the different substituents. Such an effect should be related to inter-molecular effects (i.e. better packing)[25].

The optical micrographs of these LB films at Brewster angle set-up for compounds 1 and 3 are shown in Figure 5 *a* and *b*. Polymer 1 shows a fairly rigid structure with striations running parallel to the dipping direction. The micrographs also indicate that the rigidity leads to more disorganized domain formation with different orientations in the case of polymer 3. This compares well with the mechanical studies carried out on these polymers which showed that the anisotropy in polymer 3 would decrease as the substitutents increased in size.

This work shows that these pure alkyl-substituted thiophenes form stable monolayers at air/water interface and can be transferred to solid substrates by the LB technique. It is also seen that a possible change in orientation and packing of polymeric LB films can be obtained which can find applications in conductivity[22], nonlinear optical and electronic properties, especially in light emission[3], when the polymer was fabricated into light emitting diodes .

1. Skotheim, T. A., *Handbook of Conducting Polymers*, Marcel Dekker, NY, 1986.
2. Yamamoto, T., Komamdin D., Kubota, K. and Sasaki, S., *Chem. Lett.*, 1998, 235.
3. Somanathan, N., Srinivasan, K. S. V., Ramasami, T., Vibha Rani, Prakash, R. and Santhanam, K. S. V., Novel Polythiophene Copolymers for Light Emitting Diode Applications, 1998, Indian Patent (pending), appln no. 1310/DEL/98; Prakash, R., Vibha Rani, Santhanam, K. S. V. and Somanathan, N., LEDs using Conducting Polymer/Copolymer having the Electrochemically Deposited Electron Injector, 1997, Indian patent (pending); McCullough, R. D., *Adv. Mater.*, 1998, **10**, 93; Daousty, G. and Leclerc, M., *Macromolecules*, 1991, **24**, 455; Roncali, J., Marque, P., Garreau, R., Garnier, F. and Lemaire, M., *Macromolecules*, 1990, **23**, 1347; McCullough, R. D., Eubank, P. C. and Loewe, R. S., *J. Am. Chem. Soc.*, 1997, **119**, 633; Inaoka, I. and Collard, D. M., *Synth. Met.*, 1997, **84**, 243.
4. Ballauf, M., *Angew. Chem. Int. Ed. Engl.*, 1989, **101**, 261.
5. Callender, C. L., Carere, C. A., Daoust, G. and Leclerc, M., *Thin Solid Films*, 1991, **204**, 451.
6. Jen, K. Y., Miller, G. C. and Elsenbaumer, R. L., *J. Chem. Soc., Chem. Commun.*, 1986, 1346.
7. Bennati, M., Grupp, A., Bauerle, A. and Mehering, M., *Chem. Phys.*, 1994, **185**, 221.
8. Pang, Y. and Prasad, P. N., *J. Chem. Phys.*, 1990, **93**, 2201.
9. Moulton, J. and Smith, P., *Polymer*, 1992, **33**, 2340.
10. Meille, S. V., Romita, V., Caronna, T., Lovinger, A. J., Catella, M. and Velovrzeckaga, L., *Macromolecules*, 1997, **30**, 7898.
11. Prosa, T. J., Winokur, M. J. and McCullough, R. D., *Macromolecules*, 1996, **29**, 3654.
12. Bolognesi, A., Porziowzhuo, G. and Ezquerra, T., *Eur. Polym. J.*, 1996, **32**, 1097.
13. Neal, D. B., Petty, M. C., Roberts, G. G., Ahamad, M. M., Feast, W. J., Girling, I. R., Cade, N. A., Kolinsky, K. A. and Peterson, I. R., *Electron Lett.*, 1986, **22**, 460.
14. Smith, G. W., Barton, J. W., Daniel, M. F. and Ratcliffe, N., *Thin Solid Films*, 1985, **132**, 125.
15. Nakahara, H., Nakayama, J., Hoshino, M. and Fukuda, K., *Thin Solid Films*, 1988, **106**, 87.
16. Dhathathreyan, A., Mary, N. L., Radhakrishnan, G. and John Collins, S., *Macromolecules*, 1996, **29**, 1837.
17. John Collins, S., Mary, N. L., Radhakrishnan, G. and Dhathathreyan, A., *J. Chem. Soc., Faraday Trans.*, 1997, **93**, 4021.
18. Somanathan, N. and Wegner, G., *Indian J. Chem.*, 1994, **33A**, 572.
19. Taman, K., Kodama, S., Nakajima, I. and Kumuda, M., *Tetrahedron*, 1982, **38**, 3347.
20. Sujimoto, M., Takeda, S., Su, H. B. and Yoshino, K., *Chem. Express*, 1986, **1**, 635.
21. Goedel, W. A., Somanathan, N., Enkelmann, V. and Wegner, G., *Makromol. Chem.*, 1992, **193**, 1195.
22. Somanathan, N. and Wegner, G., *Synth. Met.*, 1995, **75**, 123.
23. Gaines, G., *Insoluble Monolayers at Gas/Liquid Interface*, Wiley Inter Sci., 1966.
24. Fukuda, K. and Nakahara, H., *J. Colloid Interface Sci.*, 1984, **98**, 555; McRae, E. G. and Kasha, M., in *Physical Process in Radiation Biology* (eds Augenstein, L. G., Mason, R. and Rosenberg, B.), Academic Press, NY, 1974, p. 23.
25. Fell, H. J., Samuelson, E. J., Als Nielsen, J., Grübel, G. and Mardalen J., *J. Solid State Commun.*, 1995, **94**, 843.

