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Agrobacterium-Mediated Transformation and Regeneration of *Pharbitis nil*

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Abstract

A reliable method for the transformation of *Pharbitis* (*Ipomoea*) *nil* is described for the first time. Somatic embryos derived from immature zygotic embryos were used as starting material. These were transformed with *Agrobacterium tumefaciens* strain GV3101 (pMP90), which harbored the plasmid pIG121Hm that included genes for β-glucuronidase (GUS), kanamycin resistance and hygromycin resistance. Infected embryos were induced to undergo secondary embryogenesis and kanamycin-resistant embryos were selected and allowed to develop into plantlets. All transgenic plants were morphologically normal and fertile. Stable integration and expression of the transgene for GUS were confirmed by histochemical and Southern blotting analysis. Expression of the intron-containing reporter gene for GUS under control of the 35S promoter of cauliflower mosaic virus (CaMV 35S) was detected after staining. Southern hybridization confirmed the stable inheritance of the transgene for GUS.

1. Introduction

Pharbitis nil Choisy [*Ipomoea nil* (L.) Roth] cv. Violet is a short-day plant that has proved to be an excellent model for studies of the photoperiodic induction of flowering since plants can be induced to flower by a single short-day treatment at the seedling stage (Imamura, 1967; Vince-Prue and Gressel, 1985). *Pharbitis* is also an important horticultural plant in Japanese gardens and was used in early genetic studies (Imai, 1931).

Pharbitis nil has been considered to be a recalcitrant species for plant regeneration *in vitro* (Messerschmidt, 1974; Sangwan and Norreel, 1975; Bapat and Rao, 1977) and, our initial attempts to regenerate whole plants from protoplast-derived callus were unsuccessful (unpublished results). Matsubara and Nakahira (1966) reported the generation of active embryonic outgrowths from cultured immature embryos of *Pharbitis nil* and, using immature embryos as starting materials, Yoneda and Nakamura (1987) successfully regenerated *Pharbitis* plantlets. Several other groups have obtained similar results (Jia and Chua, 1992; O' Neill *et al.*, 1993; Otani and Shimada, 1998; Kopcewicz and Tretyn, 1998). *Pharbitis* is susceptible to infection by *Agrobacterium* and there is a report of the transformation and production of kanamycin-resistant callus (Araki *et al.*, 1989). However, to our knowledge, there have been no reports of the production of regenerated transgenic *Pharbitis* plants.

In this report, we describe a reliable method for the generation of transgenic *Pharbitis nil* plants. We optimized the protocols for both regeneration and transformation and produced fertile transgenic plants. Furthermore, we confirmed the stable integration, inheritance and expression of the foreign genes in the transgenic plants by histochemical staining and Southern blotting analysis. Our protocol appears suitable for the general introduction of foreign genes into *P. nil*, which should be very useful for future physiological and genetic studies. To our knowledge, this is the first report of the production of transgenic *Pharbitis* plants.

2. Materials and Methods

2.1 Plant material, bacterial strain and plasmid vector

Pharbitis nil Choisy cv. Violet was used through-

out the experiments. For isolation of immature zygotic embryos, we harvested immature fruits from plants that had been grown in a greenhouse or in a growth chamber (CU-255; Tomy Seiko Co., Tokyo, Japan). Plants in the growth chamber were exposed to 13 h of daily illumination from cool-white fluorescent lights (30 W m^{-2}; FLR40S, FLR20S and FLR15S; Toshiba Electronics Co., Tokyo, Japan) at 24.5 ± 2 ℃. Immature fruits were harvested from donor plants two to three weeks after flowers had opened. The immature fruits were surface-sterilized with 70% ethanol for 2 min and then with a 2% solution of sodium hypochlorite (available chloride ions) that contained 0.05% (w/v) Tween 20 for 15 min. Then they were washed four times in a large volume of sterilized water and dissected for isolation of immature embryos, which were 2 to 8 mm in length. The culture system was essentially similar to that described by Jia and Chua (1982). In all experiments we used MS medium (Murashige and Skoog, 1962) in which the organic constituents had been replaced by the organic constituents (100 mg l^{-1} *myo*-inositol, 5 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 0.5 mg l^{-1} pyridoxine hydrochloride, 0.5 mg l^{-1} thiamine hydrochloride, 0.5 mg l^{-1} folic acid and 0.05 mg l^{-1} biotin) described by Nitsch and Nitsch (1967; **Table 1**). This medium is referred to as basal medium (BM). Zygotic embryos were cultured on embryo-induction (EI) medium, which consisted of BM medium supplemented with 2 to 6 mg l^{-1} α-naphthaleneacetic acid (NAA) and 6% (w/v) sucrose (**Table 1**).

