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Localization of Persisting Agrobacteria in Transgenic Tobacco Plants

Anja Matzk[1], Sinclair Mantell[2], and Joachim Schiemann[1]

[1]Institute for Biochemistry and Plant Virology, Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11/12, 38104 Braunschweig, Germany; [2]Unit for Advanced Propagation Systems, Horticulture Section, Wye College, University of London, Wye, Ashford, Kent, TN25 5AH, UK

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The persistence of *Agrobacterium tumefaciens* cells of strain C58C1 in transformed tobacco plants (*Nicotiana tabacum*) was assessed over a period of 12 months post-transformation in vitro, and 3 to 6 months thereafter ex vitro. Three main approaches, i.e., enrichment culture followed by identification methods, tissue-print immunoblotting, and scanning electron microscopy, were combined to investigate the persistence of the agrobacteria used for transformation. Agrobacteria cells were present on the surface and within the tissues of in vitro shoot cultures at 12 months post-transformation. Moreover, persisting agrobacteria were detectable in these transgenic tobacco plants grown ex vitro in soil for at least 3 to 6 months. Results of both tissue-print immunoblotting and enrichment culture experiments showed that agrobacteria were unevenly distributed in weaned transgenic tobacco, with the majority of agrobacteria cells being located at the stem base and in the roots of the plants. There was no evidence of agrobacteria being present in either flowers or mature seeds. The implications of these results are discussed in the context of the potential risks of inadvertent introduction of persisting transgenic agrobacteria into the environment during the release of transgenic plants.

Agrobacterium tumefaciens is a soilborne, gram-negative bacterium belonging to the family of *Rhizobiaceae*. The cells are normally rod-shaped (0.6 to 1.0 by 1.5 to 3.0 μm), nonsporing, occur singly or in pairs, and are motile by one to six peritrichous flagella (Krieg and Holt 1984). Agrobacteria are found almost worldwide in soils, and especially in the rhizosphere of plants.

A. tumefaciens is known as a plant pathogen capable of inducing the plant disease crown gall, causing unregulated cell divisions and tumor formation on the host plants (Clare 1994). The crown gall disease has an impact mainly on stone fruits, ornamentals, and grapevine. In grapevine (*Vitis vinifera*), agrobacteria of biovar 3 proliferate, colonize the vascular system, and spread systemically (Lehoczky 1968, 1971). It is assumed that agrobacteria can move through the cutinfree cortex tissue, the rhizodermis, and the vascular system by "swimming" through intercellular spaces (Stellmach 1990). There are indications that *A. tumefaciens* prefers the rind tissue of the grapevine plant as a main living space (Jäger et al. 1990). Such latent infections with agrobacteria do not cause symptoms but show that *A. tumefaciens* cells are able to live in planta and furthermore have the ability to grow and multiply within various tissues of their host plants.

Corresponding author: Anja Matzk

The crown gall disease is caused by the transfer and integration of T-DNA from the bacterial tumor-inducing (Ti) plasmid to the plant nuclear genome (Hooykaas 1989). This *Agrobacterium* transfection mechanism is routinely used for transformation of higher plants. Several methods for transformation of dicotyledonous and recently of monocotyledonous species employing *A. tumefaciens* have been established over the last decade (Hooykaas and Schilperoort 1992; Hiei et al. 1994). The genes causing tumors have been replaced by several foreign genes of interest. After co-cultivation of agrobacteria with plant tissue, regeneration takes place on a medium containing antibiotics intended to eliminate the agrobacteria. However, the complete elimination of agrobacteria from transgenic tissues was difficult in many cases (e.g., van der Hoeven et al. 1991; Mogilner et al. 1993; Landsmann et al. 1995), possibly because the antibiotics used are bacteriostatic rather than bacteriocidal and/or agrobacteria are capable of forming covert relationships with plants as observed with *Enterobacteriaceae* in a number of plant tissue culture systems (reviewed recently by Holland and Polacco 1994). Critical reviews (e.g., Potrykus 1990) have suggested that there is a possibility of gene expression by *Agrobacterium* cells still attached to inoculated tissues and to the plantlets regenerated from them, which may cause "false positive" results after transformation.

The foreign genes in genetically modified agrobacteria persisting in transgenic plants could be transferred into other bacteria in the environment, particularly in the rhizosphere depending on the host range of the plasmids used for transformation. Plasmids can be exchanged between cells of different species or even different genera (Bradbury 1986). There are also possible routes for the transfer of agrobacteria from plant to plant, e.g., by phloem-feeding insects such as whiteflies (Zeidan and Czosnek 1994), and by physical spread over vegetative tissues and through soil (Mogilner et al. 1993). This could cause an undesirable spread of transgenes in the ecosystem. Therefore, it would be judicious to eliminate agrobacteria before releasing transgenic plants into the field. The possibility of transmitting genetically engineered agrobacteria into seed progeny, as well as vegetative propagules such as

tubers, also needs to be evaluated adequately. The detection of agrobacteria requires sensitive and reliable methods. Here we describe the detection of persisting *Agrobacterium tumefaciens* in transgenic tobacco plants by means of an applied enrichment culture method, and a tissue-print immunoblotting (TPIB) technique in association with scanning electron microscopy (SEM). Over a period of 18 months post-transformation, shoots were regenerated and multiplied in vitro under antibiotic selection conditions, followed by weaning and transfer into soil.