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Expression of *npt*II marker and *gus* reporter genes and their inheritance in subsequent generations of transgenic *Brassica* developed through *Agrobacterium*-mediated gene transfer

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***Npt*II marker and *gus* reporter genes were introduced in *Brassica juncea* through *Agrobacterium*-mediated transformation showing a transformation frequency of 3–5% in the best responsive medium. Seventy-one per cent regeneration from hypocotyl explants was obtained on MS medium containing 0.01 mg/l 2,4-D and 2.0 mg/l BA supplemented with 20 µM $AgNO_3$, and 0.7% agarose as the gelling agent. The presence of transgenes (*npt*II and *gus*) in T_0, T_1 and T_2 generations was confirmed by dot blot analysis, using *npt*II gene as the probe, and by histochemical assay for the *gus* gene, respectively. Mendelian inheritance of the transgene (*npt*II) was observed in the T_1 and T_2 generations. Both the marker and reporter genes co-segregated in the T_1 and T_2 generations. The kanamycin-resistant plants in the T_1 progeny were either homozygous or heterozygous for the transgene.**

AMONG the various approaches for integrative transformation, *Agrobacterium*-mediated technique is most widely used. This method of transformation has been used previously by Mathews *et al.*[1], Barfield and Pua[2],

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and Pental *et al.*[3], in different species of *Brassica*. Most of the previous investigators have reported monogenic Mendelian segregation of the transferred genes in the first generation of transgenic plants.

In the present study, *Agrobacterium*-mediated transformation has been carried out in *Brassica juncea* cv. B-85 (B-85 is the most widely grown cultivar in West Bengal) using *npt*II gene (neomycin phosphotransferase II) as a selectable marker and *gus* (β-glucuronidase) as the reporter gene. In the construct used for transformation, the two genes are in the same cassette under independent CaMV 35S promoter. The *gus* expression was checked in different plant parts in T_0, and the segregation pattern of the transgenes was observed in T_1 and T_2 progenies of the original transformants.

