3. Van Zeeland, A. A., *Mutat. Res.*, 1978, **50**, 145–151.
4. Ichikawa-Ryo, H. and Kondo, S., *Mutat. Res.*, 1980, **72**, 311–322.
5. Bhattacharjee, S. B., Chatterjee, S. and Pal, B., *Mutat. Res.*., 1982, **106**, 137–146.
6. Bhattacharjee, S. B., Bhattacharya, N. and Chatterjee, S., *Radiat. Res.*, 1987, **109**, 310–318.
7. Balansky, R., *Mutat. Res.*, 1992, **269**, 307–317.
8. Nehlig, A. and Debry, G., *Mutat. Res.*, 1994, **317**, 145–162.
9. Boothman, D., Schlegel, R. and Pardee, A., *Mutat. Res.*, 1988, **202**, 393–411.
10. Fabre, F., *Mol. Gen. Genet.*, 1971, **110**, 134–143.
11. Fabre, F., *Mol. Gen. Genet.*, 1972, **117**, 153–166.
12. Loprieno, N. and Schupbach, M., *Mol. Gen. Genet.*, 1971, **110**, 348–354.
13. Cleaver, J. E., *Teratogen. Carcinogen. Mutagen.*, 1989, **9**, 147–155.
14. Fujiwara, Y. and Tatsumi, M., *Mutat. Res.*, 1976, **37**, 91–110.
15. Kesavan, P. C. and Natarajan, A. T., *Mutat. Res.*, 1985, **143**, 61–68.
16. Kesavan, P. C., Trasi, S. and Ahmad, A., *Int. J. Radiat. Biol.*, 1973, **24**, 581–587.
17. Kesavan, P. C., Sharma, G. J. and Afzal, S. M. J., *Radiat. Res.*, 1978, **75**, 18–30.
18. Kesavan, P. C. and Powers, E. L., *Int. J. Radiat. Biol.*, 1985, **48**, 223–233.
19. Raghu, B. and Kesavan, P. C., *Indian J. Exp. Biol.*, 1986, **24**, 742–746.
20. Farooqi, Z. and Kesavan, P. C., *Mutat. Res.*, 1992, **269**, 225–230.
21. Natarajan, A. T., Obe, G. and Dulout, F. N., *Human Genet.*, 1980, **54**, 183–189.
22. Waldren, C. A. and Rasko, I., *Radiat. Res.*, 1978, **73**, 95–100.
23. Mc Cann, J., Choi, E., Yamasaki, E. and Ames, B. N., *Proc. Natl. Acad. Sci. USA*, 1975, **12**, 5135–5139.
24. Mc Cann, J. and Ames, B. N., *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 950–954.
25. Miller, J. A. and Miller, E. C., *J. Natl. Cancer Inst.*, 1971, **47**, V–XIV.
26. Sugimura, T., Yahagi, T., Nagao, M., Takeuchi, M., Kawachi, T., Hara, K., Yamasaki, E., Matsushima, T., Hashimoto, Y. and Okada, M., *Int. Agency Res. Cancer Sci. Publ.*, 1976, **12**, 81–104.
27. Zimmermann, F. K., *Z. Vererbungsl.*, 1966, **98**, 230–246.
28. Kinsella, A. R. and Radman, M., *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 6149–6153.
29. Kunz, B. A., Hannan, M. A. and Haynes, R. H., *Cancer Res.*, 1980, **40**, 2323–2329.
30. Kunz, B. A., Barclay, B. J. and Haynes, *Mutat. Res.*, 1980, **73**, 215–220.
31. Zimmermann, F. K., Kern, R. and Rasenberger, H., *Mutat. Res.*, 1975, **28**, 381–388.
32. Thompson, L. H., *Mutat. Res.*, 1996, **363**, 77–88.
33. Zimmermann, F. K., *Mutat. Res.*, 1973, **21**, 263–269.
34. Zimmermann, F. K., in *Handbook of Mutagenicity Test Procedures* (eds Kilbey *et al.*), Elsevier/North-Holland Biomedical Press, Amsterdam, 1977, pp. 119–134.
35. Clarke, C. H. and Shankel, D. M., *Bacteriol. Rev.*, 1975, **39**, 33–53.
36. Donovan, P. J. and DiPaolo, J. A., *Cancer Res.*, 1974, **34**, 2720–2727.
37. Kakunaga, T., *Nature*, 1975, **258**, 218–250.
38. Sideropolous, A. S. and Shankel, D. M., *J. Bacteriol.*, 1968, **96**, 198–204.
39. Busse, P. M., Bose, S. K., Jones, R. W. and Tolmach, L. J., *Radiat. Res.*, 1978, **76**, 292–307.
40. Zapetti-Bosseler, F. and Scott, D., *Mutat. Res.*, 1985, **143**, 251–256.
41. Painter, R. B., *J. Mol. Biol.*, 1980, **143**, 289–301.
42. Musk, S. R. R. and Steel, G. G., *Int. J. Radiat. Biol.*, 1990, **57**, 1105–1112.
43. Haynes, R. H., in *Molecular Mechanisms for Repair of DNA, Part B* (eds Hanawalt, P. C. and Setlow, R. B.), Plenum, New York, 1975, pp. 529–540.
44. Reddy, N. M. S. and Rao, B. S., *Radiat. Environ. Biophys.*, 1981, **19**, 187–195.
45. Saeki, T., Machida, I. and Nakai, S., *Mutat. Res.*, 1980, **73**, 251–265.
46. Game, J. C., *Semin. Cancer Biol.*, 1993, **4**, 73–83.
47. Prakash, S., Sung, P. and Prakash, L., *Annu. Rev. Genet.*, 1993, **27**, 33–70.
48. Ostermann, K., Lorentz, A. and Schmidt, H., *Nucleic Acids Res.*, 1993, **21**, 5940–5944.
49. Akaboshi, E., Inoue, Y. and Ryo, H., *Jpn. J. Genet.*, 1994, **69**, 663–670.
50. Bezzubova, O., Schmidt, H., Ostermann, K., Heder, W. D. and Buerstedde, J. M., *Nucleic Acids Res.*, 1993, **21**, 5945–5949.
51. Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. and Ogawa, T., *Nat. Genet.*, 1993, **4**, 239–243.
52. Morita, T., Yoshimura, Y., Yamamoto, A., Murata, K., Mori, M., Yamamoto, H. and Matsushiro, A., *Proc. Natl. Acad. Sci. USA*, **90**, 6577–6580.
53. Fahrig, R., *Mol. Gen. Genet.*, 1975, **138**, 309–314.
54. Fahrig, R., *Mutat. Res.*, 1992, **284**, 177–183.