RESULTS

Sensitivity of enrichment cultures.

Enrichment cultures, inoculated with a defined number of agrobacteria in a background of plant material or without, showed that there was no inhibition of the growth of agrobacteria caused by the plant material. Even cultures inoculated with a single agrobacterium cell and incubated in a background with 0.1 g of tobacco tissue could be enriched to the late exponential phase after 3 days. When no agrobacteria were inoculated the OD_{600} did not increase.

Because of its sensitivity, this method was used as a routine detection method and for the isolation of agrobacteria in combination with localization studies.

Localization of agrobacteria in in vitro shoot cultures by SEM at 2 months after transformation.

Leaf, stem, and root tissues excised from in vitro plants were prepared for SEM observation and, in parallel, materials were used for enrichment culture. From all transgenic tobacco tissues bacteria were isolated by enrichment. All bacteria were identified as agrobacteria by means of the slide agglutination test, plating on selective media, and the ketolactose test. No bacteria were isolated from untransformed tobacco plants.

Examination of the leaf and stem samples by SEM revealed bacteria (1 by 0.3 μm rods) in all of the 5 transgenic tobacco plants tested. The external surfaces were always found to be colonized by bacteria but their distribution was not uniform (Fig. 1Aa–c). The agrobacteria mainly appeared in colonies, often associated with an extracellular matrix. Within tissues exposed by freeze-fracturing, agrobacteria were rarely observed. A few bacteria were seen as discrete microcolonies. They could be found in the epidermal layer (Fig. 1Ba–c), the intercellular spaces beneath the epidermis (Fig. 1Ca–c), and within the vascular tissue (Fig. 1Da–c). Freeze-fractured root material was also examined. The presence of dried agar around roots that remained from the in vitro culture stage made examinations of the external surfaces of these roots impossible. No bacteria were detected on the internal surfaces of the tested root specimens of transgenic tobacco plants. The same number of samples from untransformed tobacco plants was examined for agrobacteria cells by SEM but no agrobacteria-like structures were found in all of the plants tested.

Localization of agrobacteria in in vitro shoot cultures by TPIB and SEM at 12 months after transformation.

After cultivating the transgenic tobacco plants under in vitro conditions and on antibiotic containing media for 12 months, bacteria could be isolated by enrichment culture in approximately 35% of all tested plants, and these bacteria were identified as the agrobacteria used for transformation by the three methods previously described.

In cases in which agrobacteria had been isolated by enrichment culture the distribution of the bacteria in planta was determined by means of TPIB. The various transgenic tobacco plants tested revealed different numbers of agrobacteria as shown by immuno-dot-blot (Fig. 2A). Plants were found in which agrobacteria were distributed uniformly throughout the plant tissue (Fig. 2B,C). The external surfaces often showed higher signals due to a higher density of agrobacteria. In some plants weaker signals occurred, and the distribution was not even (Fig. 2D). Agrobacteria could always be localized in stem sections and often in leaf sections. No root material of the in vitro tobacco plants was tested by TPIB. Stem and leaf tissue from untransformed tobacco showed no reaction. To prove that there was no carry-over of agrobacteria detected by TPIB from outer to inner parts of tissues, a razor blade was used to cut tissue of a highly positive plant, and then of an untransformed plant. Weak or no positive TPIB reaction was obtained in tissues of the latter (data not shown).

Samples of different tissues of in vitro transgenic tobacco plants were used for enrichment culture and fixed in parallel for SEM observation. Samples of five transgenic plants from which agrobacteria were isolated and identified were then examined by SEM. Their external surfaces were less densely colonized by bacteria than at 2 months post-transformation, and the bacteria were mostly present in the bases of indentations of the leaf and stem surfaces (Fig. 3Aa–c, Ba–c). Freeze-fractured specimens revealed a few bacteria arranged in discrete microcolonies, and several rod-shaped bacteria were found within an epidermal (Fig. 3Ca–c) and a mesophyll cell (Fig. 3Da–c). No bacteria could be isolated by enrichment from the untransformed tobacco plants tested, and no agrobacteria-like structures were found by SEM observation.

Localization of agrobacteria in ex vitro plants by TPIB at 15 to 18 months after transformation.

After 12 months of in vitro cultivation, plants were weaned and transferred into soil. Three to 6 months thereafter, flowering transgenic tobacco plants were tested for the presence and distribution of agrobacteria by enrichment culture. No agrobacteria could be isolated from seeds, flowers, and young or old leaves. However, agrobacteria were concentrated in the roots and stem bases of 16 plants tested (Fig. 4). Identification of the isolated bacteria was carried out as previously described. In the case of untransformed tobacco no agrobacteria could be isolated after enrichment in selective bacterial growth media.

Plants containing persisting agrobacteria in vitro were monitored for agrobacteria after planting in soil. After 2 weeks under greenhouse conditions agrobacteria could be enriched from all tested leaves and roots (100%) but after 4 to 6 weeks in soil there was already an uneven distribution of agrobacteria within the transgenic plants, i.e., agrobacteria were always isolated from roots but only in 20% of leaves (data not shown).

