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Histology and chimeral segregation reveal cell-specific differences in the competence for shoot regeneration and *Agrobacterium*-mediated transformation in *Kohleria* internode explants

Thomas Geier [1] **and Rajbir S. Sangwan** [2]

[1] Fachgebiet Botanik, Forschungsanstalt Geisenheim, Von-Lade-Str. 1, D-65366 Geisenheim, Germany
[2] Laboratoire Androgenèse et Biotechnologie, Faculté des Sciences, Université de Picardie Jules Verne, 33 rue Saint-Leu, F-80039 Amiens Cédex, France

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Summary. Internode explants of *Kohleria* sp. (*Gesneriaceae*) are capable of regenerating large numbers of adventitious shoots. Regeneration of green shoots from explants of an albino periclinal chimera with genetically green L1, as well as microsurgical removal of the epidermis revealed that shoots originate only from the epidermis. Histological studies further showed that shoots arise from a particular epidermal cell type, *viz* the basal cell of young glandular trichomes. On the other hand, cells competent for *Agrobacterium*-mediated transformation are mainly located in vascular tissues, as could be shown by histochemical localization of ß-glucuronidase (GUS) expression in explants that had been inoculated with *A. tumefaciens* strains carrying binary plasmids with GUS and kanamycin resistance (NPTII) genes. Only 3% of GUS expression events took place in the epidermis. Consequently, shoot regeneration in the presence of kanamycin was very poor. Moreover, most of those shoots proved GUS-negative and did not survive subcultivation on kanamycin-containing medium. Six regenerants, however, were most probably transgenic, as suggested by the ability to produce adventitious shoots in the presence of kanamycin and by polymerase chain reaction (PCR) analysis. To our knowledge, this is the first positive result towards genetic transformation in a taxon of the *Gesneriaceae*.

Abbreviations: BA: N^6-benzyladenine; ct: cefotaxime; GUS: ß-glucuronidase; IAA: indole-3-acetic acid; km: kanamycin; NPTII: neomycin phosphotransferase II; PCR: polymerase chain reaction.

Introduction

Gene transfer to plants has been achieved through various approaches (for a review see Potrykus, 1991), *Agrobacterium*-mediated transfer being the most widely and successfully applied. Most of the currently used transformation protocols are modified from the "leaf disk technique" (Horsch *et al.* 1985) which consists of co-cultivation of complex explants with *Agrobacterium* and subsequent plant regeneration under selective conditions. To obtain transgenic plants through this technique requires that explants contain at least some cells that share competence for regeneration and transformation. Competence for transformation may be absent or low in recalcitrant materials, but can sometimes be enhanced by pre-cultivation in the presence of growth regulators (e.g. Sangwan *et al.* 1991a,b, 1992; Jacq *et al.* 1993; Hamza and Chupeau 1993). Further, transformation can be improved by selecting the appropriate explant type and duration of co-cultivation with *Agrobacterium* (e.g. Pawlicki *et al.* 1992). Recalcitrance to transgenic plant production may also result from differential competence of cells for transfomation and regeneration, as has been described in regeneration systems of *Linum* (Jordan and McHughen, 1988), *Vitis* (Colby *et al.* 1991) and *Dendranthema* (Lowe *et al.* 1993); in these examples, shoots regenerated only from cells not competent for transformation. Another undesirable effect of specific regeneration patterns may be the production of chimeras, when regenerants are of multicellular origin. Chimeras have been observed e.g. after *Agrobacterium*-mediated transformation in tobacco (Schmülling and Schell, 1993) and biolistic transformation in soybean (Christou, 1990).

In a previuos paper, a system of highly efficient direct adventitious shoot regeneration from internode segments of *Kohleria* (*Gesneriaceae*) and its use in clonal propagation and production of induced mutants has been described (Geier 1988). Here, we detail the precise origin and mode of shoot regeneration in this system as revealed by histological methods, regeneration from chimeric explants and effect of microsurgical separation of tissues. Further, we report on the localization of cells competent for *Agrobacterium*-mediated transformation, using histochemical demonstration of ß-glucuronidase (GUS) reporter gene activty, and describe the regeneration of putatively transgenic shoots.