Received 11 September 1997; revised accepted 27 April 1998

Agrobacterium rhizogenes-mediated gene transfer in mungbean (*Vigna radiata* L. (Wilczek))

Pawan K. Jaiwal*, Christof Sautter† and Ingo Potrykus†

Department of BioSciences, M.D. University, Rohtak 124 001, India
†Institute of Plant Sciences, Swiss Federal Institute of Technology, CH-8092, Zurich, Switzerland

Stable transformation and expression of transgenes was achieved in mungbean using *Agrobacterium rhizogenes*-mediated system. Seedling hypocotyls, on cocultivation with different *A. rhizogenes* strains containing Ri plasmid and a binary vector (pBin9GUSint) with neomycin phosphotransferase II and GUS (with a intron in coding region) as marker genes, produced roots which showed GUS expression and rapid growth on hormone free-medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. Transformation frequency varied with the age of the explants, duration of pre-induction of explants, cocultivation time and medium, concentration and strain of *Agrobacterium* but did not vary too much with different genotypes of mungbean. The transgenic roots produced calli on callusing medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The calli showed expression of *nptII* and *Gus* genes. Transfer of GUS DNA into mungbean genome was confirmed by Southern blot analysis. However, neither transgenic roots nor their calli could regenerate shoots on either the B_5 or B_5-containing different cytokinins or auxins alone or in combination.