Plants known to contain persisting agrobacteria were used for TPIB experiments. There was a high concentration of agrobacteria in the stem base of the transgenic plants (Fig. 5), but no signals in the upper and middle part of the stem. Bacteria were simultaneously isolated from the stem base by enrichment and again identified as the agrobacteria used for

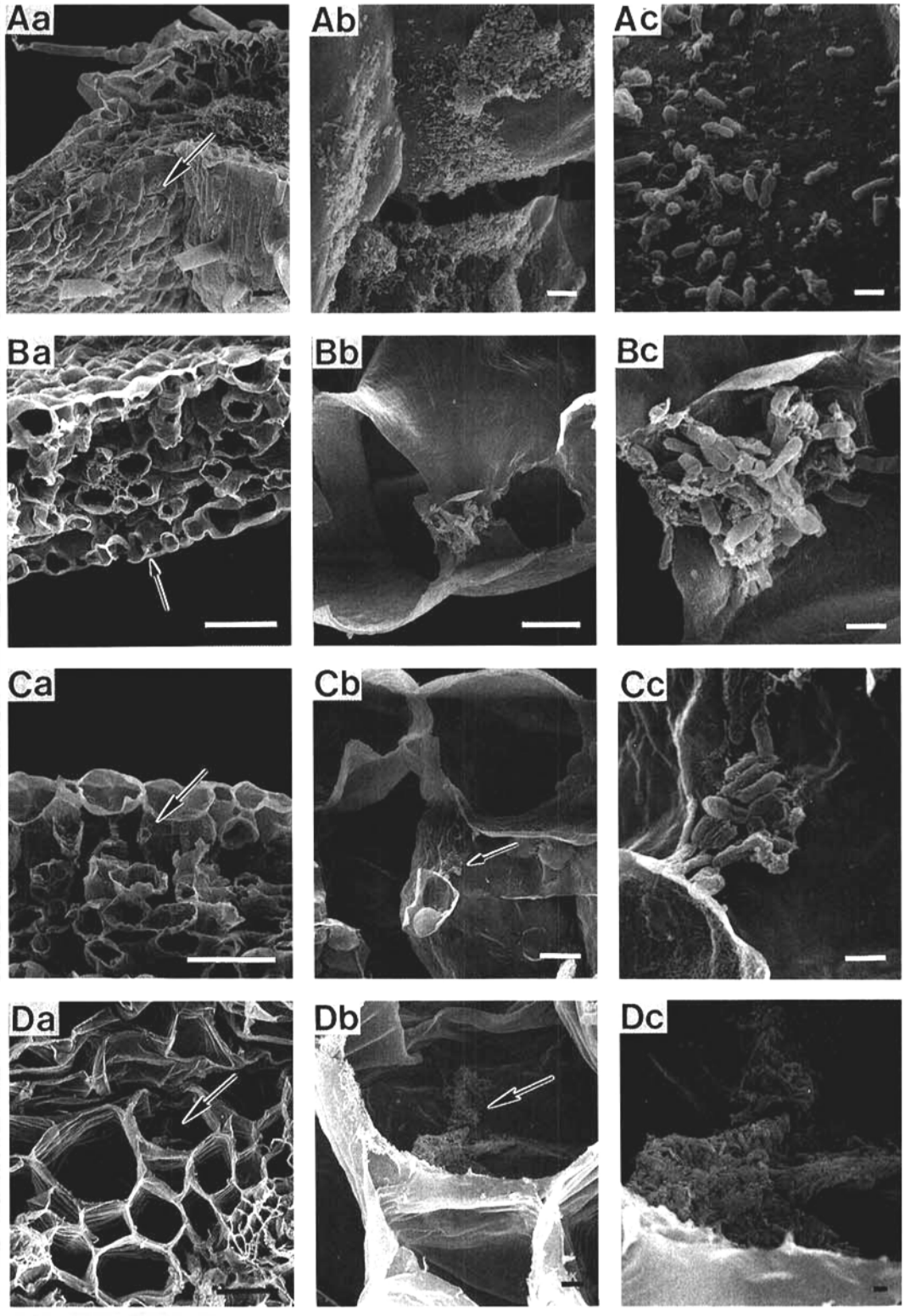


Fig. 1. Scanning electron micrographs of leaf specimens of transgenic tobacco viewed at 2 months after transformation. Rod-shaped bacteria present on the external surface (**A**); in the epidermal layer (**B**); in an intercellular space in outer layers beneath epidermis (**C**); and in xylem embedded in a reticulated matrix (**D**). Each line (**A–D**) represents the same detail in different magnifications (a–c). Arrows in low (a) magnification micrographs indicate the areas enlarged in the medium (b) and high (c) magnification micrographs. Bar = 50 μm (a), 5 μm (b), 1 μm (c).

transformation by the three methods described. In the case of untransformed tobacco plants grown under the same conditions no positive signals could be obtained by TPIB, and no agrobacteria could be isolated following enrichment culture. In addition, randomly taken samples of isolated agrobacteria were confirmed to contain the construct used for transformation by dot-blot cell hybridization by means of a specific probe for the *pat* gene located on the T-DNA of the binary vector. To be sure that the positive signals of the dot blot hybridization showed the construct of the genetically modified agrobacteria, all positive samples were assured by polymerase chain reaction (PCR) with one primer in the CaMV 35S promoter region and the other primer in the coding region of the *pat* gene. The resulting 734-bp fragment proved that we reisolated the agrobacteria used for transformation.