Materials and Methods

Plant Material and in vitro Culture. Internode segments (5-7mm long) were cut from in vitro shoot cultures of two interspecific hybrid idiotypes ('KII-6' and 'B-29') of *Kohleria* (Geier, 1988). In addition to explants of homohistic green (GGG) shoots of 'B-29', explants of an albino periclinal chimera (GWW) derived from 'B-29' through mutation were cultivated to

Correspondence to: T. Geier

trace the origin of adventitious shoots. For transformation experiments, explants of 'KII-6' were prepared in different manner (normal segments, longitudinally halfed segments, abraded segments) from 8 week-old shoots, while in 'B-29' (GGG only) normal segments were prepared from shoots of different age (8 and 28 weeks). Shoot cultures were maintained by cultivating node segments on Nitsch's (1969) basal medium (BM). For shoot regeneration from internode segments, BM supplemented with 2.5 µM indole-3-acetic acid (IAA) and 2.5 µM 6-benzyladenine (BA) was used (=SRM). For selection and propagation of transformants, BM and SRM media were supplemented with 50 mg/l kanamycin (km50) and 100 or 250 mg/l cefotaxime (ct100 or ct250). Cultures were kept under fluorescent light (ca. 40 µE/m²/s for 14 h/day) at 24°C.

Agrobacterium Inoculation. Internode segments were inoculated with two different strains of *Agrobacterium tumefaciens* carrying the binary plasmids pGS Gluc 1 (Gl) or p35S-GUS INT (GIn), both harbouring GUS and neomycin phophotransferase (NPTII) genes as detailed before (Sangwan *et al.* 1991a, 1992 , Vancanneyt *et al.* 1990). The non-oncogenic plasmid pGV 2260 (Deblaere *et al.* 1985), supplied by M. van Montagu (State University Gent, Belgium), was used as a control in the transformation experiments. All strains of *A. tumefaciens* were grown on Luria broth (LB) medium with appropriate antibiotics. Colonies of bacteria, picked from selection plates were grown overnight in 5 ml of LB liquid medium at 28°C on a rotary shaker at 200 rpm and were then diluted to a final density of A600 = 1.0 (Sangwan *et al.* 1991a, 1992). Explants freshly cut or pre-cultivated (2, 4 or 6 days) on SRM were incubated in the bacterial suspension for 5 min, blotted on sterile filter paper and were co-cultivated with agrobacteria on SRM for either two or three days at 27°C. Explants were then rinsed during 20 min in liqid BM + 750mg/l cefotaxime and subsequently transferred to SRM+km50+ct250 in petri dishes. After 8 weeks, regenerated shoots were isolated and transferred to BM+km50+ct100. Nodes of such shoots were subcultured to the same medium and later to BM or BM+km50.

Histological Techniques. Explants grown on SRM were fixed at weekly intervals in 5% acrolein in 0.025 M phosphate buffer pH 6.8 or in 4% glutaraldehyde plus 1.5% sucrose in 0.1 M cacodylate buffer pH 7, dehydrated and embedded in glycol methacrylate according to O'Brien and McCully (1988). Microtome sections were cut at 2 µm thickness and stained with toluidine blue. GUS expression in explants that had been inoculated with *Agrobacterium* was histochemically revealed as blue color development using X-Gluc as a substrate for ß-glucuronidase (Jefferson 1987). Explants stained for GUS activity were fixed either in formalin-acetic-alcohol (FAA) for macroscopic inspection only, or in glutaraldehyde for sectioning. Ten µm thick sections were cut and counterstained with safranine.

Analysis of Putatively Transformed Regenerants. In the course of subculturing through node segments, parts of putatively transformed and control plantlets were retained and subjected to histochemical GUS assays as described above. After 1 year of subculturing, entire plantlets (ca. 200 mg) of selected clones were extracted and GUS activity was measured fluorimetrically in terms of production of 4-methyl umbelliferone (MU), using 4-methyl umbelliferyl glucuronide (MUG) as substrate (Jefferson, 1987). Further, putative transformants and controls were tested for NPTII expression by examining the ability of internode segments to regenerate shoots on SRM+km50 or roots on BM+km50 supplemented with 10 µM IAA. Finally, for PCR analysis, total DNA was isolated from plantlets of cloned putative transformants and controls following Dellaporta *et al.* (1983). From each sample, 0.8µg of genomic DNA was used as template DNA for PCR. The presence of the transferred NPTII was demonstrated by using the standard PCR techniques as described by Sambrook *et al.* (1989) as well as Kwok and Higuchi (1989). The NPTII gene was amplified using the primers: 201-222: 5'-GAG GCT ATT CGG CTA TGA CTG-3'; 900-879 :5'-ATC GGG AGG GGC GAT ACC GTA-3' (Beck *et al.* 1982). Expected size of fragment was 700bp.