The *Agrobacterium tumefaciens* strain LBA 4404 containing the binary vector, pPH26 (11.55 kb), was constructed from pPZP vector family containing *npt*II gene which confers kanamycin resistance, and *gus* gene which controls the expression of β-glucuronidase. Both the genes were under the control of a separate CaMV 35S promoter with opposite orientation and situated within left and right border sequences (Figure 1 *a*). The construct was a gift from P. Maliga, Waksman Institute, Rutgers University, USA. The bacteria were cultured overnight at 28°C Luria broth (LB: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.2) medium supplemented with 200 mM $MgCl_2$ and 50 mg/l kanamycin. The bacteria were then centrifuged at 4000 rpm, suspended in hormone-free MS medium[4] and the density was adjusted to $A_{600} = 0.1$.

The hypocotyl explants were precultured in regeneration medium (without $AgNO_3$) for one or two days before co-cultivation with *Agrobacterium*. Pooled lots of the explants were suspended in inoculum for 10 min, blotted dry on sterile blotting paper, and placed over regeneration medium without $AgNO_3$. After co-cultivation with *Agrobacterium* for 48 h, the explants were washed overnight in liquid, (hormone-free) MS medium, blotted dry, and placed over regeneration medium containing 250 mg/l cefotaxime and 20 µM $AgNO_3$. The medium was changed at 10–14 d interval. Within 2–4 weeks, the shoot primordia emerged, and the explants were then transferred to regeneration medium containing 25 mg/l kanamycin, 250 mg/l cefotaxime, and 20 µM $AgNO_3$. The explants were placed in fresh medium at intervals of 10–12 d. Green, putative-transformed shoots were repeatedly transferred to fresh hormone-free MS medium containing 25 mg/l kanamycin and 200 mg/l cefotaxime.

GUS activity was tested histochemically in various plant parts like leaf, root, stem, sepal, petal, anther, pistil, pollen, pod of different transgenic plants as well as their subsequent progenies. The histochemical determination was carried out according to the procedure

