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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Protocol for transformation of the apple rootstock Jork 9 with the *rol*B gene and its influence on rooting

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Abstract A suitable protocol for transformation has been developed for the apple rootstock Jork 9 using *Agrobacterium tumefaciens* strain EHA101A(pEHA101A) (pSCV1.6). Root formation was increased by transforming the rootstock with *A. tumefaciens* strain C58C1 (pGV3850)(pB-B:GUS), which contains the *npt*II, *rol*B and *gus* genes on the T-DNA. Transformation for all of the introduced genes was confirmed by polymerase chain reaction and Southern blot analyses. Of the 18 independent shoot lines obtained after transformation only ten contained at least one copy of intact T-DNA, while six lines were missing the *gus* gene and two lines were missing both the *gus* and *rol*B genes. The rooting experiments showed that introduction of the *rol*B gene increased root percentage and root number, giving 13.8 roots per shoot compared to 2.3 for untransformed shoots. More than two copies of the *rol*B gene decreased the number of roots and percentage of rooted shoots.

Keywords *Agrobacterium* · Genetic transformation · *rol*B · Apple rootstock · Jork 9

Abbreviations *BAP*: 6-Benzylaminopurine · *IBA*: Indole-3-butyric acid · *NAA*: α-Naphthaleneacetic acid · *TDZ*: Thidiazuron

Introduction

Large-scale vegetative propagation of woody plants is based on a high rooting capacity. However, in several tree species adventitious root formation is a major problem. Furthermore, rooting ability declines with maturation. In general, plants in the juvenile stage root easily, whereas cuttings from mature plants are difficult or impossible to root. Auxin is the key hormone for inducing adventitious root formation, which usually occurs after a dedifferentiation process in which plant cells become competent to the hormone. Subsequent treatment with auxin can induce the differentiation of plant cells, ultimately leading to the formation of root meristems (Christianson and Warnick 1983).

It is possible to improve root formation and root architecture by means of a new "tool" – transformation or inoculation with root-inducing bacteria. The phytopathological soil bacterium *Agrobacterium rhizogenes* triggers root formation in infected plants by transferring the T-DNA fragment of the Ri (root-inducing) plasmid into plant cells (Tepfer 1984). Four root loci (*rol*), A, B, C and D, have been identified in the T_L-DNA of the Ri plasmid, of which the *rol*B gene is the most effective in promoting rooting in different plant species (White et al. 1985; Spena et al. 1987). The expression of *rol*B is known to increase auxin sensitivity (Delbarre et al. 1994; Filippini et al. 1994) and is believed to involve changes in the auxin perception pathway (Maurel et al. 1994).

The root-inducing and root growth-stimulating characteristics of the *rol* genes in *A. rhizogenes* have already been successfully used for increasing the rooting ability in many fruit tree species such as: kiwi (Rugini et al. 1991), almond (Damiano et al. 1995), walnut (Caboni et al. 1996), cherry (Druart et al. 1998) and the apple rootstock Jork 9 (N. Pawlicki, submitted). The *rol*B gene used separately increased rooting in the apple rootstock M26 (Welander et al. 1998) and M.9/29 (Zhu et al. 2001).

The apple rootstock Jork 9 is very recalcitrant for both regeneration and transformation. An efficient system for adventitious shoot regeneration was developed by Pawlicki and Welander (1994). They found that different factors, such as pre-treatment of the mother shoots, hormone concentrations, the carbohydrate source and the gelling agent, influenced the regeneration capacity of Jork 9. The replacement of sucrose by sorbitol increased the rate of

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adventitious shoot regeneration and the number of shoots per explant (Pawlicki and Welander 1994). The efficiency of sorbitol as a carbon source for in vitro shoot multiplication has also been shown for other *Malus* species (Pua and Chong 1984).

The apple rootstock Jork 9 has been chosen by many scientists (Welander and Pawlicki 1993; van der Krieken et al. 1993; de Klerk 1995; Butler and Gallagher 1999) as a model plant to study root formation at the anatomical, physiological and molecular levels. However, until now, it has not been possible to transform Jork 9.

The aim of the investigation reported here was initially to optimize the conditions for transformation of the apple rootstock Jork 9 with strain EHA101 by testing various combinations of hormone supplements to the regeneration medium. Using the results of this preliminary study our final aim was to transform Jork 9 with the *rol*B gene and investigate how this gene and its integrated copy number can influence rooting ability with respect to that shown by untransformed shoots.

Materials and methods

Plant material

The plant material consisted of in vitro cultivated shoots of the apple (*Malus domestica*) rootstock Jork 9. The shoot cultures were maintained on shoot multiplication medium consisting of MS salts (Murashige and Skoog 1962) with 4.4 µ*M* BAP, 0.49 µ*M* IBA and 30 g/l sorbitol. Before autoclaving the pH was adjusted to 5.5 and the medium solidified with 4 g/l agar (Bacto-Difco) and 1 g/l Gelrite. Subculturing occurred every 6 weeks. The plants were grown at 23°/18°C (day/night) under a 16/8-h (day/night) photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 33 µmol $m^{-2}s^{-1}$.