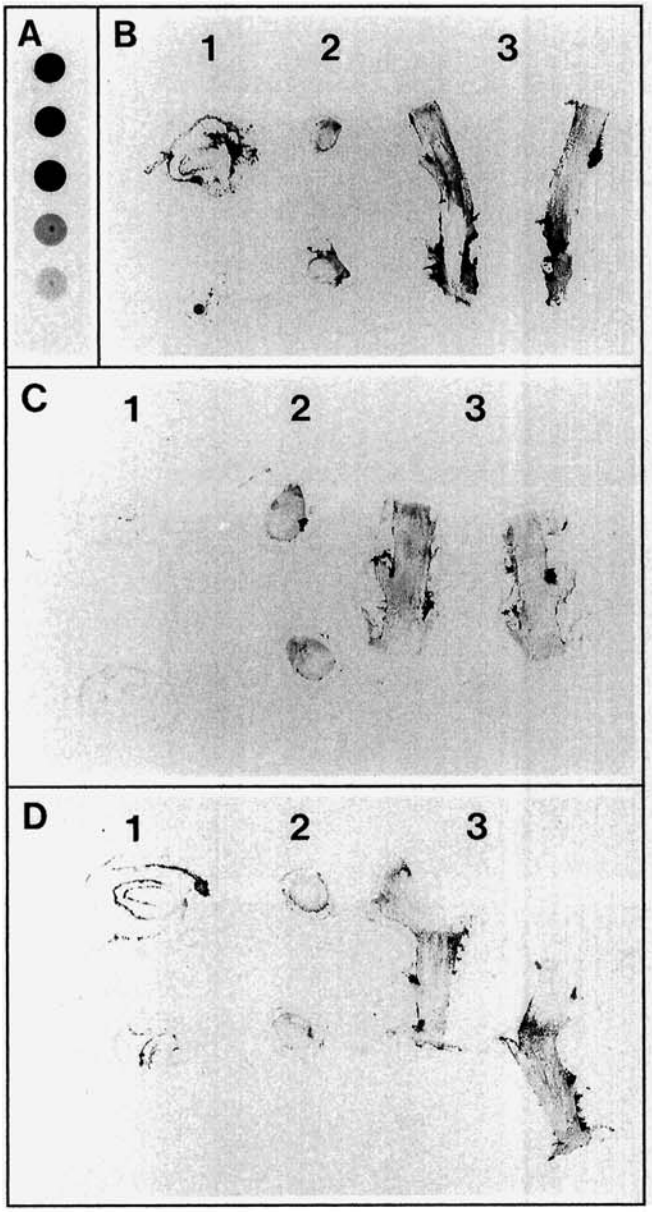


Fig. 2. Tissue-print immunoblots of transgenic tobacco plants cultivated for 12 months in vitro. **A,** Immuno-dot-blot of decreasing numbers of *A. tumefaciens* C58(pEHA101)(pOCA18/Ac) cells. Numbers of microorganisms (MO) used per dot (from top to bottom): 1×10^6; 5×10^5; 1×10^5; 5×10^4; 1×10^4; 1×10^3 MO. **B–D,** Tissue-print immunoblots of different transgenic tobacco plants (1 = cross sections of leaves, 2 = cross sections of stem, 3 = longitudinal sections of stem). The tissues in **B–D** have been outlined in pencil. **A** × 0.8; **B–D** × 1.2.

DISCUSSION

Our results confirm that *Agrobacterium* cells containing the binary vector pOCA18/Ac persisted for at least 18 months post-transformation in both transgenic in vitro and ex vitro tobacco plants.

The data obtained proved unequivocally that *Agrobacterium* cells survived the standard antibiotic treatments of cefotaxime and carbenicillin applied alternately in the tissue culture medium for 12 months at 300 mg per liter. Cells of the disarmed strain not only survived but multiplied on and within their transgenic hosts and spread systemically in the in vitro–cultivated tobacco plants. Such findings are in agreement with those of van der Hoeven et al. (1991) who also found persistence of engineered *A. tumefaciens* in transformed plants for a few months post-transformation. Our observations of in vitro plants indicate that *Agrobacterium* strains carrying binary vectors widely used for transfection are able to persist despite the repeated antibiotic treatments used in standard laboratory protocols.

During the current investigations we employed different sensitive detection strategies. Selective enrichment of *Agrobacterium* from macerated plant material is highly sensitive and appropriate for routine testing of large numbers of samples. Cultivation-based methods take advantage of selectable resistance markers. The chromosomally encoded rifampicin resistance turned out to be extremely useful for the selection of persisting agrobacteria from plants grown in soil after a selective enrichment step due to a low bacterial background with a rifampicin-resistant phenotype. Bacteria obtained after selective cultivation can be identified as the agrobacteria used for transformation by standard microbiological techniques and immunological methods. In addition, we used the dot-blot hybridization combined with a PCR technique for the identification of bacteria that were isolated after enrichment of samples from ex vitro–grown plants. The probe used for dot-blot screening was that of the *pat* gene localized on the T-DNA of the agrobacteria used for transformation. To exclude positive results caused by nonspecific hybridization signals we screened all dot-blot positive strains by PCR. One primer was located in the promoter region (CaMV 35S) and the other in the coding sequence of the *pat* gene. Positive PCR results therefore showed the construct of the genetically engineered agrobacteria had been detected. Under in vitro conditions there were never any bacteria isolated by enrichment culture from untransformed tobacco plants, and all the enriched bacteria from transgenic tobacco were identified as agrobacteria showing the triple combination of the expected antibiotic resistance. Because it was considered unlikely that bacteria other than the agrobacteria used for transformation were present in the in vitro tobacco plants, no additional molecular identification methods were included in the current studies. As an alternative method for detecting nonculturable agrobacteria PCR-based analyses are under investigation (data not shown).