Results and Discussion

Origin and Mode of Adventitious Shoot Regeneration

After explantation onto SRM, internode segments showed visible swelling at the cut ends within one week. About three weeks after explantation, shoot initials became macroscopically discernible. Usually, shoot regeneration started from the swollen parts and then spread over the entire surface of the explants (Fig.1). Internode segments of the chimeric albino type 'B-29'-GWW regenerated almost exclusively homohistic green (GGG) shoots (Fig.2); among more than 3,000 shoots formed during cultivation on SRM, only one chimeric albino (GWW) regenerant was observed. Notably, this pattern of shoot regeneration did not change (no albino shoots were formed), when the growth regulator supplement of SRM was substantially altered (data not presented). After complete microsurgical removal of the epidermis, GWW explants stayed viable and occasionally produced callus, but never regenerated shoots. These findings demonstrate that in *Kohleria* internodes, adventitious shoots generally arise from the epidermal layer. As an extremely rare exception, cells of the subepidermal layer(s) may be integrated into the apex of developing adventitious shoots.

Histological investigations were carried out using homohistic green (GGG) explants of idiotypes 'KII-6' and 'B-29'. These studies confirmed the epidermal origin of adventitious shoots and revealed the importance of glandular trichomes of a certain developmental stage as starting points for regeneration. The responsive stage of trichomes was characterized by a four-celled glandular head, a short stalk cell and a large basal cell. Divisions of basal cells of such trichomes started within the first week of cultivation on SRM and led to groups of small cells with high cytoplasmic content and prominent nuclei (Fig.3). This response first became evident close to the cut surfaces and then spread over the entire explant, involving virtually all the trichomes of the respective stage. Notably, basal cells of older trichomes in which the stalk had elongated and become multicellular had lost the capability to respond in this way. Also, no other cell type within the explant gave rise to comparably small and highly cytoplasmic cells, except normal epidermal cells in the immediate neighbourhood of the cut surfaces of explants and cells of vascular parenchyma including the pericycle from which root primordia were formed. In explants fixed after three weeks of cultivation, shoot primordia in various stages of development were present. On top of younger primordia, the collapsed stalk and head of a glandular trichome was repeatedly observed, evidencing the origin of those primordia from basal cells of trichomes (Fig.4). Where remnants of trichomes could not be revealed, as was generally the case in older shoot initials (Fig.5), they may have fallen apart or may have been overlooked because of the difficulty to obtain a complete series of sections. Nevertheless, the results suggest that the basal cell of young glandular trichomes constitutes the preferential (if not exclusive) origin of adventitious shoots from *Kohleria* internode segments. Essentially similar observations on shoot regeneration from basal cells of trichomes have been made by Bigot (1970) in *Begonia* leaf segments. More recently, Ohki (1994) reported on the role of basal cells of glandular hairs in determining the origin of adventitious shoots in *Saintpaulia* leaf explants.