For genetic transformation, we used *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986) that carried the binary vector pIG121Hm (Hiei *et al.*, 1994). pIG121Hm includes marker genes for kanamycin resistance (NPTII; neomycin phosphotransferase II), hygromycin resistance (HPT; hygromycin phosphotransferase) and β-glucuronidase (GUS) in the T-DNA region. The gene for GUS, which includes an intron at the 5' end of the coding sequence, allows for expression of GUS activity in plant cells but not bacterial cells and is under the control of the 35S promoter of cauliflower mosaic virus (CaMV; Ohta *et al.*, 1990).

2.2 Transformation and regeneration

The time required from the induction of secondary embryos to the production of transgenic plantlets was approximately five months. The steps are outlined schematically in **Fig. 1** and all media used are described in **Table 1**.

The suspension of *Agrobacterium* used for infections was prepared as follows. Cells were cultured in Luria broth that contained 50 mg l^{-1} kanamycin at 28 ℃ for 16 h. The cells, at the late-logarithmic phase, were collected, washed with secondary embryo-formation (SEF) medium that had been prepared without Gelrite™ and adjusted to a concentration that gave an optical density at 600 nm (OD_{600}) of 1 in the same medium. For infection with *Agrobacterium*, the secondary embryos were transferred to a petri dish that contained 5 m*l* of the suspension of *Agrobacterium* and gently shaken for 5 min at room temperature. The embryos were then transferred to plates of SEF plus 40 mg l^{-1} acetosyringone (SEF + AS), solidified with Gelrite™ (2,000 mg l^{-1}; Monsanto Co., St. Louis, MO, USA), for co-cultivation for two days. The embryos were then washed five times in a petri dish that contained 5 m*l* of SEF medium plus 500 mg l^{-1} carbenicillin and 200 mg l^{-1} claforan (SEF + KCC) and transferred to plate of SEF medium that contained 25 mg l^{-1} kanamycin and 200 mg l^{-1} claforan (SEF + KLC). The secondary embryos that formed on the original infected embryos were transferred to embryo-maturation and germination medium supplemented with 50 mg l^{-1} kanamycin and 200 mg l^{-1} claforan (EMG + KHC). After about a month, regenerated kanamycin-resistant shoots were excised

Table 1. Composition of media with concentration of all components given in (mg l^{-1})

BM	EI	SEF	SEF+AS	SEF+CC	SEF+KLC	EMG+KHC	1/2MS+KLC
MS salts & Nitsch's organic components	4 NAA	0.5 NAA	0.5 NAA	0.5 NAA	0.5 NAA	0.2 IAA 2 BA	(1/2 BM)
				500 CB	25 Km	50 Km	25 Km
			40 AS	200 Cla	200 Cla	200 Cla	100 Cla
	60,000 Suc	60,000 Suc	60,000 Suc	60,000 Suc	60,000 Suc	30,000 Suc	15,000 Suc
	2,000 GR	2,000 GR	2,000 GR		2,000 GR	2,000 GR	12,000 Agar

AS, Acetosyringone; BA, benzylaminopurine; BM (basal medium), MS salts supplemented with Nitsch's organic components; CB, carbenicillin; Cla, claforan; GR, Gelrite™; IAA, indoleacetic acid; Km, kanamycin; NAA, α-naphthaleneacetic acid; Suc, sucrose.