SEM studies revealed agrobacteria on the surface and in both intercellular and intracellular positions in the epidermis

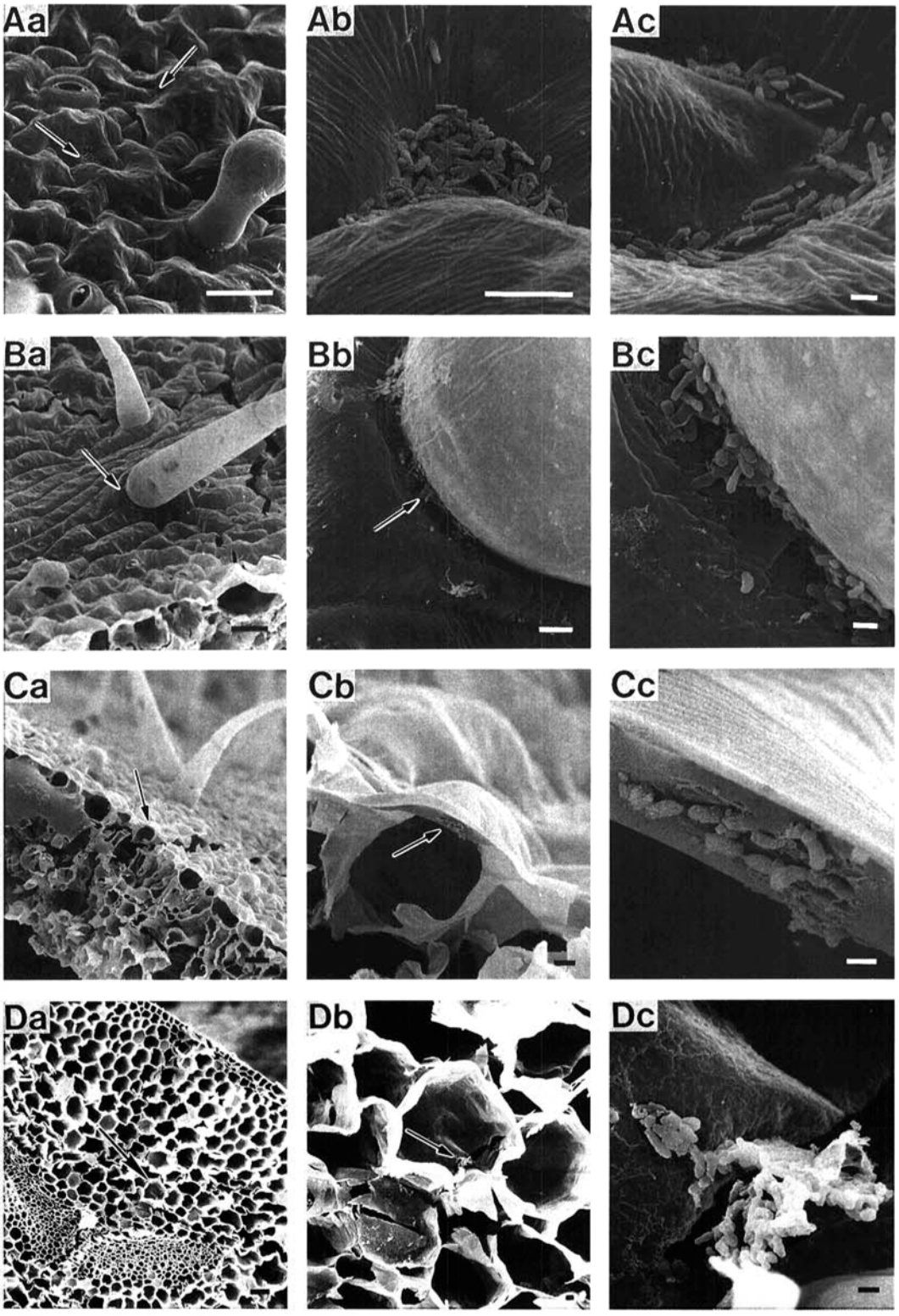
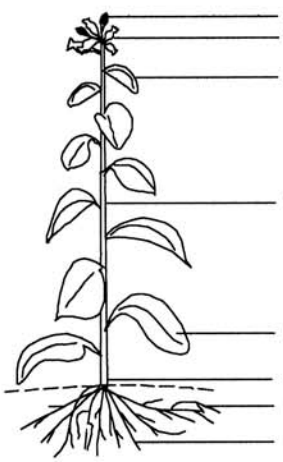


Fig. 3. Scanning electron micrographs of leaf and petiole specimens of transgenic tobacco viewed at 12 months after transformation. Rod-shaped bacteria present on the external surfaces (**A, B**); in the epidermal layer (**C**); and within cortical tissue (**D**). Each line (**A–D**) represents the same detail in different magnifications (a–c). Arrows in low (a) magnification micrographs indicate the areas enlarged in the medium (b) and high (c) magnification micrographs. Bar = 50 μm (a), 5 μm (b), 1 μm (c).