Bacterial strain and vector

The *Agrobacterium tumefaciens* strains EHA101A(pEHA101A) (pSCV1.6), kindly provided by Dr G. Edwards (Shell Research Limited), and C58C1(pGV3850)(pCMB-B:GUS), kindly provided by Dr C. Maurel (CNRS Gif/Yvette, France), were used. The vector pSCV1.6 carries the gene for neomycin phosphotransferase II (*npt*II) and the intron-containing gene for β-glucuronidase (GUS-intron), both under the cauliflower mosaic virus (CaMV) 35S promoter. The vector pCMB-B:GUS contains the *npt*II gene, the *rol*B gene driven by its own promoter and the *gus* gene under the *rol*B promoter (Fig. 1). Both strains were grown on LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0). For the EHA101A strain, 25 mg/l neomycin, 50 mg/l chloramphenicol and 10 mg/l gentamycin was added to the medium and for the C58C1 strain, 200 mg/l kanamycin plus 75 mg/l neomycin.

Genetic transformation

For the transformation experiments, young expanded leaves from 6-week-old micropropagated shoots were used as explants. The mother shoots were dark- and cold-treated at 4°C for 1 week prior to the transformation. The leaves were wounded with a scalpel prior to inoculation with *Agrobacterium*. For inoculation, bacteria were grown overnight at 28°C, under shaking, in liquid LB medium supplemented with the appropriate antibiotics. The bacteria were then pelleted by centrifugation at 5,126 *g* for 15 min and resuspended in a MS20 medium (MS salts and 20 g/l sucrose) to an OD_{420} of 0.5–1.0. The wounded leaves were gently shaken in the bacterial suspension for 20 min and then cultured on callus induction medium (MS salts, 30 g/l sucrose, 5 µ*M* NAA, 15 µ*M* TDZ, 2.5 g/l Gelrite) for 3 days in dark. After co-cultivation, the leaves were rinsed in MS20 medium containing 500 mg/l cefotaxime, dry-blotted on sterile filter paper, transferred onto callus induction medium supplemented with 500 mg/l cefotaxime and 50 mg/l kanamycin and placed under dark conditions. The explants were transferred to fresh callus induction medium containing 200 mg/l cefotaxime and 50 mg/l kanamycin every 2 weeks. After 8 weeks, when transformed callus appeared, the explants were transferred to regeneration medium (MS salts, 40 g/l sorbitol, 3.5 g/l agar and 1.2 g/l Gelrite) supplemented with 200 mg/l cefotaxime and 50 mg/l kanamycin. In the first transformation experiment, 20 different combinations of TDZ, BAP and NAA in the regeneration medium were tested (Table 1). One of the best combinations – 1 µ*M* NAA and 20 µ*M* TDZ – was then used for further transformation experiments. The leaves were continuously transferred to fresh regeneration medium every second week. After 4 weeks, when shoots had been formed, the explants were transferred to light conditions; they were covered with a nylon cloth during the first 2 weeks to reduce the light intensity. The shoots were excised from the explants and propagated on shoot multiplication medium containing 50 mg/l kanamycin. The growth conditions were the same as those described for shoot multiplication. Each leaf explant was numbered, and all shoots regenerated from the same leaf explant were kept separated. Only one shoot per leaf was taken for further molecular analysis.

Polymerase chain reaction and Southern analysis

Plant genomic DNA was extracted from 6-week-old shoots of *rol*B-transformed and untransformed control plants using the CTAB (hexadecyltrimethyl-amonium bromide) extraction meth-