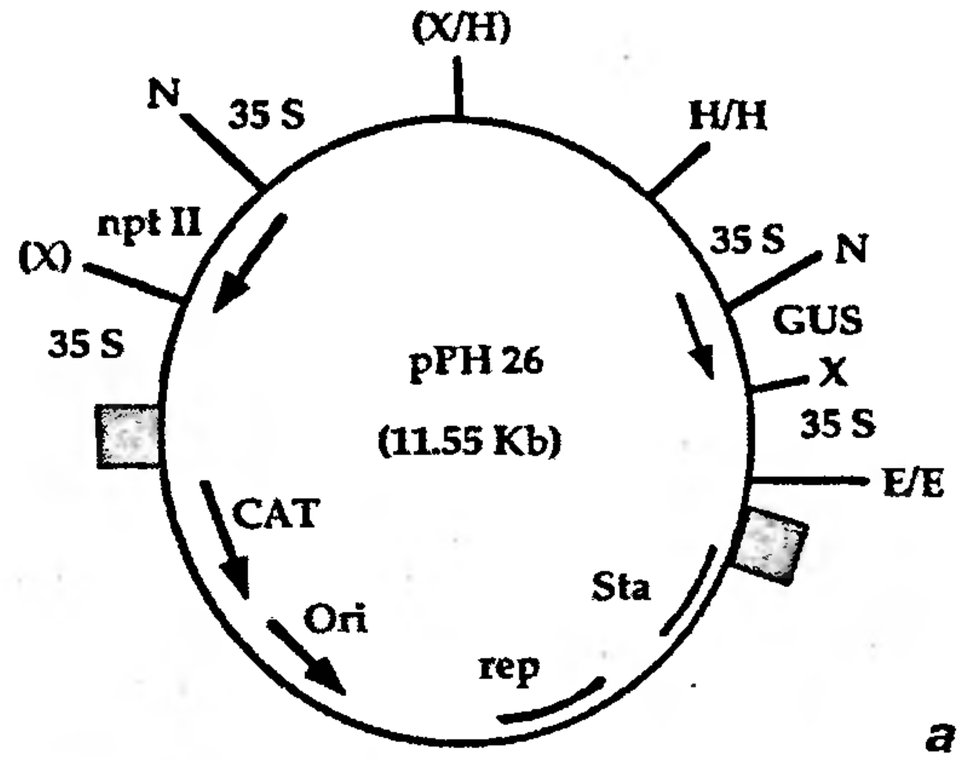

Figure 1. pPH26 construct and dot blot for molecular characterization of the transgenics: *a*, Construct used in transformation experiment (pPH26); *b*, Dot blot using *npt*II gene as marker probe. Lane A; 1–12, all putative transformants. Lane B; 1–12, all putative transformants. Lane C; 1–6, all putative transformants along with negative control (untransformed plant) at C_7 position. Lane D; 1–6, putative transformants along with negative control at D_7 position. Lane E_{12}; positive control.

of Jefferson[5]. Tissue sections or organs were incubated overnight at 37°C in x-gluc solution made up of 10 mM EDTA, 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (w/v) x-gluc. Chlorophyll was removed by treating the tissue in 1:3 aceto-ethanol mixture. A blue colour was observed in 70% ethanol in transformed shoots in comparison to white colour in untransformed control plant.

The green and healthy rooted plants were transferred to soil for acclimatization after proper washing away of agar from the root surface. The potted plants were initially covered with a plastic bag, for 7–10 d and later slowly exposed to the ambient environment of the glasshouse at 20–22°C with 60–70% humidity and natural photoperiod.

The presence of transgene (*npt*II) was detected through dot blot analysis. Plant DNA was extracted

using urea buffer according to the method of Sambrook *et al.*[6]. The final pellet was treated with RNAase and dissolved in Tris–EDTA buffer. The total genomic DNA was blotted on nitrocellulose membrane after denaturing the DNA by treating with 4 M NaOH. Pre-hybridization and hybridization procedures were followed according to Sambrook *et al.*[6]. The probe was prepared using *npt*II gene cut from the vector, labelled with [α-^{32}P]dATP (Amersham), using PRIME IT TM random primer kit from Stratagene. After hybridization, washing was carried out under high stringency condition (65°C, 0.1 × SSC, 0.5% SDS).

Among the different media tested for regeneration from hypocotyl explants, 0.01 mg/l 2,4-D and 2.0 mg/l BA were found to be the best hormone combination (9.0% regeneration). Use of 20 µM $AgNO_3$, an ethylene inhibitor, increased the plant regeneration to 31.8%. Additionally, when 0.7% agarose was used as the gelling agent instead of agar agar, the plant regeneration frequency increased to 71%.

The ability of hypocotyl tissues to regenerate shoots was significantly reduced by infection with *Agrobacterium*, and was delayed by 5–6 d. Presence of kanamycin (25 mg/l) in the selection medium had adverse effect on shoot induction. Hence, kanamycin selection was applied after 10–14 d of culture after induction of shoot primordia. Two types of shoots, green and white with occassional purple pigment in the young leaves were produced on kanamycin-containing medium. The green shoot buds were considered to be the resistant, and putative transformants. The green shoot buds were repeatedly transferred to fresh kanamycin (25 mg/l)-containing, hormone-free MS medium. From two different sets of experiments it was observed that all the green shoots raised from the kanamycin-containing shoot-induction medium could not produce roots in the same selection medium. Some turned white, while others showed rooting but turned white after several passages, and did not produce healthy green plants. In the first experiment, 13 out of 25 selected shoots (52%) and in the second experiment 24 out of 43 selected shoots (57%) of the regenerated green shoots showed rooting and normal growth in further selection medium. The transformation frequency was calculated as:

$$\frac{\text{No. of explants produced transgenic shoots} \times 100}{\text{No. of total explants used in the experiment}}$$

The calculated transformation frequency was 3.2 and 5.0% in the two independent experiments (Table 1).

The plant DNA was isolated from the leaf tissue of all kanamycin-resistant plants together with the negative control. One-kb fragment of *npt*II gene was used as the probe to check for presence of the *npt*II gene among the selected plants. Among the 36 selected plants tested, 24 showed clear presence of *npt*II gene; negative control did not hybridize in the same blot (c 7 and d 7 position in Figure 1 *b*).