and vascular tissues of leaves and stems of tobacco plants at 2 and 12 months post-transformation. From our SEM observations we cannot say whether *Agrobacterium* invaded dead or living plant cells. Smith et al. (1912) suggested that *Agrobacterium* may have the potential to be an intracellular plant parasite. Escudero et al. (1995) showed that *Agrobacterium* can function as an intracellular infectious agent in plants. We shall be investigating this possibility at the cellular level by immuno-fluorescence and transmission electron microscopy, which will allow an unequivocal identification of agrobacteria and of the state of the invaded plant tissue. The low number of agrobacteria observed by SEM compared with the TPIB studies may be due to the possibility that many agrobacteria may be washed off during preparation of tissue for SEM. Graves et al. (1988) have shown that agrobacteria not firmly attached to the plant cells were removed by stringent washing.

We have adapted the TPIB method, previously used to localize viruses (Kaufmann et al. 1992; Lin et al. 1990), to study the persistence of agrobacteria. An advantage of the TPIB method was that comparatively low numbers of agrobacteria could be detected and their specific locations on plants determined. However, TPIB is not an empirical method: for example, differences in the intensity of signals may be due to differences in the pressure applied to a tissue. Nevertheless, it is a method of choice for localization studies not depending on technical equipment, such as in situ localization methods based on electron microscopy or laser microscopy (Assmus et al. 1995). It is significant that *Agrobacterium* tends to be present throughout in vitro plants while it is only found at the bases of ex vitro plants, i.e., close to the soil level and the rhizosphere where it usually occurs in nature. The uniform distribution of agrobacteria in tissue culture plants is probably due to the fact that in vitro–cultured plants have high moisture contents that are an ideal environment for the survival and growth of plant-associated bacteria. In the ex vitro situation after weaning of plants, the moisture content normalizes. In tobacco plants that had been weaned for 4 weeks agrobacteria were detected in more basal positions on the plants and were more prevalent on roots than on leaf tissues. These observations are in agreement with those of Stellmach (1990), who also found a high mobility of agrobacteria in growing grapevine. It is also of interest and of some significance that the persistence of agrobacteria did not extend to the flowers and seeds (Fig. 4).



tested part of the plant	number of tested samples	% of samples containing agrobacteria
seeds	29	0
flowers	78	0
young leaves	26	0
upper part of the stem	45	0
old leaves	29	0
stem base	13	31
root hairs	27	96
main roots	19	89

Fig. 4. Distribution of *A. tumefaciens* in transgenic tobacco plants grown for 3 to 6 months in soil. Agrobacteria were isolated by enrichment culture and identified as the agrobacteria used for transformation.

The accumulation of persisting agrobacteria in the basal parts of tobacco plants would indicate that there is a low probability of agrobacteria contaminating the progeny of seed-propagated plants like tobacco. On the contrary, in the case of vegetatively propagated plants such as potato and other tuber crops, the current findings emphasize that agrobacteria are likely to accumulate in those parts of the plant commonly used for vegetative propagation. The potential for the inadvertent introduction into the environment of laboratory strains of agrobacteria during the process of transgenic plant release is higher for vegetatively propagated than for seed-propagated plants. However, it should be stressed that the aforementioned potential risks are dependent upon the strain of agrobacteria used for transformation, and especially upon the nature of the plasmid constructs employed and the genes transferred.

In conclusion, the fact that agrobacteria can survive and persist in transgenic plants for at least 1 year, despite the continual presence of the antibiotics cefotoxime and carbenicillin, emphasizes the need for constant vigilance in monitoring transgenic plants, particularly of vegetatively propagated crop species, prior to their release into the field.

MATERIAL AND METHODS

Agrobacterium strain.

A. tumefaciens C58C1 containing the T-DNA-free Ti plasmid pEHA101 (Hood et al. 1986) and the binary vector pOCA18/Ac was obtained from P. Eckes (AgrEvo, Frankfurt/Main, Germany). pOCA18/Ac consists of pOCA18 (Olszewski et al. 1988) in which the synthetic phosphinothricin-N-acetyl transferase gene (*pat*) driven by CaMV 35S control sequences was integrated (Donn and Eckes 1992). This strain contains chromosomal rifampicin resistance, the resident plasmid harbors a kanamycin resistance gene (*npt*II), and the binary vector can be selected by means of tetracycline.

Plant transformation.

Tobacco leaves (*Nicotiana tabacum* L. var. Gatersleben) were used for *Agrobacterium*-mediated leaf disk transformation according to Horsch et al. (1985). Transformants were selected on 50 mg of kanamycin per liter (Duchefa, Haarlem, The Netherlands). The antibiotics cefotaxime and carbenicillin (Duchefa) were used alternately at concentrations of 500 mg/liter (on regeneration media) and 300 mg/liter (for further cultivation) to inhibit growth of agrobacteria. Regenerants were subcultured every 4 to 6 weeks by means of shoot tip propagation. After 1 year of in vitro cultivation on antibiotics, 34 weaned plants were grown in soil in the greenhouse.

Antibodies.