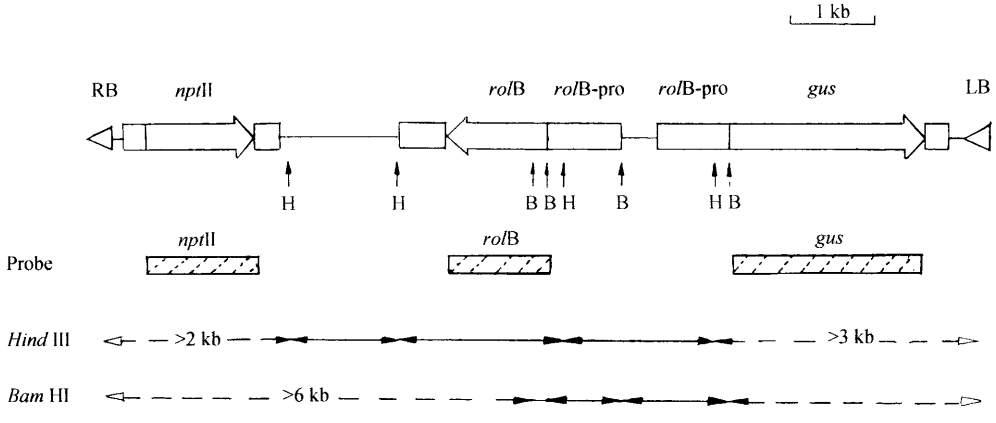

Fig. 1 Diagrammatic representation of the T-DNA region of pB-B:GUS. The positions of the *Hin*dIII (*H*) and *Bam*HI (*B*) restriction sites within the T-DNA are indicated by *arrows*. *Shaded boxes below* the map represent the neomycin phosphotransferase II (*npt*II), *rol*B and β-glucuronidase (*gus*) probes. *Lines below* these denote the anticipated sizes of the *Hin*dIII and *Bam*HI restriction fragments. *Solid lines* indicate internal fragments, and *broken lines* indicate fragments generated from sites outside the border sequences

Table 1 Effects of hormone concentration in the regeneration medium on the transformation frequency. For every hormone combination 60 leaf explants were infected with *Agrobacterium tumefaciens* strain EHA101A

Auxin (μ*M*)	Cytokinin (μ*M*)		Number of regenerated shoots	Transformation frequency (%)
NAA	BAP	TDZ		
0.1	22	0	0	0
0.1	0	5	0	0
0.1	0	10	0	0
0.1	0	15	1	1.67
0.1	0	20	0	0
0.5	22	0	0	0
0.5	0	5	0	0
0.5	0	10	0	0
0.5	0	15	2	3.33
0.5	0	20	2	3.33
1	22	0	0	0
1	0	5	0	0
1	0	10	0	0
1	0	15	0	0
1	0	20	2	3.33
5	22	0	0	0
5	0	5	0	0
5	0	10	0	0
5	0	15	0	0
5	0	20	0	0

od described by Aldrich and Cullis (1993). Plasmid DNA was isolated using the alkaline lysis method of Sambrook et al. (1989).

The PCR was carried out in 10-μl volumes, each containing 0.2 m*M* of each dNTP, 0.25 μ*M* of each oligonucleotide primer, 0.25 U *Taq* polymerase and 50 ng of sample DNA. The reactions were amplified in a thermal cycler (Gene AmpR System 2400, Perkin Elmer) using the following program: an initial denaturing at 95°C for 5 min; 30 cycles of 95°C for 15 s 55°C for the *rol*B or 65°C for both the *npt*II and the *gus* for 15 s, 72°C for 30 s; a final extension at 72°C for 5 min. Amplified DNA fragments were electrophoresed on a 3% agarose gel, stained with ethidium bromide and visualized under UV light. The primers used for amplification of the fragment of the *npt*II gene were 5′-GCCCT GAATGAACTGCAGGACGAGGC-3′ and 5′-GCAGGCATCG CCATGGGTCACGACGA-3′, generating a 411-bp product; those used for amplification of the fragment of the *rol*B gene were 5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′ and 5′-TTAG GCTTCTTTCTTCAGGTTTACTGCAGC-3′, generating a 779-bp product; the primers used for amplification of the fragment of the *gus* gene were 5′-CCTGTAGAAACCCCAACCC GTG-3′ and 5′-CCCGGC AATAACATACGGCGTG-3′, generating a 365-bp product.

Southern blots were prepared by digesting 10 μg genomic apple DNA either with *Hin*dIII or *Bam*HI, separating the DNA fragments on a 1% agarose gel and transferring them to a positively charged nylon membrane (Boehringer Mannheim). The membrane was hybridized with one of the three different digoxigenin-11-dUTP-labeled probes for *npt*II, *rol*B and *gus*; these were constructed by PCR using pCMB-B:GUS as a DNA template and the primers described above. Hybridization was carried out using Boehringer Mannheim's protocol (van Miltenburg et al. 1995) in standard buffer overnight at 68°C; the probe concentration was 25 ng/ml. After the membrane had been washed, hybridized DNA bands were visualized following the immunochemiluminescence detection protocol of Boehringer Mannheim (van Miltenburg et al. 1995). X-ray films were exposed for 3 h.

Rooting experiment

Five-week-old transformed and untransformed shoots were used for the rooting experiments. Shoots longer than 1.5 cm were cut from the mother shoot and placed on rooting medium consisting of half-strength Lepoivre macro-nutrients, full-strength Lepoivre micro-nutrients, Walkey vitamins, 30 g/l sucrose, 100 mg/l proline, pH 5.5, and solidified with 7 g/l agar. Two types of rooting experiments were performed. In the first experiment, one clone of transformed plants (clone 14) was compared with untransformed plants. The shoots were incubated in the dark on rooting medium supplemented with 0, 2.4, 4.9 and 9.8 μ*M* IBA. After 5 days the shoots were transferred to hormone-free medium and placed in the light. In the second experiment, eight clones of Jork 9 were used (NT, C14, C9, C17, C8, C6, C1 and C3), each containing a different copy number of the *rol*B gene. All of the shoots were incubated on hormone-free medium, first in the dark and then, after 5 days, in the light. The percentage of rooted shoots and number of roots per rooted shoot were recorded after 3 weeks.