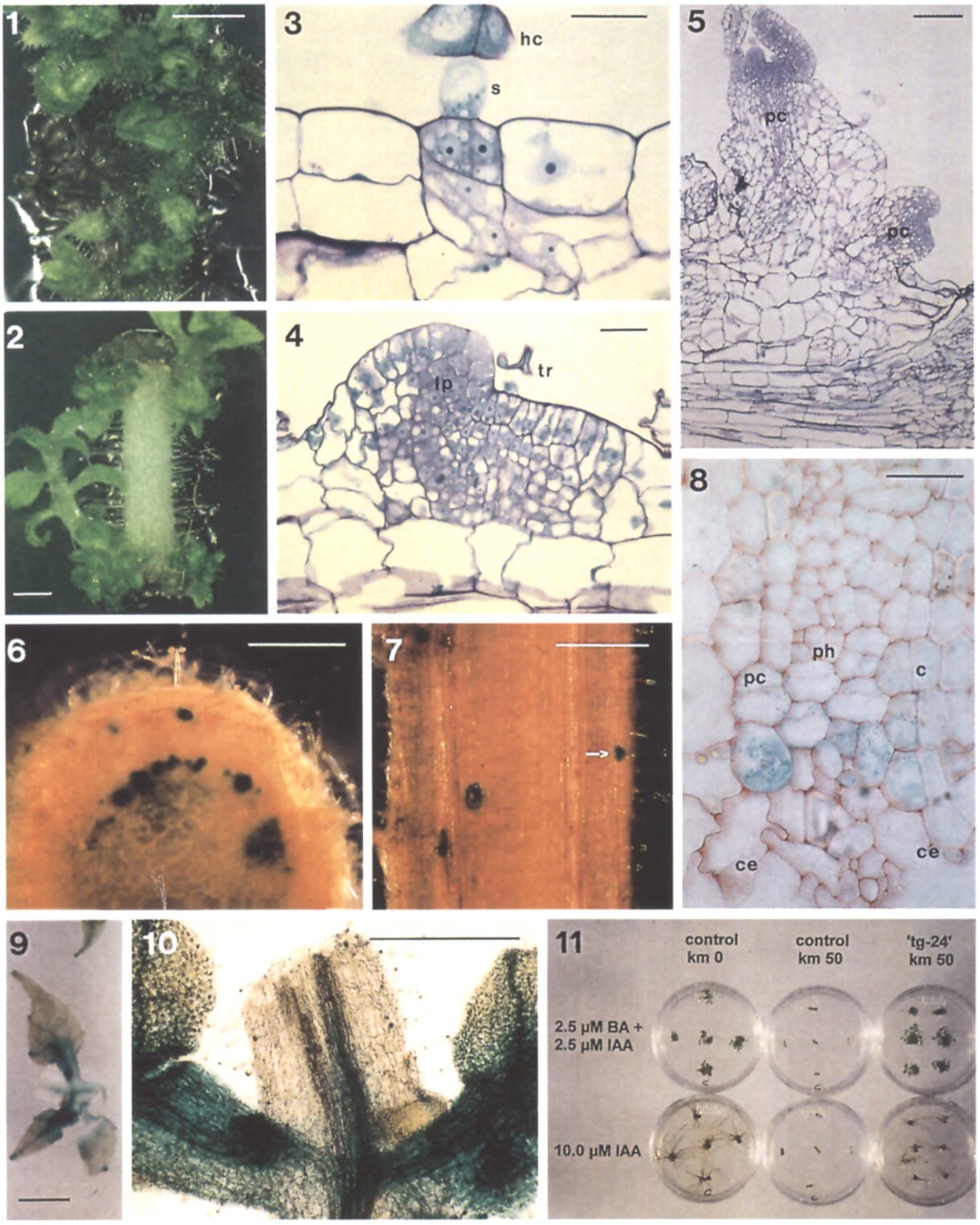

Fig.1. An internode explant of idiotype 'B-29', covered with adventitious shoots; bar = 1mm. **Fig.2** Green shoots regenerating from an albino periclinal chimeral form (GWW) of 'B-29'; bar = 1mm. **Figs.3-5.** Histology of adventitious shoot formation. Shoots are initiated from the basal cell of glandular trichomes, early divisions giving rise to small, highly cytoplasmic cells (Fig.3); hc = head cells, s = unicellular stalk of trichome; bar = 25µm. More advanced shoot initial still bearing the collapsed head and stalk of the trichome (tr) from whose basal cell it was derived (Fig.4); lp = leaf primordium; bar = 25µm. At later stages of development, procambial tissue (pc) is formed (Fig.5); bar = 100µm. **Figs.6-8.** GUS expression in explants is found in cells close to the transversal (Fig.6) and longitudinal (Fig. 7) cut surfaces and is mostly confined to vascular tissues. Fig.7 shows a rare event in the epidermis (arrow; the explant is oriented slightly oblique, thus creating the impression of a subepidermal event); bars = 500µm. Microtome sections (Fig.8) revealed GUS expression in cambium (c), phloem (ph) and pericycle (pc); bar = 25µm. **Figs.9, 10.** Part of a GUS-positive regenerant (tg-22) exhibiting tissue-specific GUS activity (Fig.9); bar = 1mm. Higher magnification (Fig.10) discloses high expression in vascular tissues and head cells of glandular trichomes; bar = 500µm. **Fig.11.** Test for kanamycin resistance. While adventitious organogenesis from 'B-29' control explants is completely inhibited by kanamycin (compare left and central column), clone 'tg-24' regenerated shoots and roots in the presence of 50mg/l kanamycin and respective growth regulator supplements (right column).