Polyclonal antibodies against whole cells of *Agrobacterium tumefaciens* C58C1(pEHA101)(pOCA18/Ac) were raised in rabbits by means of a standard protocol (Niepold and Huber 1988). The antibodies reacted strongly with all C58C1 strains tested but weakly with *A. rhizogenes*, *A. vitis*, and *E. coli*. The

antibodies were conjugated with alkaline phosphatase as described by Clark and Adams (1977).

TPIB.

TPIB was performed according to Kaufmann et al. (1992). In vitro–cultivated leaf and stem tissues of tobacco were cut and the freshly exposed surfaces pressed firmly onto a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). The membrane was incubated in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 2 h to block free binding sites. The bacterial antigens were detected by means of the alkaline phosphatase-labeled *A. tumefaciens* C58C1-specific antibodies. To prevent nonspecific binding of the antibodies to the plant tissue, antibodies were diluted 1:20 in the sap of untransformed *N. tabacum* leaves, and the mixture was kept for 2 h at 4°C before low-speed centrifugation. The supernatant was further diluted 1:100 with PBS and incubated with the membrane for 2 h. The binding of alkaline phosphatase-labeled *Agrobacterium*-specific antibodies was detected on the washed blots (PBS containing 0.05% Tween 20) by means of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Lin et al. 1990).

SEM.

Leaf, stem, and root explants were aseptically removed from in vitro cultures and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) overnight at 4°C, then washed twice in phosphate buffer. After dehydration in an acetone series, the internal surfaces of explants were exposed by freeze-fracture in liquid nitrogen. The specimens were dried to critical point in liquid carbon dioxide, gold-coated, and examined under a scanning electron microscope (Hitachi S430) at 15 kV accelerating voltage.

Enrichment cultures.

Approximately 0.1 g of plant tissue was homogenized and transferred into 20 ml of liquid bacterial growth medium (0.5% sucrose, 0.5% tryptone [Difco, Detroit, MI], 0.1% yeast extract [Difco], 2 mM $MgSO_4$, pH 7.5). The cultures were cultivated by shaking (200 rpm, 28°C) for 2 to 7 days.

Enrichment with in vitro plant material. Plant material was taken from different leaves, homogenized under sterile conditions, and incubated until the medium became turbid (but no longer than 7 days).

Enrichment with ex vitro plant material. Different tissues of a plant (i.e., seeds, flowers. leaves, stem, roots) were sampled separately, homogenized by grinding, and incubated for 3 days in a bacterial growth medium containing 50 mg of rifampicin (Duchefa) per liter and 100 mg of cycloheximide (Sigma, Deisenhofen, Germany) per liter. Seeds were tested in batches of 1,000 to 1,500 seeds per capsule (approximately 0.1 g).

Enrichment in parallel to SEM experiments. Leaf, stem, and root tissue samples were taken from in vitro plants. Half of the sample was fixed for SEM observation while the other half was left intact and placed separately in bacterial growth medium.

Enrichment in parallel to TPIB experiments. In the case of in vitro plants, the shoot tips were used for further propaga-