Histochemical assay for presence of *gus* gene was carried out in all the putative transformants; small pieces of leaves from each plant were used for the assay. The experiment revealed that many of the transgenic shoots were chimeric in nature. The few constitutively expressing GUS-positive plants were tested for the

Table 1. Transgenic plants of *Brassica juncea* using pPH26 construct containing *gus* reporter gene and *npt*II marker gene

Number of	Expt. no. 1	Expt. no. 2
Explants cultured	156	168
Explants that developed green shoots	16	28
Green shoots recovered	25	43
Transgenic shoots	13	24
Explants that produced transgenic shoots	5	8
Transformation frequency	3.2%	5.0%

Table 2. GUS expression in different parts of transgenic plants

Plant parts	Positive (+)/Negative (–)
A. Root	
(i) germinating seedling root	
(a) apical region	++
(b) other regions	–
(ii) mature root	
(a) apical region	++
(b) other regions	+
B. Stem	
(a) epidermis	+
(b) cortex	+
(c) vascular bundle	++
(d) pith	–
C. Leaf	
(a) young leaf	++
(b) mature leaf	
(i) margin/tip	++
(ii) vascular region	+
(iii) petiole	–
D. Flower	
(a) sepal	++
(b) petal	
(i) vascular region	+
(ii) other regions	–
(c) androecium	
(i) filament	–
(ii) anther lobe	++
(d) gynoecium	
(i) ovary	–
(ii) style	+
(iii) stigma	++
E. Fruit	
(a) pod wall	+
(b) funicle	++
(c) mature seed coat	–
F. Cotyledonary leaf	++

++, indicates dark blue; +, indicates light blue.