GENETIC transformation of grain legumes has, so far, been a difficult and challenging problem probably due

*For correspondence.

to the lack of efficient and reproducible regeneration systems[1]. Of the various transformation techniques, *Agrobacterium*-mediated transformation system has been the most efficient and widely employed one for the production of transformed plants with predictable patterns of DNA integration[2]. In this system, the phytopathological response depends on the type of the plasmid. Tumour-inducing (Ti) plasmids of *A. tumefaciens* cause crown gall tumours whereas root-inducing (Ri) plasmids of *A. rhizogenes* cause hairy roots on susceptible dicotyledonous plants[3]. Root induction is due to the integration of the Ri-T DNA (transferred DNA) into the plant genome and its subsequent expression[4]. Not all plant cells are uniformly transformed in crown gall tumour tissues, but all tissues are uniformly transformed in hairy roots[3]. Transformed roots are genetically stable, hormone autotrophic and are easily regenerated into shoots spontaneously or on exposure to suitable plant growth hormones in a number of plants[5]. However, there are only a few reports of recovery of transgenic plants from transformed roots in legumes[6]. In addition to this, transformed roots have been used in physiological, biochemical and molecular studies on *Rhizobium* and mycorrhizal interactions, adaptation to drought, heavy metal uptake, secondary product system and *in situ* hybridization (see Ramsay and Kumar[7]).

Mungbean is an important grain legume of the Indian subcontinent which accounts for more than two-thirds of the total world's production. There are, so far, no published reports on stable integration and expression of transgenes using any of the transformation techniques in mungbean[8,9]. In the present report, the production of transgenic roots and calli of mungbean using *Agrobacterium rhizogenes* has been described for the first time. The stable transfer of transgenes and their expression have also been shown by Southern analysis and enzyme assays.

Seeds of different cultivars, ML-332, K-851, Pusa-105 and PS-7 were obtained from Pulse Research Lab, Division of Genetics, IARI, New Delhi. Three strains of *A. rhizogenes*, LBA 9402, ATCC 15834 and Ar 8196 carrying the respective Ri plasmid and the binary vector pBin9Gusint[10] containing *nos-npt-nos* and *35S-uidA-nos* genes were used in transformation studies. The GUS contains an intron in the coding region to ensure that the observed GUS expression is in plant cells and not in the *agrobacterial* cells. These strains were grown overnight in liquid TY medium containing 50 mg l^{-1} kanamycin at 28°C. Bacterial cells were pelleted at a speed of 4000 rpm for 10 min and resuspended in liquid B_5 plant culture medium with the density of 10^9 cells ml^{-1} ($OD_{600} = 1$) (unless otherwise mentioned).

Entire mature cotyledons and hypocotyl explants (10 mm in size) were excised from 2-day-old-etiolated *in vitro*-raised seedlings[8] and immersed in bacterial suspension for 30 min to 24 h with gentle shaking. Infected explants were blot-dried on sterile filter paper and cocultivated on basal B_5 medium or B_5 containing BAP (0.1 or 1.0 mg l^{-1}) under dark at 25 ± 2°C. After cocultivation, explants were washed 3–4 times with sterile distilled water with vigorous shaking and blotted dry on sterile filter paper and cultured on basal B_5 medium or basal B_5 medium + BAP (0.1 mg l^{-1}) containing kanamycin (50 mg l^{-1}) and cefotaxime (500 mg l^{-1}). The roots that developed at both the cut ends of the hypocotyl explants were excised and subcultured on a fresh medium with the same antibiotics after every 2 weeks till 8 weeks. Transgenic roots (GUS +) developed calli on B_5 + BAP (1.0 mg l^{-1}) + 2,4-D (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) containing kanamycin (50 mg l^{-1}) and cefotaxime (500 mg l^{-1}). The calli were subcultured on the same medium with the same antibiotics after every 2 weeks till 6–8 weeks. Regeneration from the transgenic roots and their calli was tried on medium containing different concentrations of BAP (1, 10, 20 mg l^{-1}) or kinetin (1, 10 mg l^{-1}) or TDZ (1, 2 mg l^{-1}) either alone or in combination with an auxin, IBA (0.05, 0.5 mg l^{-1}). Genomic DNA from transformed and untransformed calli was extracted by cetyltrimethyl ammonium bromide (CTAB) method[11]. For Southern analysis, 20 µg of DNA samples was digested with *Hin*dIII and was separated on 0.8% agarose gel, blotted to nylon membrane and probed with DIG (Boehringer, Mannheim, Germany)-labelled coding regions of GUS following supplier's instructions. Neomycin phosphotransferase activity in transformed calli (grown on selection medium with 50 mg l^{-1} kanamycin) was assayed by dot blot method using (γ^{32}-P) ATP and kanamycin as substrate[12]. GUS activity in transformed roots and calli was determined by histochemical assay[13]. The DNA isolated from *A. rhizogenes* strain 9402, transformed and untransformed calli were subjected to PCR analysis using virA primers to amplify a 1.0 kb fragment of virulence region (virA) of *A. rhizogenes*.