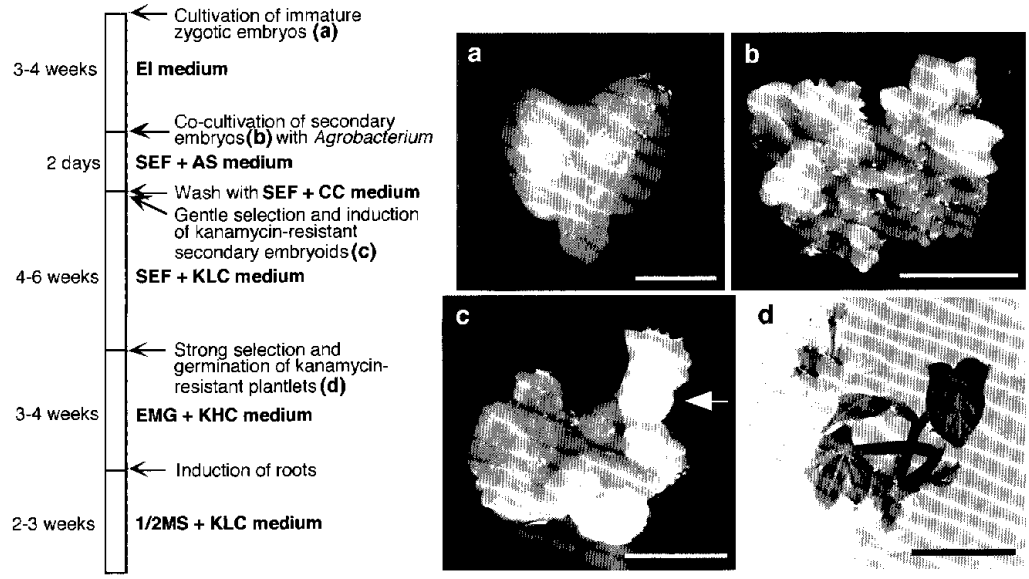

Fig. 1 Summary of the method for transformation and regeneration of *Pharbitis nil*. The time schedule is indicated on the left. The photographs on the right show an immature zygotic embryo (a), secondary embryos used for transformation (b), a kanamycin-resistant embryo (c; indicated by an arrow), and regenerated shoots (d). Scale bars: (a), 1 mm; (b), 5 mm; (c), 5 mm; and (d), 2 cm.

and rooted on plates of half-strength BM medium that contained 25 mg l^{-1} kanamycin and 100 mg l^{-1} claforan (1/2MS + KLC). 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.

2.3 Assay of GUS activity

The expression of GUS was analyzed histochemically in callus tissue, embryos and excised organs of mature plants as described elsewhere (Jefferson *et al.*, 1987; Dai *et al.*, 1996). Tissues were treated first with 90% acetone for 1 to 5 h at -20 ℃. They were rinsed twice with 50 mM sodium phosphate buffer (pH 7.0) and then incubated in 50 mM sodium phosphate buffer (pH 7.0) that contained 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 1 mM EDTA (ethylenediaminetetraacetic acid), 0.05% (v/v) Triton X-100, 0.1 mM potassium ferrocyanide and 0.1 mM potassium ferricyanide. Samples were briefly vacuum-infiltrated and then incubated at 37 ℃ in darkness for 6 to 24 h for optimal coloring. Reactions were stopped and tissues were cleared by sequential treatments with 70% and 95% ethanol at 45 ℃ for 2 to 6 h. The tissues were stored in 75% ethanol at 4 ℃. For a comparison of patterns of expression of GUS under the control of the CaMV 35S promoter, we examined transgenic tobacco plants (*Nicotiana tabacum* cv. Maryland Mammoth) that had been transformed by the same vector.