Statistical analysis

The rooting experiments were performed in two blocks with 20 replicates per clone in each block. To test the differences in rooting percentage, we used logistic regression [the procedure GENMOD in SAS(1999)], and to compensate for many pair-wise comparisons, we used the significance level P=0.01. The analysis of variance model and Tukey's method for multiple comparisons together with the significance level P=0.05 was used to compare the roots per rooted shoots.

Results

Genetic transformation and regeneration

In the experiment for optimizing the transformation protocol, the apple rootstock Jork 9 was transformed with *Agrobacterium tumefaciens* strain EHA101(pEHA101A)(pSCV1.6). Twenty different hormonal combinations were tested in the regeneration medium using 60 leaf explants for every combination. Transformed shoots were obtained only from the four following combinations: 0.1 μ*M* NAA and 15 μ*M* TDZ gave a transformation frequency of 1.67%; 0.5 μ*M* NAA and 15 μ*M* TDZ, 0.5 μ*M* NAA and 20 μ*M* TDZ, 1 μ*M* NAA and 20 μ*M* TDZ all gave a transformation frequency of 3.33% (Table 1). PCR analysis confirmed the integration of both the selection and marker genes, *npt*II and *gus*, in all regenerated shoots (data not shown). Based on these results, we chose the combination of 1 μ*M* NAA and 20 μ*M* TDZ for further transformation experiments.

The *rol*B gene was introduced into the experimental system by infecting 475 young expanded leaves with *A. tumefaciens* strain C58C1(pGV3850)(pCMB-B:GUS). Thirty-one leaves regenerated one or more shoots, resulting in a transformation frequency of 6.5%. Shoot yield was 1–6 shoots per leaf explant. The transformed shoots were multiplied several times on multiplication medium supplemented with 50 mg/l kanamycin before selection. From all of the kanamycin-resistant shoots, we chose the 18 best growing ones, from 18 different leaf explants, for further analysis.

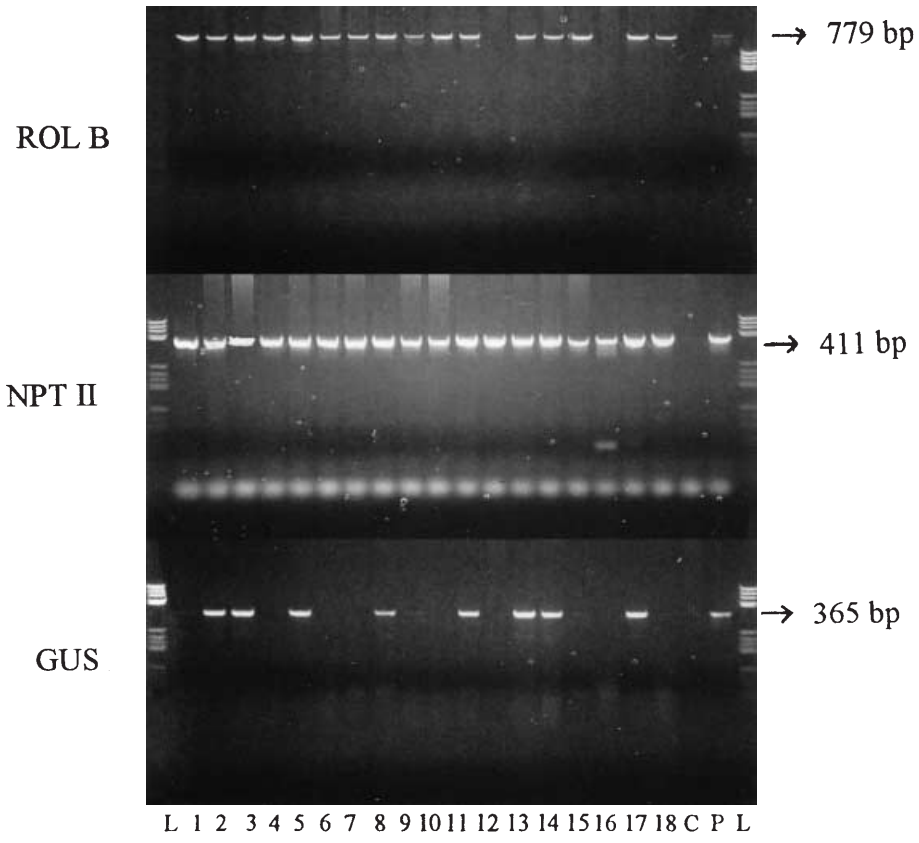

Fig. 2 Agarose gel of PCR-amplified fragments showing: 779-bp *rol*B fragment, 411-bp *npt*II fragment and 365-bp *gus* fragment. *Lanes*: *L* DNA molecular-weight markers, *1–18* representative clones of transformed Jork 9 shoots, *C* untransformed control, *P* pBB:GUS plasmid DNA