Out of three strains of *A. rhizogenes*, LBA 9402, ATCC 15834 and Ar 8196 used in transformation studies, LBA 9402 was more effective than ATCC 15834 and Ar 8196 in inducing higher transient GUS expression in both the explants after 72 h of cocultivation (Figure 1). *Agrobacterium* LBA 9402 induced blue spots in 80% and 100% of the cotyledon and the hypocotyl explants respectively, after 3 days of cocultivation. The GUS expression in cotyledons was confined to the distal end on adaxial surface away from the regenerating proximal end (results not shown) while in hypocotyl explants it was localized to vascular tissues and epidermal cells either at one (in a few explants) or at both the cut ends in most of the explants (Figure 2 *a*). Other factors influencing the transient GUS expression in hypocotyl explants were also optimized. Hypocotyl explants of 2-day-old seedlings were more responsive compared to

those from 4 to 6-days-old seedlings to infection with strain LBA 9402 (Figure 3). Bacteria at a concentration of 10^8 cells ml^{-1} and cocultivation for 3 days gave an optimal combination for higher transient transformation rates and controllable bacterial growth (Figures 4 and 5). Cocultivation medium also affected transformation. Explants cultured on B_5 with low BAP concentration (0.1 mg l^{-1}) showed higher GUS expression than those cultured either on B_5 alone or B_5 with high BAP concentration (1.0 mg l^{-1}). Prior to cocultivation, inoculation of explants in bacterial suspension for 1 to 12 h also affected the transient transformation frequency. Six hours preinduction was found to be optimal (Figure 6). Four genotypes, ML-332, K-851, Pusa-105 and PS-7 which were screened for their susceptibility to *A. rhizogenes* showed GUS expression with minor variations.

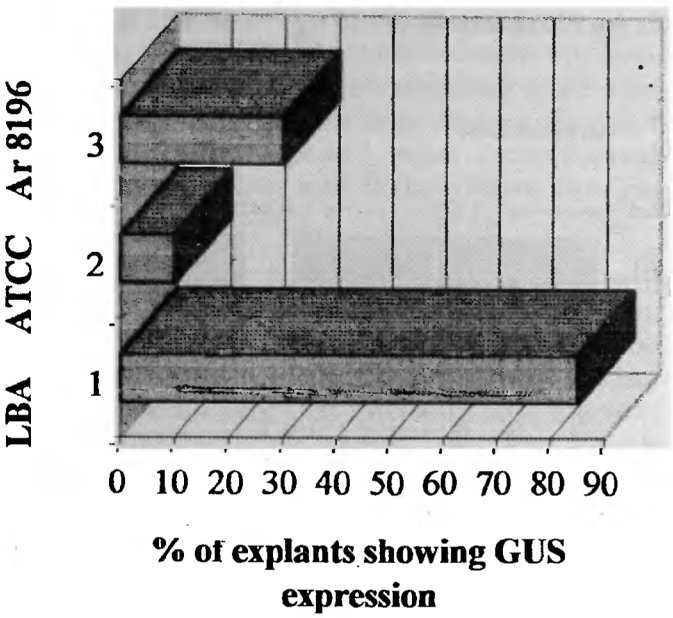

Figure 1. Effect of various bacterial strains on transient transformation frequency of mungbean hypocotyl explants.