2.4 Southern hybridization analysis

Genomic DNA was isolated from leaves of Pharbitis with a plant DNA extraction kit (PhytoPure™; Amersham-Pharmacia, Tokyo, Japan). The isolated DNA was digested with *Eco*RI. Aliquots of 10 μg of digested DNA were fractionated by electrophoresis on a 1% agarose gel and then bands of DNA were transferred to a nylon membrane (Biodyne B; Nihon Pall Ltd., Tokyo, Japan). The DNA on the membrane was allowed to hybridize with a ^{32}P-labelled DNA fragment of the GUS-coding sequence. The conditions for hybridization, washing and analysis were the same as those described previously by Sage-Ono *et al.* (1998).

2.5 Segregation analysis

Trangenic plants were self-pollinated and seeds were harvested. As a consequence of the relatively low yield of seeds in our growth chamber, we could only harvest between 10 and 50 seeds per plant. Segregation ratios were determined from T2 populations and tips of cotyledons were used for assays of GUS activity.

3. Results and Discussion

3.1 Agrobacterium-mediated transformation and regeneration of transgenic plants

We optimized our protocol for plant regeneration, which exploited the high regenerative potential of the immature zygotic embryos of *Pharbitis nil*

described by Jia and Chua (1992), for the generation of transgenic plants. Details of the protocol are described in Materials and Methods and a summary is shown in **Fig. 1**. The compositions of various media used are given in **Table 1**.

Immature zygotic embryos, which we used as starting material, were cultured in EI medium for induction of secondary embryos. We compared the effects of different concentrations of NAA, as did Jia and Chua (1992), and concluded that 4 mg l^{-1} was the optimal concentration for induction of secondary embryos. The secondary embryos were subsequently used for transformation by *Agrobacterium*. We found that secondary embryogenesis in *Pharbitis* was very sensitive to antibiotics, namely, kanamycin and hygromycin, which, at concentrations of 50 mg l^{-1} and 25 mg l^{-1}, respectively, completely suppressed embryogenesis. After several trials, we concluded that inclusion of a lower concentration of kanamycin (25 mg l^{-1}) at the beginning of secondary embryogenesis was both sufficient and essential for the selection of transgenic embryos. We also tested hygromycin as a selective antibiotic but no transgenic plants could be obtained. Hygromycin may be too strong as a selective reagent since secondary embryogenesis in *Pharbitis* appears to require undamaged adjacent cells.

The secondary embryos that had formed on SEF + KLC medium (**Table 1**) were transferred to EMG + KHC medium (**Table 1**) for maturation of embryos, germination and stringent selection. Within one week after the transfer of these embryos to EMG + KHC medium, most of the non-transformed embryos had lost their chlorophyll. Three to four weeks later, resistant embryos became dark green in color and developed mature leaves. Cotyledons were rarely observed and no roots developed on EMG + KHC medium, as also reported by Jia and Chua (1992). When shoots had reached 4 cm in length, they were cut at the first node and transferred onto 1/2MS + KLC medium (**Table 1**). Roots emerged from one to two weeks after transfer and plantlets were acclimatized by transfer to moist vermiculite. When shoots had started to grow, the transgenic plantlets were repotted in soil. All regenerated plants were morphologically normal (**Fig. 2h**) and were not visibly different from those of parental cultivar, Violet. The frequency of transformation varied from 2% to 10% with respect to the number of infected secondary embryos. In the future, it may be possible to increase the frequency of transformation by use of different strains of *Agrobacterium* and different vectors.

3.2 Expression of the transgene for GUS

To examine the proliferation of transformed cells during secondary embryogenesis, we stained embryos with X-Gluc three weeks after infection (**Fig. 2a**). Developing embryos that strongly expressed GUS were detected among non-stained calli and embryos. Jia and Chua (1992) noted that secondary embryos originated from both peripheral cells (epidermal and subepidermal) and internal meristematic cells (adjacent to the xylem of the vascular bundle) of the hypocotyl axis. Although each of these types of cell might have the capacity for embryogenesis, only a limited number of cells actually developed into embryos. For formation of a transgenic secondary embryo, a transformed cell must be more active in embryogenesis than the untransformed surrounding cells. The low concentrations of kanamycin that we used might have suppressed embryogenesis of untransformed surrounding cells and supported the development of transformed cells.