PCR and Southern analysis

Eighteen transformed shoots (numbered from 1 to 18) selected on kanamycin media were tested with PCR for the presence of the introduced genes. Ten of these shoots showed the predicted bands for the *npt*II, *rol*B and *gus* genes (Fig. 2, lanes 1–3, 5, 6, 8, 11, 13, 14, 17). Even though the bands in lanes 1 and 6 for the *gus* gene were very weak, the presence of the *gus* gene was confirmed by Southern blot analysis. Six shoots showed bands for the *npt*II and *rol*B genes (Fig. 2, lanes 4, 7, 9, 10, 15, 18) but no band for the *gus* gene, and two plants showed only the *npt*II band (Fig. 2, lanes 12, 16). No DNA band was detected in the untransformed control shoots (Fig. 2, lane C). These results show that almost 50% of the transgenic plants contained only part of the T-DNA. In such cases, the kanamycin-resistant gene (*npt*II) was always inserted because of selection on kanamycin, but the *rol*B and *gus* genes may have been deleted or modified. Integration of the T-DNA containing the *npt*II, *rol*B and *gus* genes and their copy number was confirmed by Southern hybridization analysis (Fig. 3). The autoradiographs of the genomic blots showed a wide variation in the copy number of the introduced genes in individual transgenic Jork 9 shoots. Digestion with *Hin*dIII and hybridization with the *npt*II probe resulted in bands larger than 2 kb for all transgenic plants (lanes 1–18). These bands were expected to contain not only the *npt*II gene and the right T-DNA border but also a plant DNA fragment that varies in size depending on the insertion site. Between one and seven bands were detected in the different transgenic plants, suggesting that one to seven copies of the *npt*II gene were integrated. Digestion with *Bam*HI and hybridization with the *rol*B probe resulted in one to seven bands with high-molecular-weight bands that corresponded to the copy number of the *rol*B gene in 16 transgenic plants. Two shoots did not show any bands for *rol*B (Fig. 3, lanes 12, 16). Digestion with *Hin*dIII and hybridization with the *gus* probe produced bands representing the DNA fragment containing the *gus* gene and the part of plant genomic DNA located outside the left T-DNA border. Only ten transgenic plants contained the *gus* gene in one, two or four copies (Fig. 3, lanes 1–3, 5,

Table 2 Number of roots per rooted shoot and percentage of rooted shoots of untransformed shoots (NT) compared with one transformed clone (C14) containing one copy of the introduced genes. The shoots were incubated on rooting medium with four different IBA concentrations

IBA concentration (μ*M*)	Number of roots per rooted shoot		Rooting percentage (%)	
	NT	C14	NT	C14
0	1.5	16.8	40	100
2.4	10.32	14.25	95	100
4.9	10.85	12.25	100	100
9.8	13.55	8.73	100	95

