). However, no genes isolated from *Pharbitis* have yet been characterized by the analysis of its expression in transgenic *Pharbitis* plants. Our protocol for the production of transgenic *Pharbitis* plants should allow a more detailed analysis of the functions of specific genes in *Pharbitis*.

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