We analyzed five transgenic plants from independent transformation experiments, namely, IG1 through IG5, for their patterns of GUS expression. The results were similar in each case. As shown in **Fig. 2b** and **2c**, leaf and cotyledon tissue was strongly stained by X-Gluc. In the case of roots, only apices were stained (**Fig. 2d**). The florally induced shoot tips, 10 days after the short-day induction of flowering, were also strongly stained (**Fig. 2e**). Furthermore, developing flower buds, petals, ovules and calyxes were also stained to varying degrees (**Fig. 2f**), and anthers were strongly stained. By contrast, most pollen grains were not stained (**Fig. 2g**). It seemed likely that some of the stained pollen grains that we observed might have been the result of the transfer of pigment from the strongly stained anthers (**Fig. 2g**). We obtained essentially similar results with transgenic tobacco plants (data not shown), which suggested that the tissue specificity of expression of the CaMV 35S promoter might be similar in *Pharbitis* and tobacco plants.

3.3 Southern hybridization analysis of transgenic lines

We confirmed the presence of the GUS transgene in the genomes of transgenic plants by Southern hybridization (**Fig. 3**). We used a DNA fragment that contained the GUS-coding region (indicated as F1 in **Fig. 3b**) to probe the *Eco*RI-digested genomic DNA of transgenic plants. In such analysis, the number of bands detected showed indicate the number of integrated copies of the transgene in the plant genome since there is only one *Eco*RI restric-