Histochemical Localization of GUS Expression in Explants

Histochemical localization of GUS activity was performed 5, 7 and 10 days after inoculation with *Agrobacterium* in explants of idiotype 'KII-6'. Macroscopical inspection showed that after 5 days, blue spots indicative of GUS activity were present in 8 out of 16 explants examined. The number of spots increased by the time, and after 10 days, spots were found in 13 out of 16 explants. In total 260-270 spots were detected in the 48 explants examined, most of the spots (84%) being located in vascular tissues, 7% in pith, and only 3% each in adventitious roots, cortex and epidermis (Figs.6,7). Of the various parameters tested (type of explant preparation, duration of pre- and co-cultivation, *Agrobacterium* strain), only the bacterial strain had a significant effect on mean number of spots per explant, G1 being more effective than GIn. More precise localization of GUS-positive cells at the microscopic level was impeded by low concentraton of the reaction product. Even in 10 µm thick sections no staining was visible, except in vascular tissues (Fig.8). Thus, in the rare cases of GUS staining in the epidermis, neither macroscopic, nor microscopic inspection allowed to establish whether or not staining had occurred in basal cells of glandular trichomes.

Regeneration of Shoots on Selective Medium

After inoculation with *Agrobacterium* and subsequent 6 weeks of cultivation on selective medium (SRM+ km50+ct250), none of 189 explants of idiotype 'KII-6' had regenerated shoots. In one explant small green spots, reminiscent of shoot initials were observed, but did not develop further and finally died. Thus, while GUS assays had revealed numerous events of transgene expression in explants of 'KII-6', no transgenic regenerants could be obtained from this idiotype. It appears that no or no sufficient number of cells competent for shoot regeneration had been transformed. In contrast, explants of idiotype 'B-29' were able to regenerate shoots on selective medium. After 6 weeks in culture, 10 out of a total of 60 explants had produced buds or shoots at a maximum of 10 per explant. Internode segments cut from young (8 weeks old) stock plants were clearly more responsive than those from aged (28 weeks old) stock plants. In the former, 9 out of 30 explants had produced shoots, while in the latter only one out of 30 explants showed regeneration. As opposed to pre-cultivation which had no effect on regeneration, duration of co-cultivation with *Agrobacterium* was of importance. Three days were superior to 2 days, each treatment yielding 8 and 2 regenerating explants, respectively. Also vectors were found to differ in their efficiency to trigger regeneration on selective medium. Out of 30 explants inoculated with G1, 8 showed regeneration, while after inoculation with GIn, only 2 out of 30 explants had produced shoots. This difference is in accordance with the results of GUS assays in explants of idiotype 'KII-6'.

A total of 38 adventitious shoots regenerated on selective medium from explants of idiotype 'B-29' were isolated, a basal node or leaf of each was taken and histochemically assayed for GUS expression, while the remaining part was subcultured as a whole or as nodal sections on BM+km50+ct100. Twenty-four shoots, all without GUS expression, died during the first subculture. Ten shoot clones survived three subcultures on BM+km50+ct100, 6 of them growing as vigorous as control shoots on BM. However, lateral shoot growth from node explants turned out to be less sensitive to kanamycin than was regeneration from internodes. Therefore, internode segments of the 6 mentioned clones were examined for their ability to regenerate adventitious organs on selective media. All 6 produced shoots on SRM+km50 as well as roots on BM+km50+10µM IAA (Table 1, Fig.11).

Table 1. Kanamycin resistance, in terms of shoot and root regeneration from internode segments, and GUS expression, of 'B29' control and putatively transformed clones examined one year after *Agrobacterium* inoculation

clone/ vector	kanamycin [mg/l]	mean no. of shoots[1]	mean no. of roots[2]	GUS expression [nmolMU/gFW/min]
control/-	0	24.5 ab	16.1 ab	0.6 c
control/-	50	0.0 e	0.0 c	-
tg-22/G1	50	26.3 a	8.7 b	10.5 c
tg-24/G1	50	19.8 bc	17.7 a	105.8 b
tg-26/GIn	50	19.4 cd	13.8 ab	1.4 c
tg-29/GIn	50	20.1 bc	13.7 ab	1.2 c
tg-37/GIn	50	20.3 bc	13.3 ab	916.2 a
tg-38/GIn	50	14.5 d	14.3 ab	0.1 c

[1] media supplemented with 2.5 µM BA and 2.5 µM IAA; [2] media supplemented with 10 µM IAA; means within the same column and followed by the same letter are not significantly different at p=0.05; LSDs (Duncan) at p=0.05 are 4.96 for shoots, 7.50 for roots, and 44.97 for GUS expression, respectively.