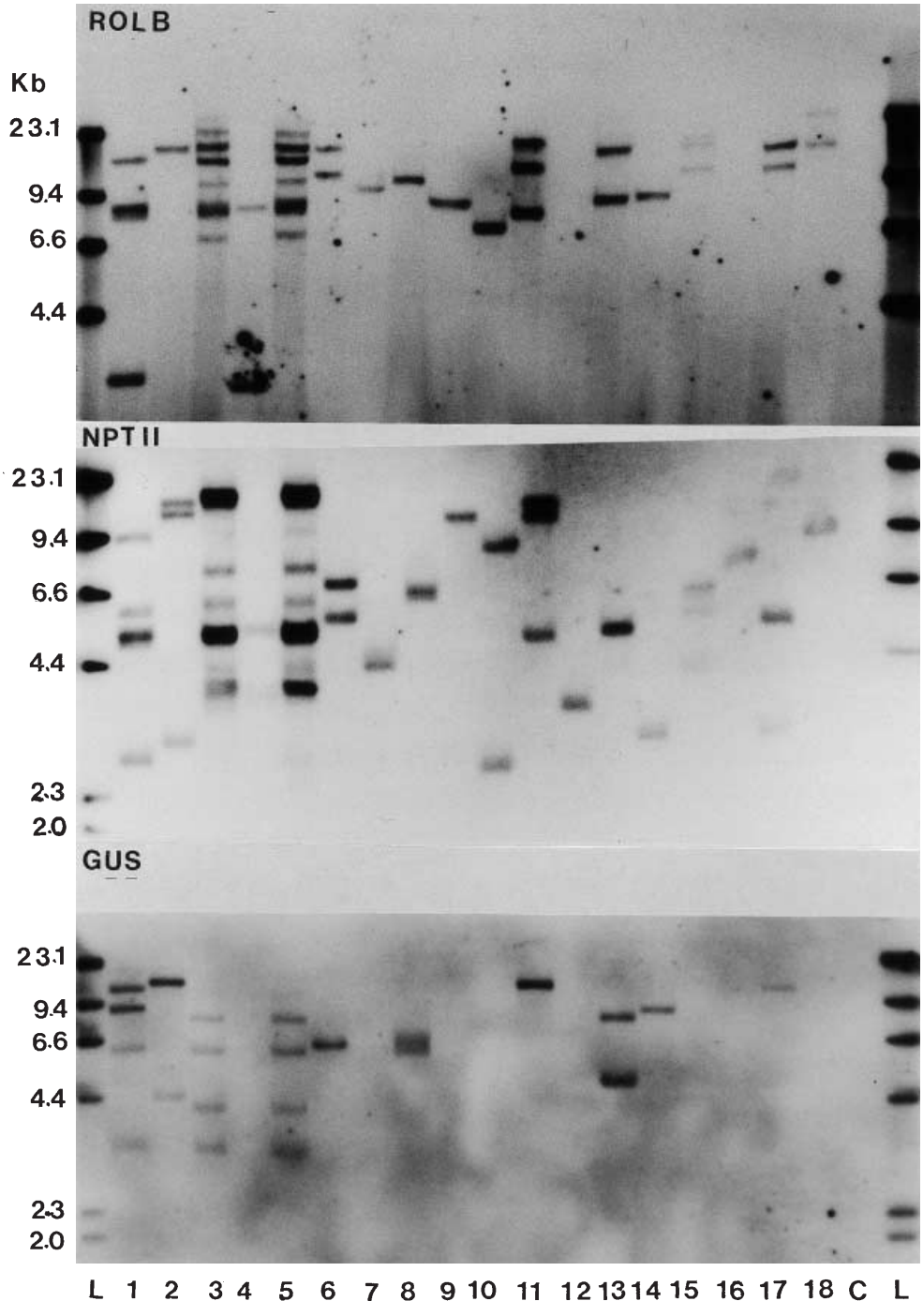

Fig. 3 Southern blot analysis of transgenic apple shoots. Ten micrograms of the plant genomic DNA was digested with either *Bam*HI (*rolB*) or *Hin*dIII (*nptII* and *gus*). Filters were hybridized with the DIG-labeled, PCR-generated *rol*B fragment, *npt*II fragment and *gus* fragment. *Lanes*: *1–18* Individual transformants, *C* an untransformed plant, *L* the DIG-labeled DNA molecular-weight marker λ/*Hin*dIII

6, 8, 11, 13, 14, 17). Except for clones 3 and 5 (Fig. 3, lanes 3 and 5), all of the transformed shoots showed different band patterns, which provides the evidence for independent transformation events. No hybridization bands were present in the untransformed shoots (lane C).

Rooting experiments

Table 2 shows that the highest percentage of rooted shoots (100%) and the highest number of roots per cutting (16.8) were obtained on the medium without hormones. Increasing the IBA concentrations reduced both the rooting percentage and the number of roots and also increased callus formation. Callus formation was also observed during rooting. The frequency of callus formed on *rol*B-transformed shoots increased with higher concentrations of auxin. Almost no callus was formed on untransformed shoots. The morphology of the roots was also influenced by the transformation process. Figure 4 shows that the transformed shoots are more sensitive to exogenously applied auxin, giving rise to more