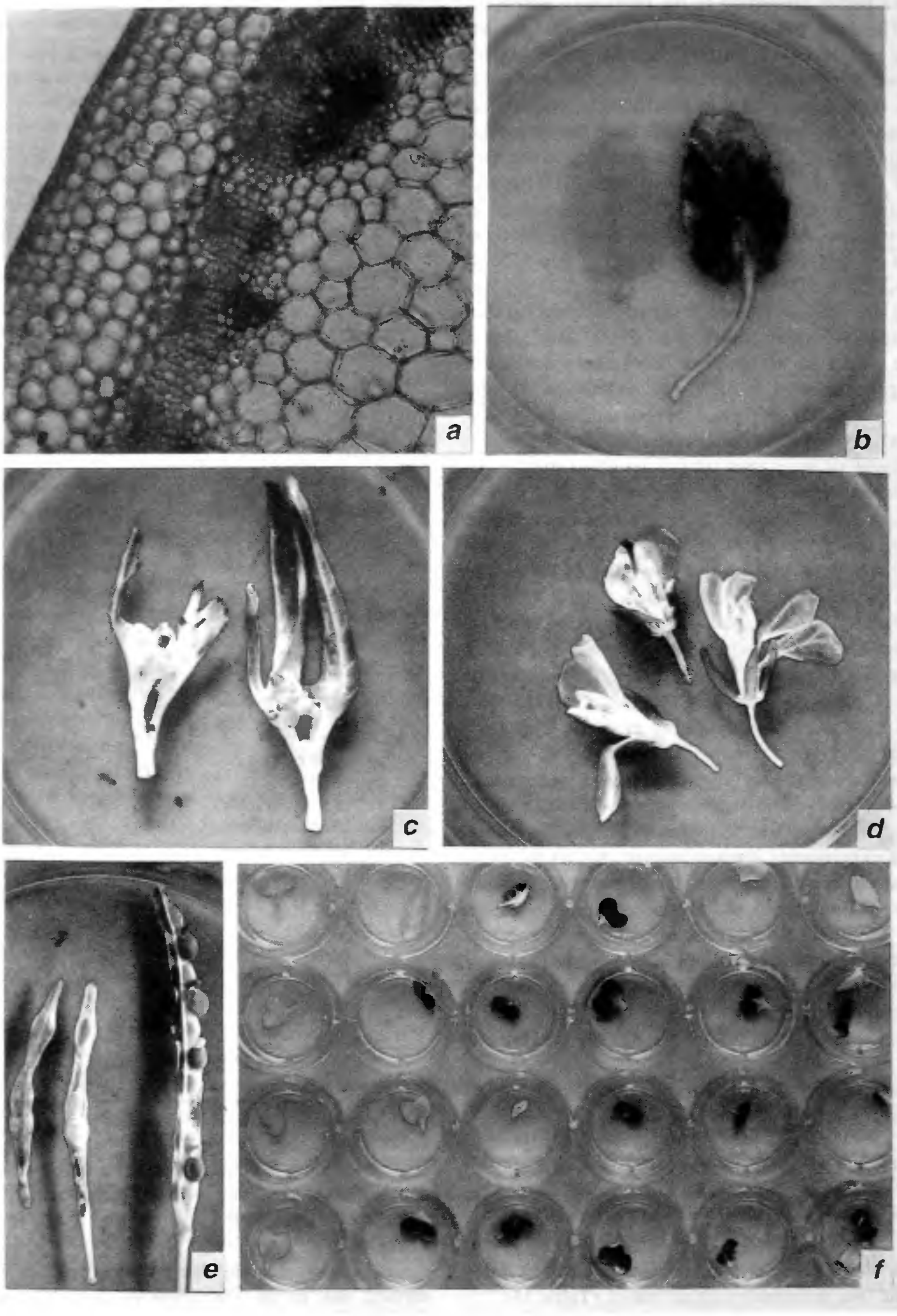

Figure 2. Histochemical assay for presence of *gus* gene in transgenic plants. (*a–e*, T_0; f : T_1). *a*, Stem section showing *gus* gene expression in the cortical region and vascular tissue (× 120); *b*, Young leaf of transgenic plant showing GUS expression (right) and the young leaf from control plant not showing any GUS expression (left) (× 1); *c*, Inflorescence axis of transgenic plant bearing flower buds (FB) and young leaves (YL), all showing GUS expression (× 2); *d*, Flowers of transgenic plant showing *gus* gene expression in sepals (S), petals (P), anther lobes (AL), style and stigma (SS) (× 2); *e*, Pods bearing the seeds showing presence of *gus* gene expression on the pod wall (PW) and funicles (F) (× 1.5); *f*, Segregation of *gus* gene evidenced by blue coloration of cotyledonary leaves in a small population of T_1 progeny when stained with x-gluc (× 1).

presence of *gus* gene in different plant parts (Figure 2 *a–e*). Table 2 gives the GUS expression in different organs of transgenic plants.

Seeds were obtained from the bagged inflorescences of three different nonchimeric transgenic plants which were transferred to soil. On germination in kanamycin (50 mg/l)-containing, hormone-free MS medium; the selfed seeds showed segregation of resistant (green) and sensitive (white) seedlings. The statistical data given in Table 3 prove that the gene for kanamycin resistance showed dominant, monogenic Mendelian inheritance. In addition, all the seedlings were tested histochemically for the presence of *gus* gene which was found to be present in all the kanamycin-resistant seedlings, but was absent in all the kanamycin-sensitive seedling (Figure 2*f*). The experiment proved the co-segregation of both the marker and the reporter genes. Some of the green seedlings of the T_1 generation were transferred to pots to produce the T_2 seeds. GUS expression was examined in different plant parts like flowers, seeds, pods, etc. in the T_1 plants. The flowers showed the same expression as in T_0 transgenic progeny (Figure 2*d*). When a random population of T_1 transgenic plants was maintained in culture in presence of 100 mg/l kanamycin, after prolonged culture in kanamycin-containing media the plants showed differential growth pattern: out of 20 plants, 4 showed deep green colour in leaf and normal growth, but the other 16 showed pale appearance with normal growth and proper rooting. Potrykus *et al.*[7] had reported similar segregation for dark and light green leaf colour in transgenic tobacco plants with the *npt*II gene, which the authors attributed to dosages effect of the transgene. After 8 weeks of *in vitro* culture in kanamycin-containing medium, *gus* gene expression was not observed in 25% of the plants that were checked histochemically. Occasional instability or loss of the marker gene in the progeny has been reported by Potrykus *et al.*[7].