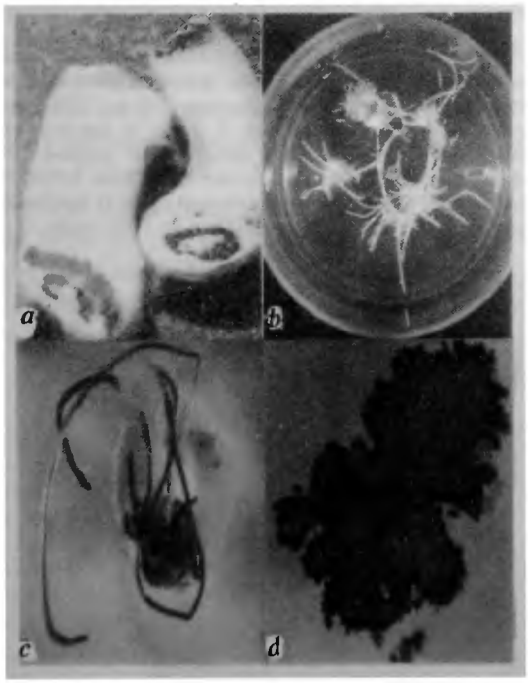

Figure 2 *a–d*. Transient expression of GUS on hypocotyl explants after 72 h cocultivation with LBA 9402 (pBin9GusInt) (*a*), regeneration of roots from hypocotyl inoculated with LBA 9402 (*b*), GUS staining of transgenic roots (*c*) and their calli (*d*) selected on kanamycin medium.

Hypocotyls of 2-day-old etiolated seedlings after 3 days of cocultivation with 10^8 cells ml^{-1} of LBA 9402 were cultured on B_5 supplemented with BAP (0.1 mg l^{-1}) and cefotaxime (500 mg l^{-1}) medium. On culture, 52% of the explants either directly or through a slight callus produced straight growing thicker roots at one or both the cut ends after 10–14 days (Figure 2 *b*). The roots were excised from explants and cultured on RM containing kanamycin (50 mg l^{-1}) where they grew faster with limited root hair and extensive lateral branching. Twelve per cent of the putative transformed roots were GUS positive (Figure 2 *c*). Roots excised from untrans-

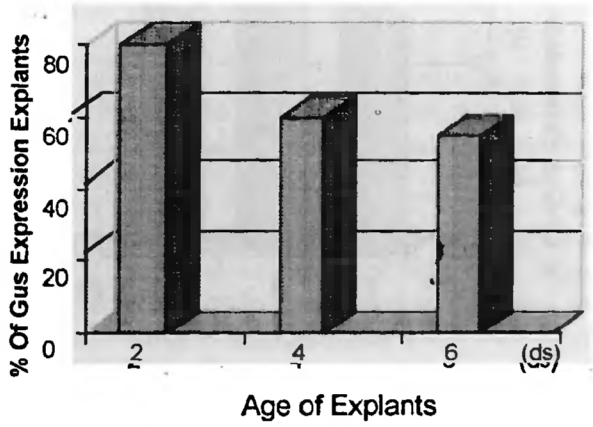

Figure 3. Effect of age of explant on transient GUS expression.

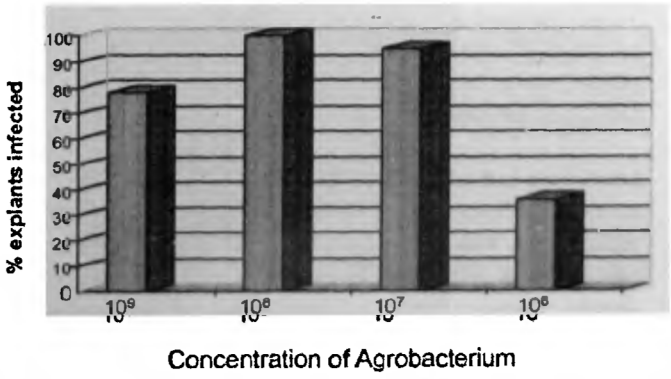

Figure 4. Effect of bacterium concentration on frequency of explant infection.