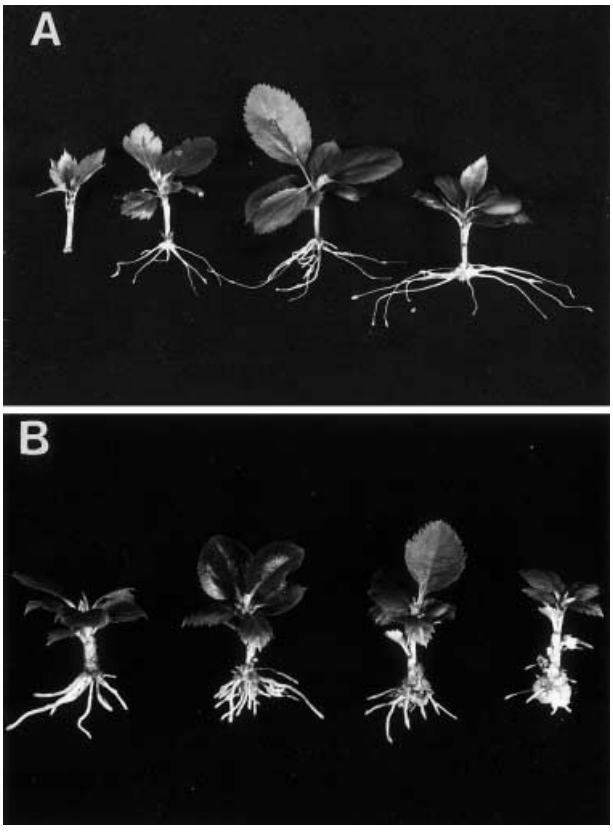

Fig. 4 Rooting of untransformed shoots (**A**) and transformed shoots (**B**) containing one copy of the *rol*B gene. The shoots were grown on rooting medium containing (from *left* to *right*) 0, 2.4, 4.9 and 9.8 μ*M* indole-3-butyric acid

callus and shorter and thicker roots than the untransformed ones.

In the second experiment, eight clones with different copy numbers of the *rol*B gene, including one clone lacking the *gus* gene and one untransformed clone, were checked for rooting ability on the rooting medium without hormones. Figure 5 shows that 80% of the shoots from all of the transformed shoots rooted after 3 weeks, while only 47.5% of the untransformed shoots rooted. Furthermore, plants transformed with the *rol*B gene produced up to 13.8 roots per shoot compared to 2.3 roots per shoot for the untransformed shoots. However, the number of roots per shoot varied between shoots containing different copy numbers of the *rol*B gene. Optimum rooting ability was obtained for shoots containing two copies of intact T-DNA, whereas four and seven copies of incorporated T-DNA resulted in a lower percentage of rooted shoots and a lower number of roots per shoot. The plant carrying a single but partly deleted T-DNA insert (the *gus* gene was missing) gave a better rooting result than the plant with one intact copy of the T-DNA.

Discussion

Up to now the apple rootstock Jork 9 has been very recalcitrant to transformation. Several initial experiments using different types of *Agrobacterium* strains and vectors were carried out without any success but eventually we obtained successful transformation using the *Agrobacterium tumefaciens* strain C58C1(pGV3850)(pB-BGUS). The high transformation frequency – 6.5% – was the result of an optimization of the regeneration and transformation protocol.

One important factor of our successful transformation protocol is the replacement of sucrose by sorbitol. The beneficial influence of sorbitol on callus initiation and shoot multiplication in apple has also been shown by Chong and Taper (1972). It is possible that the replacement of sucrose by sorbitol in callus-promoting medium can also result in high percentages of regeneration; this is currently being investigated. The positive effect of sorbitol on shoot regeneration was also enhanced when the shoots were subjected to a dark and cold treatment (Pawlicki and Welander 1994).

On the basis of previous results (Pawlicki and Welander 1994), the best hormonal combination for regenerating adventitious shoots from untransformed Jork 9 is a low NAA concentration (0.1 μ*M*) and a high concentration of BAP (22 μ*M*). However, in the transformation experiments reported here the most important factor for the regeneration of transformed plants was TDZ (thidiazuron), a potent cytokinin-like substance for woody plant tissue culture (Huetteman et al. 1993). TDZ stimulated callus formation and enhanced shoot production when added to both the callus-promoting medium (15 μ*M*) and the regeneration medium (20 μ*M*).

The copy numbers of the introduced genes varied from one to seven for *npt*II, zero to seven for *rol*B and zero to four for the *gus* gene. However, the intensities of the bands obtained in the Southern blot analysis were quite variable. Some of the faint bands may have occurred as a result of an incomplete digestion of DNA; T-DNA transfer was sometimes incomplete, and the T-DNAs may have been rearranged following the gene transfer. This rearrangement could be affected by an excessively long T-DNA fragment (approx. 10.7 kb) or by the presence of tandem *rol*B promoters orientated in both sense and antisense, making it easy for rearrangement to occur at this point. Incomplete T-DNA transfer has also been shown for the apple rootstock M26 transformed with the same gene construct (Welander et al. 1998). Over the years various investigators have shown the correlation between copy number and transgene expression to be negative (Jones et al. 1987; Hobbs et al. 1990; Finnegan and McElroy 1994), indeterminate (Shirsat et al. 1989), positive or negative (Hobbs et al. 1993) and positive (Gendloff et al. 1990). In our study, copy number strongly affected the influence of the *rol*B gene on rooting. Two copies of the T-DNA showed the highest influence on rooting. However, one of the three clones with two copies of the *rol*B gene varied from the other two. This variabili-