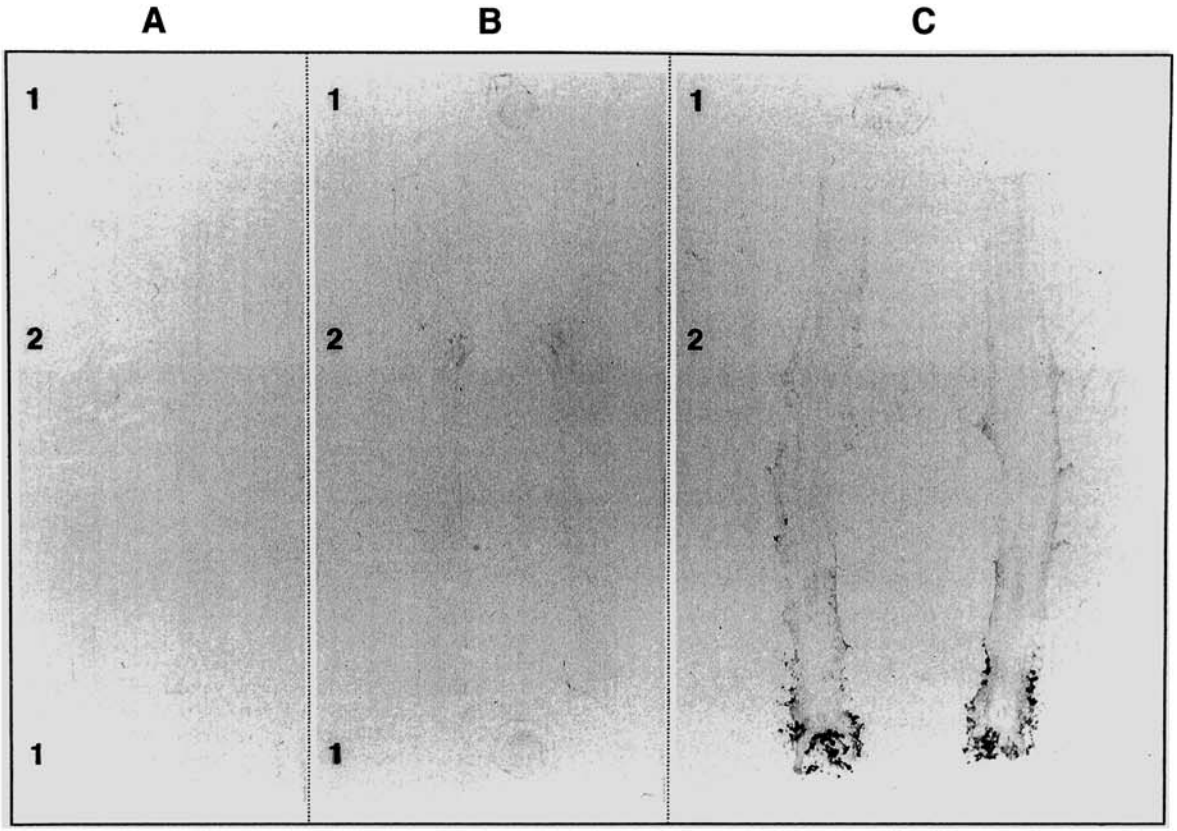


Fig. 5. A representative tissue-print immunoblot of a transgenic tobacco plant grown ex vitro in soil for 3 months. **a**: upper region, **b**: middle region, and **c**: base of the stem. (1 = cross sections of stem, 2 = longitudinal sections of stem). × 0.8.

tion, and the stem and two leaves were used for TPIB. The remaining plant material was used for enrichment culture as described above. In the case of plants grown in soil, tissue samples were taken from regions adjacent to the tissues used for TPIB and cultivated as described previously.

Identification of isolated bacteria.

Selective plating. Isolated bacteria were plated on selective media containing 50 mg of rifampicin per liter, 25 mg of kanamycin per liter, and 25 mg of tetracycline (Duchefa) per liter. Samples from ex vitro material were plated on the same media supplemented with 100 mg of cycloheximide per liter. Plates were incubated at 28°C for 3 days.

Slide agglutination test. The third bleed of an antiserum raised against *A. tumefaciens* C58C1(pEHA101)(pOCA18/Ac) was conjugated with attenuated *Staphylococcus aureus* (American Type Culture Collection, U.S.A., code 12598) cells according to Lyons and Taylor (1990). Five microliters of this working reagent and 5 µl of a suspension of the test bacteria were mixed; a positive reaction was seen as granular clumping of the fuchsin-stained working reagent. As a negative control, bacterial suspension and working reagent containing normal serum were used.

Ketolactose test. Bacteria were inoculated at 28°C for 2 to 3 days on medium containing 1% α-lactose, 0.1% yeast extract, and 2% agar. Then, the agar surface was flooded with Benedict's reagent (Bernaerts and de Ley 1963) and left at room temperature for 1 h. If 3-ketolactose was present, a yellow ring of Cu_2O became visible around the cell masses.

Dot blot analysis. Putative *A. tumefaciens* C58C1 (pEHA101)(pOCA18/Ac) colonies were picked from the selective medium and incubated overnight in liquid bacterial growth medium. One hundred microliters of the late exponential phase cultures was treated with 50 µl of buffer (4 mg of lysozyme per ml + 200 µg of RNAse per ml in 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) in a microtiter plate and applied to a Hybond-N Nylon membrane (Amersham-Buchler, Braunschweig, Germany) with a multichannel pipette. Membranes were then treated as described for colony blots by Sambrook et al. (1989) and hybridized with a probe for the *pat* gene. The digoxigenin-labeled probe was synthesized by PCR with a Boehringer (Mannheim, Germany) kit (cat. no. 1636090). Detection was carried out with a Boehringer kit (cat. no. 1363514) according to the manufacturer's instructions.

Polymerase chain reaction. Plasmid DNA extracted according to Birnboim and Doly (1979) was employed as template in PCR reactions. Detection of the *pat* gene was achieved by amplification of a 734-bp fragment by means of the following primer pair: (i) CAG AAC TCG CCG TAA AGA CT, and (ii) CAA CTC CCA CAA CAC CGA CC. The PCR solution contained 1× reaction buffer, 2.5 mM $MgCl_2$, 200 µM each of dNTPs, 25 ng of template DNA, 0.2 µM each primer, and 2 U of Stoffel fragment (Perkin Elmer, Norwalk, CT). After 5 min of incubation at 94°C, 35 cycles of 1 min at 94°C, 1.5 min at 53°C, and 2 min at 72°C were performed. Aliquots were analyzed on agarose gels.

Determination of the sensitivity of detection methods.

Bacteria cell counting. The total number of bacteria cells was counted with the use of a Thoma chamber (Hecht, Sondheim, Germany). The number of living cells was detected by plating on nonselective medium and by counting the grown colonies as CFUs. All dilutions were plated in 5 individual replicates, and the mean values taken.

Sensitivity of enrichment cultures. A defined number of *A. tumefaciens* C58C1(pEHA101)(pOCA18/Ac) cells (i.e., 1, 5, 10, 50 based on the counted number of viable bacteria) was mixed with 20 ml of liquid bacterial growth medium supplemented with 0.1 g of homogenized, untransformed, in vitro tobacco leaf tissue. As a control, the same number of bacteria was cultivated in medium without plant material. The optical densities (OD_{600}) of cultures were measured every 3 h, starting 48 h after inoculation. The experiment was carried out 5 times in parallel, and the mean values taken.

Immuno-dot-blot. A defined number of *A. tumefaciens* C58C1(pEHA101)(pOCA18/Ac) cells was incubated on a nitrocellulose membrane within a dot blot apparatus for 30 min, then the membrane was treated as described for TPIB.

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