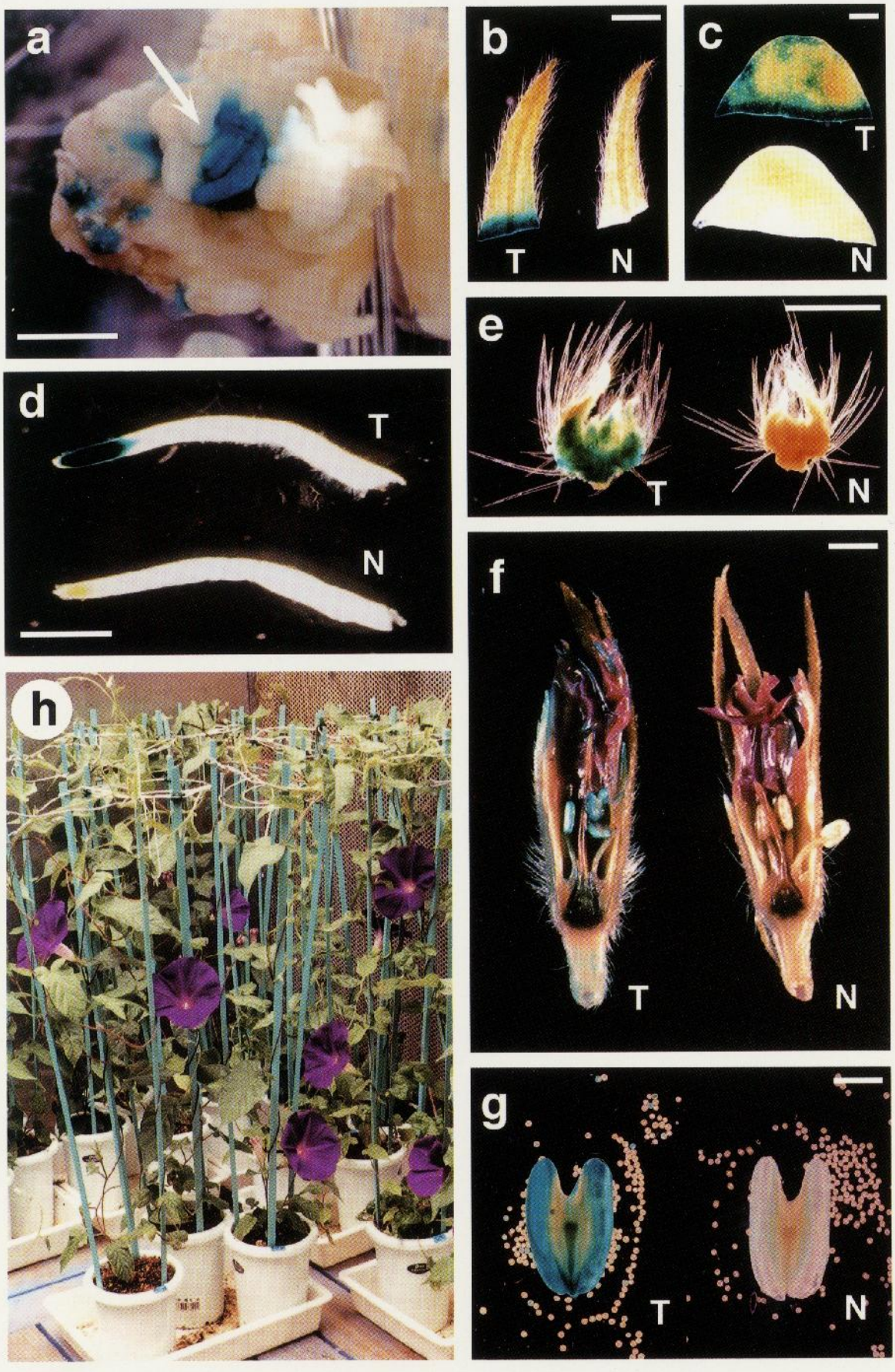

Fig. 2 Expression of the GUS reporter gene in transgenic *Pharbitis nil*. (a) Developing secondary embryos. GUS-positive embryoids are indicated by an arrow. Panels (b) through (g); tissues of transgenic (T) and non-transgenic (N) plants. (b) Tips of mature laeves, (c) tips of cotyledons, (d) tips of roots, (e) tips of shoots 10 days after short-day induction, (f) longitudinal sections of flower buds, (g) anthers and pollen, and (h) transgenic plants two months after acclimatization. Scale bars: (a) through (d), 2 mm; (e), 1 mm; (f), 5 mm; and (g), 1 mm.