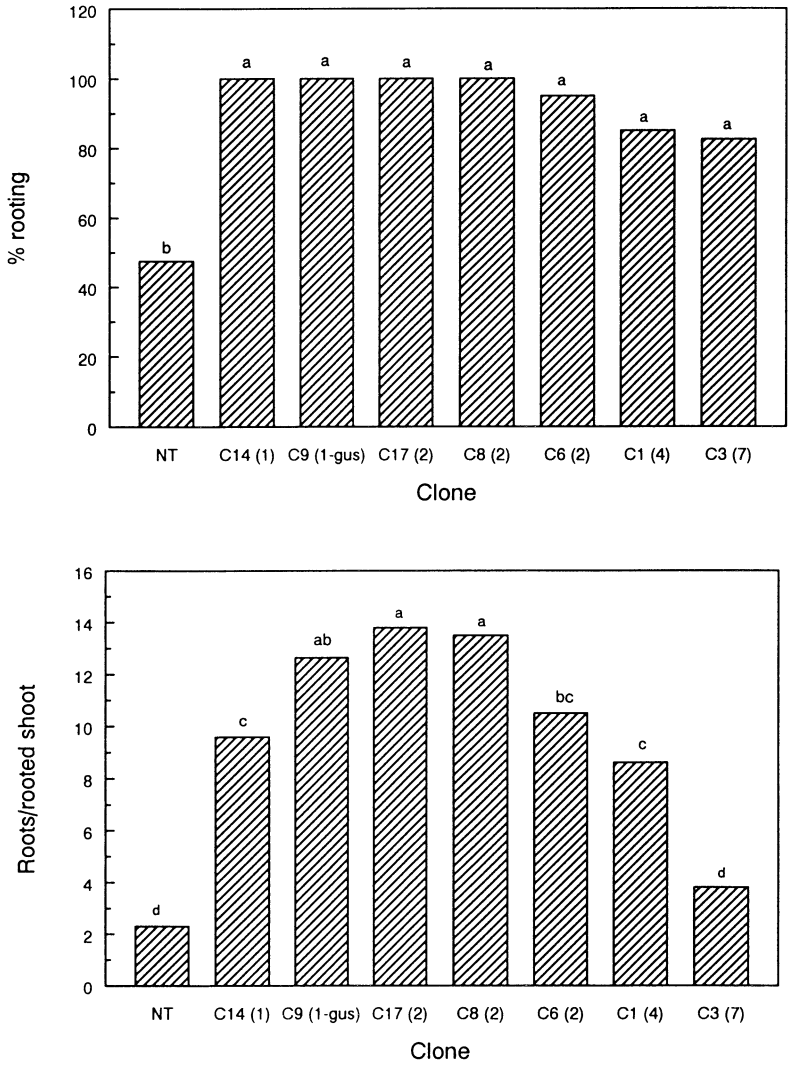

Fig. 5 Rooting of shoots on hormone-free medium from eight clones of Jork 9 containing different copy numbers of the *rol*B gene. The *upper bars* show the percentage of rooting; *different letters* indicate differences at P=0.01 upon pair-wise comparisons in a logistic regression. The *lower bars* show the least-squares means for the number of roots per rooted shoot. The *letters* refer to a multiple comparison with Tukey's method of analysis at a significance level of P=0.05. *NT* Untransformed shoots, *C14 (1)* clone with one copy of *rol*B gene, *C9 (1-gus)* clone with one copy of the *rol*B gene but lacking the *gus* gene, *C17 (2)*, C8 *(2)*, *C6 (2)* clones with two copies of the *rol*B gene, *C1 (4)*, *C3 (7)* clone with four copies and seven copies, respectively, of the *rol*B gene

ty can possibly be ascribed to different target sites of integration into the host genome. More than two copies of the introduced genes had a negative influence on rooting. Transgene inactivation generally occurs at the highest frequency when multiple copies of the gene are integrated. This inactivation is associated with increased methylation of the T-DNA (Hobbs et al. 1990).

There is very little information available on the changes that occur in gene expression during the initiation and development of adventitious roots, particularly with respect to tree species. A number of genes have been identified that are expressed specifically in roots, but these are found mostly in annual plants. A cDNA clone encoding a novel 2-oxoacid-dependent dioxygenase that is upregulated during the induction phase of adventitious root formation has been isolated in the apple rootstock Jork 9. The corresponding gene, designated ARRO-1, may function as a component of auxin-triggered, rooting-specific gene cascades (Butler and Gallagher 1999). Rooting-related genes expressed in the early processes of root development in *Pinus* have recently been isolated and characterized (Lindroth 2000).

The transformation with the *rol*B gene reported here makes Jork 9 even more attractive as a model plant, especially in studying gene expression during root induction. This is because of a higher sensitivity to auxin followed by increased rooting and a higher number of produced roots. Most woody cuttings require the treatment of exogenous auxin to form adventitious roots. The use of genetically modified *rol*B plants abolishes the need for additional hormones in culture. This is a great advantage in studies on root-inducing genes since the addition of auxin triggers a number of other genes that are not involved in the root induction phase.

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