The T_2 seeds (selfed seeds) of the transgenic plants were obtained from the T_1 plants. The seeds from two different T_1 plants were germinated in the presence of 200 mg/l kanamycin. The seeds from one plant (T_0-22-4) showed the segregation of green and white seedlings in a ratio of 3:1 (Table 4) when germinated in the selection medium, indicating that the particular plant was heterozygous in respect of marker gene in T_1 generation. All the seedlings from another plant (T_0-22-5) remained green, and did not show segregation. Hence the plant T_0-22-5 of T_1 generation was homozygous in respect of the marker gene. When all these plants were tested in 300 and 500 mg/l kan, they all showed proper rooting in both the concentrations, but few of them bleached. When GUS expression was checked from a random population, it showed co-segregation of both the genes.

This report of transformation of Indian mustard, an important oil seed crop of this subcontinent, with the *gus* reporter gene and its stable inheritance in 2nd and 3rd generations is an essential step towards transfer of agronomically useful gene(s). Here the protocols for transferring foreign genes into *Brassica juncea* at a high transformation frequency have been developed that can be utilized further for introgression of agronomically important gene(s).

Table 3. T_1 progeny analysis of *B. juncea* transgenic plants developed through *Agrobacterium*-mediated gene transfer of pPH26 plasmid containing *gus* reporter gene and *npt*II marker gene

Plant no.	T_0-1	T_0-22	T_0-31
No. of seeds tested	56	81	68
No. of seeds germinated	27	50	38
No. of plants that showed gus-positive reaction	20	38	29
Expected no. of GUS-positive plants according to Mendelian 3:1 ratio	20.25	37.5	28.5
*Chi square value	0.0122	0.0266	0.0241
Kanamycin positivity test	All GUS positive plants showed karamycin positivity at 50 mg/l kan		

*Probability lies between 80 and 95%.

Table 4. T_2 progeny analysis of two different T_1 transgenic *B. juncea* plants

Plant no. T_0-22-4	
No. of seeds tested	176
No. of seeds germinated	160
No. of plants that showed kan resistance at 100 mg/l kan	124
Expected no. of kan^r plants according to Mendelian 3:1 ratio	120
*Chi square value	0.533
Plant no. T_0-22-5	
No. of seeds tested	201
No. of seeds germinated	196
All the plants were kan resistant and could tolerate up to 200 mg/l kan	

*Probability lies between 50 and 20%.

1. Mathews, H., Bharathan, N., Litz, R. E., Narayanan, K. R., Rao, P. S. and Bhatia, C. R., *Plant Sci.*, 1990, **72**, 245–252.
2. Barfeild, D. G. and Pua, E. C., *Plant Cell Rep.*, 1991, **10**, 308–314.
3. Pental, D., Pradhan, A. K., Sodhi, Y. S. and Mukhopadhyay, A., *Plant Cell Rep.*, 1993, **12**, 462–467.
4. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
5. Jefferson, R. A., *Plant Mol. Biol.*, 1987, **5**, 307–405.
6. Sambrook, J., Fritsh, E. F. and Maniatis, T., *Molecular Cloning*, Cold Spring Harbour Laboratory Press, New York, 1989, 2nd edn.
7. Potrykus, I., Paszkowski, J., Saul, M. W., Petruska, J. and Shillito, R. D., *Mol. Gen. Genet.*, 1985, **199**, 249–268.

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