Initially, GUS expression had been revealed in all parts of positive samples, suggesting a non-chimeric nature of the regenerants, staining intensity, however, strongly varied in a tissue-specific pattern (Fig.9). Strongest staining was associated with vascular tissues and head cells of glandular trichomes (Fig.10). During successive subcultures, GUS expression substantially declined in most clones, visible staining often being restricted to few glandular trichomes and cells close to the cut surfaces of the samples. After one year of subculturing, significant GUS expression could be detected in only two of the clones (Table 1). PCR analysis using NPTII-specific primers was performed in 4 clones (Fig.12). All the tested plants (lanes 3-6) showed the predicted 700bp band, while this band was absent in the untransformed control plant (lane 1). We are aware of the possibility that a positive PCR signal may originate from residual agrobacteria. This possibility, however, cannot explain the high expression of the GUS-Intron construct in tg-37 (Table 1), all the more considering that no bacteria could be recovered, when liquid LB medium was inoculated with fragmented shoots of putative transformants. Taking into account all the available evidence, the results are strongly suggestive of stable transformation.

Since cells of vascular tissues are most easily transformed (Fig.6) and it is this region where roots are initiated, an

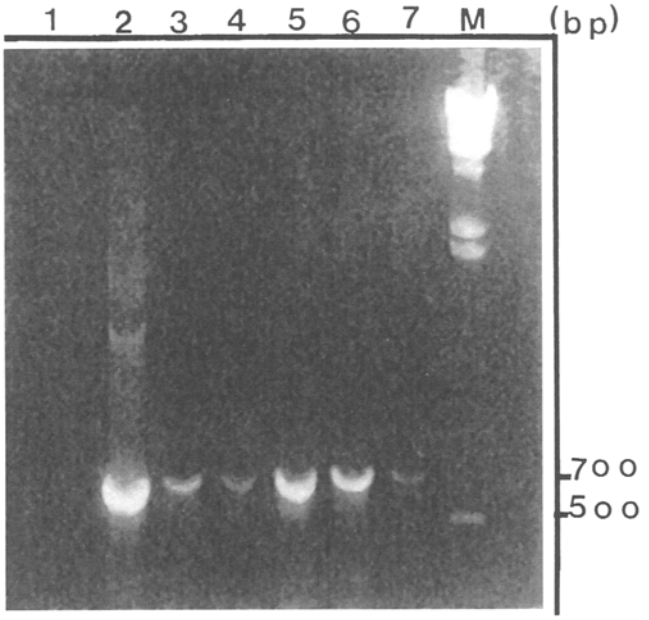

Fig.12. Agarose gel electrophoresis of PCR products using NPTII-specific primers. Lane 1, DNA from untransformed control plant. Lane 2, DNA from plasmid pGS Gluc 1. Lanes 3-6, DNA from independent putative transformants (tg-22, tg-24, tg-29 and tg-37, respectively). Lane 7, DNA from a transgenic tobacco plant transformed with pGS Gluc 1 vector. Lane M, lambda DNA digested with Hind III. Note the expected 700bp band was present in the putatively transformed lines (lanes 3-6) but not in the untransformed control plant (lane 1).

attempt was made to obtain transformed plants via root and subsequent shoot regeneration. However, no transformed roots could be obtained from explants co-cultivated with the same *Agrobacterium* strains as above and transferred to root induction media containing 10μM IAA or IBA. Correspondingly, examination of explants treated in this way revealed very little GUS expression (data not shown). A possible explanation could be that the growth regulator supplements employed for root induction strongly reduce transformation competence.

Conclusion

The present findings underline that a highly efficient regeneration system is not necessarily well suited for the routine production of transformed plants. In *Kohleria* internodes, shoot regeneration occurs at high frequency, but only from a particular epidermal cell type, *viz* the basal cell of young glandular trichomes, which proved hardly susceptible to *Agrobacterium* infection. On the other hand, cells highly competent for *Agrobacterium*-mediated transformation were found to be concentrated in the vascular tissues. Regeneration of non-transformed shoots under selective conditions is an undesirable feature of systems using kanamycin resistance as selectable marker. Due to its highly cell-specific and invariable origin of adventitious shoots, the present system seems particularly suited for studying the extent of cross-protection of non-transformed cells under selective culture conditions.

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