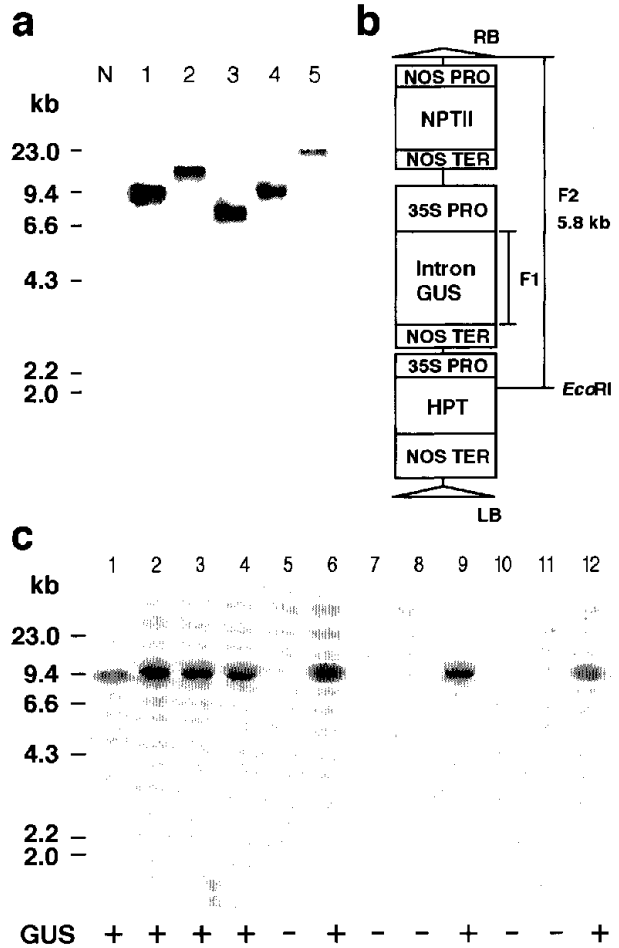

Fig. 3 Southern hybridization analysis of transgenic *Pharbitis* plants. (a) Analysis of genomic DNA from five GUS-positive T1 plants (lane 1, IG1; lane 2, IG2; lane 3, IG3; lane 4, IG4; lane 5, IG5) and a non-transgenic control plant (N). DNA was digested with EcoRI and the transgene was detected with a ^{32}P-labelled DNA fragment (F_1) of the GUS gene (indicated in b). (b) Physical map of the T-DNA region of the vector pIG121Hm. F_1, Probe DNA; F_2, minimum length of the detectable *Eco*RI fragment from the single *Eco*RI site to the right border (RB; 5.8 kb); NOS PRO, promoter of the gene for nopaline synthase; NPTII, gene for neomycin phosphotransferase II; NOS TER, terminator of the gene for nopaline synthase; 35S PRO, 35S promoter of cauliflower mosaic virus; Intron GUS, the gene for β-glucuronidase with an intron; HPT, the gene for hygromycin phosphotransferase; RB, right border of T-DNA; LB, left border of T-DNA. (c) Analysis of the genomic DNA from twelve T2 plants of the IG1 transgenic line together with results of assays for GUS activity (+, positive; -, negative).

tion site within the T-DNA region. All five lines tested, namely, IG1 through IG5, carried a single copy of the T-DNA in their genomes (**Fig. 3a**). All detected bands corresponded to fragments of at least 8 kb in length, and such fragments were long enough to include the 5.8-kb region of the T-DNA that contained the kanamycin-resistance gene (indicated as F2 in **Fig. 3b**).

Table 2. Segregation analysis of transgenic plants

Line (T2)	No. of plants	GUS-positive	GUS-negative	Ratio	Number of copies of the transgene[a]
IG1	15	10	5	2:1	1
IG2	15	11	4	2.8:1	1
IG3	14	10	4	2.5:1	1
IG4	14	11	3	3.7:1	1
IG5	12	9	3	3:1	1

[a] From Fig. 3.

3.4 Segregation analysis

We examined 15 T2 seeds of each of the five lines IG1 through IG5 for segregation analysis (**Table 2**). Histochemical analysis of the expression of GUS revealed that the GUS transgene was heritable. The segregation ratios in the T2 generation ranged from 2:1 to 3.7:1, resembling the ratio of 3:1 expected from a single integration event. Even though we tested a limited number of plants, our results suggest the Mendelian inheritance of the transgene.

Fig. 3c shows the results of Southern hybridization analysis of 12 randomly selected T2 plants of line IG1. All GUS-positive plants generated a 9.4-kb fragment that hybridized with the probe. The stronger intensity of the hybridized bands in lanes 2 and 6 in **Fig. 3c** suggests that IG1.2 (lane 2) and IG1.6 (lane 6) might have been homozygous for the GUS transgene.

In conclusion, we have established a relatively efficient method for the transformation of *Pharbitis nil*, that allows the stable integration and expression of a GUS transgene, as confirmed by histochemical and molecular analysis and the heritability of the transgene.

Genes related to photoperiodic responses and to the morphogenesis and pigmentation of flowers, as well as genes in mobile genetic elements, have been isolated from *Pharbitis* and characterized (e.g., Sage-Ono *et al.*, 1998; Ono *et al.*, 2000; Fukada-Tanaka *et al.*, 1997; Inagaki *et al.*, 1994). However, no gene isolated from *Pharbitis* has yet been characterized by analysis of its expression in transgenic *Pharbitis* plants. Our protocol for the production of transgenic *Pharbitis* plants should allow more detailed analysis of the functions of specific genes from *Pharbitis*.

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