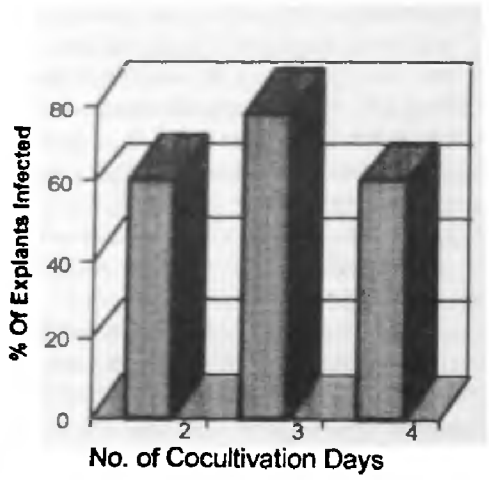

Figure 5. Effect of cocultivation days on explant infection.

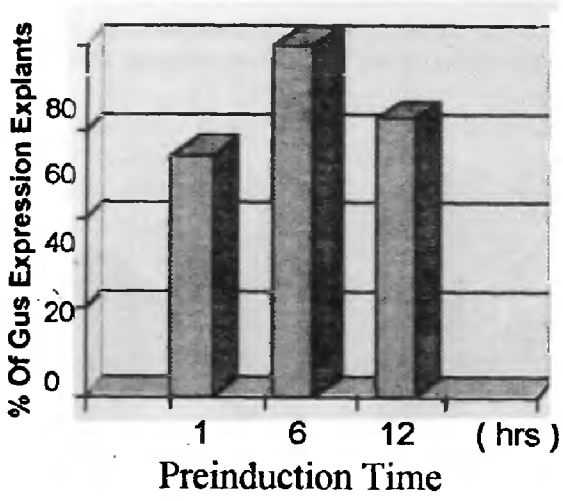

Figure 6. Effect of preinduction of explants in bacterial suspension on transient GUS expression.

formed explants failed to grow in the presence of kanamycin. Eighty per cent of the cotyledon explants which showed transient GUS expression on cocultivation with LBA 9402 failed to produce roots either on B_5 or B_5 with BAP (0.1 mg l^{-1}). The transgenic GUS-positive roots (check by staining a small portion of roots with X-gluc.) cultured on B_5 or B_5 with BAP (1, 10 and 20 mg l^{-1}) or kinetin (1, 10 mg l^{-1}) or TDZ (1, 2 and

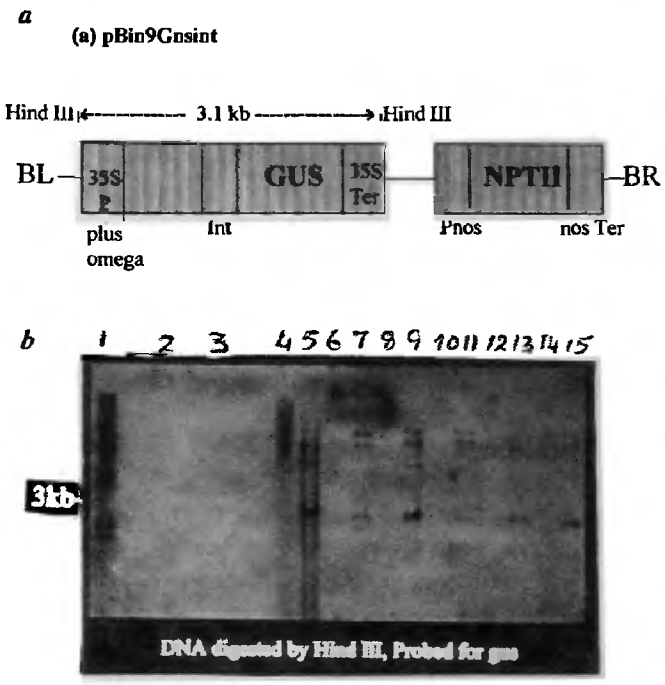

Figure 7. *a*, T-DNA region of pBin9Gusint. BR, right border; BL, left border; Gus, β-glucuronidase; nptII, neomycin phosphotransferase; 35S, 35S-promoter plus omega; int, intron; 35S ter, 35S terminator; nos, nopalin synthase promoter; pnos, 3 signal of nopaline synthase. *b*, Southern blot of transgenic root calli. *Hind*III digested DNA untransformed calli (lane 3) and transformed calli (lanes 5, 7, 9, 11, 13, 15) and undigested DNA from untransformed (lane 2) and transformed calli (lanes 4, 6, 8, 10, 12, 14) hybridized to GUS probe.

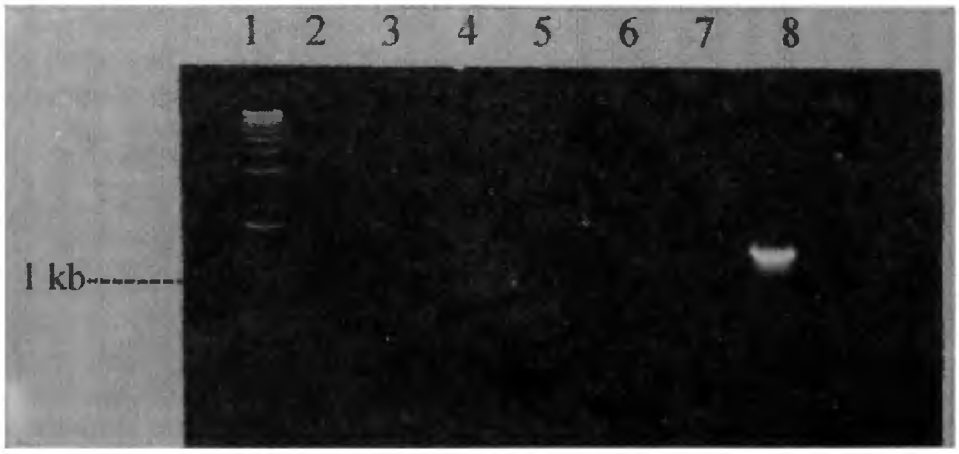

Figure 8. PCR analysis of calli transformed by *A. rhizogenes* LBA 9402. DNAs were primed with oligonucleotides specific to the *virA* gene of *A. rhizogenes*. Marker DNA lane 1, positive control (lane 2), water (lane 3) negative control (lane 4) and transgenic calli (lanes 5 to 8).

5 mg l^{-1}) alone or in combination with IBA (0.05 or 0.5 mg l^{-1}) (SRM) turned green with slight proliferation but could not regenerate shoots even on prolonged incubation for 2–3 months. Regeneration from transformed roots indirectly via callus was also tried. The transgenic roots on C (MS salts + B_5 vitamins) medium containing 1 mg l^{-1} of BAP, 0.5 mg l^{-1} each of NAA and 2,4-D, 50 mg l^{-1} of kanamycin and 500 mg l^{-1} cefotaxime started callusing within 2 weeks. The roots with newly initiated callus were transferred to the same fresh medium containing the same level of antibiotics after every 2 weeks till 4 to 8 weeks. The calli turned dark blue when stained with X-gluc. (Figure 2 *d*). The growth of transgenic calli was normal up to 200 mg l^{-1} kanamycin. To confirm the absence of *Agrobacterium* in transformed calli after 3–4 subcultures, they were grown in the absence of cefotaxime. Bacterial growth was not observed even after 2 more subcultures on cefotaxime-free medium. Southern hybridization was carried out in DNA from GUS-expressing calli. The GUS DNA was detected as the fragment of expected size 3.1 kb (Figure 7). Since the GUS DNA probe only hybridizes to DNA from transformed calli, the results indicated that GUS DNA was transferred to mungbean genome. To rule out the possibility of GUS DNA from *agrobacterial* contamination, a PCR reaction was set up using the virA primers. The fragment of 1 kb corresponding to the sequence of *virA* gene was not detected in the transformed calli (Figure 8). Southern hybridization of DNA from GUS-expressing calli with *Agrobacterium* virE2 probe showed no hybridizing bands. These results indicate that GUS (+) kanamycin-resistant calli do not contain a significant amount of residual *Agrobacterium*. GUS-positive calli also showed the expression of nptII activity by dot blot analysis (data not shown), suggesting simultaneous insertion of both the genes in the host genome. The transformed roots and their calli cultured on SRM could not regenerate shoots even after prolonged (6–8 weeks) incubation. Standardization of tissue culture conditions for regeneration should permit the generation of transgenic mungbean plants using *A. rhizogenes*.

1. Vasil, I. K., *Nature Biotechnol.*, 1996, **14**, 702–703.
2. Dillen, W., DeClercq, J., Gossens, A., Van Montagu, M. and Angenon, G., *Theor. Appl. Genet.*, 1997, **94**, 151–158.
3. Petit, A., Berkaloff, A. and Tempe, J., *J. Mol. Genet.*, 1986, **202**, 388–393.
4. Chilton, M. D., Tepfer, D. A., Petit, A., Casse-Delbart, F. and Tempe, J., *Nature*, 1982, **295**, 432–434.
5. Tepfer, D., *Gene Transfer to Plants* (eds Potrykus, I. and Spangenberg, G.), Springer, Berlin, 1995, pp. 45–52.
6. Kumar, V. and Davey, M. R., *Euphytica*, 1991, **55**, 159–169.
7. Ramsay, G. and Kumar, A., *J. Exp. Bot.*, 1990, **41**, 841–847.
8. Jaiwal, P. K. and Gulati, A., *Euphytica*, 1995, **86**, 167–181.
9. Christou, P., *Field Crops Res.*, 1997, **53**, 83–87.
10. Holtorf, S., Apel, K. and Bohlmann, H., *Plant Mol. Biol.*, 1995, **29**, 637–646.
11. Potrykus, I. and Spangenberg, G. (eds), *Gene Transfer to Plants*, Springer, Berlin, 1995, pp. 45–52.
12. Scheid, O. M. and Neuhaus-Url, G., in *Methods in Molecular Biology: Agrobacterium Protocols* (eds Gartland, K. M. A. and Davey, M. R.), Humana Press Inc., Totova, NJ, 1994, pp. 201–206.
13. Jefferson, R. A., *Plant Mol. Biol. Rep.*, 1987, **5**, 387–405.

ACKNOWLEDGEMENT. P.K.J. thanks DBT, New Delhi for financial assistance.

Received 3 November 1997; revised accepted 30 April 1998

Variants of *Trichophyton rubrum*

S. Das and B. K. Choudhury

Botany Research Laboratory, P.G. Department of Botany, Government College, Rourkela 769 004, India

Variants of *Trichophyton rubrum* prevalent at Rourkela were identified following the study of their macromorphology and micromorphology and by special tests like urease and hair perforation. Four distinct variants were isolated and identified. One of the variants was completely new.

TRICHOPHYTON RUBRUM is an anthropophilic fungus and infectious only to man[1]. It is one of the most common and widely distributed cutaneous human pathogens[2]. *T. rubrum* was first described by Castellani[3] who had isolated the organism from a case of *Tinea cruiris* and named it *Epidermophyton rubrum*. Later in the same year, Bang[4] described a fungus and named it *T. purpureum*. Sabouraud[5] studying the fungi of Castellani and Bang, concluded that they were identical and their correct nomenclature is *T. rubrum*.

Before the Second World War, the fungus was endemic to far-east[6]. Since then, it has spread across Asia to Europe, East and South America and Australia. It occurs at Rourkela, an industrially important town of Orissa, India. The warm and humid climate of this part of the world and due to general deterioration of hygiene caused by widespread migration and concentration of population after the establishment of the steel plant in the 1950s, the rate of incidence of the mycotic desease has increased rapidly[7].

T. rubrum is distinguished from other red-pigmented dermatophytes by its usually white fluffy or fuzzy surface and dark red non-diffusing pigment under the thallus. The microscopic morphology of its fluffy mycelia consists of long strands of hyphae with small lateral, characteristically tear-shaped or peg-shaped microaleuriospores. Macroaleuriospores are absent or rarely present[8]. However, due to the occurrence of pleomorphism, it is difficult to define the species[9–12]. In the present study,