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Use of *Agrobacterium rhizogenes* to create transgenic apple trees having an altered organogenic response to hormones

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Summary. The apple rootstock, M26, was genetically and phenotypically transformed using the *Agrobacterium* wild-type strain, A4. First, chimeric plants were obtained having transformed roots and normal aerial parts. Transformed plants were then produced through regeneration from transformed roots. Transformation was demonstrated by molecular hybridization and opine analysis. The effects of hormones on organogenesis was altered in transformants: cytokinins were required to form roots, whereas auxin was toxic at the concentration used to induce rooting in the control.

Key words: Apple – Root development – Genetic transformation – *Agrobacterium rhizogenes*

Introduction

The genetics of the control of morphogenesis in higher plants is poorly understood, partly due to the difficulties in cloning the genes regulating growth and differentiation. One source of such genes is the soil bacterium, *Agrobacterium rhizogenes*, which engages in natural genetic transformation. The transforming genes are carried on 'transferred DNA' (T-DNA) and are inserted into the plant nuclear DNA. Their presence causes the formation of genetically transformed roots which, in many species, give rise to whole plants carrying the foreign genes. A transformed phenotype which includes increased branching, shortened stature, wrinkled leaves, modified flowering and root systems (Tepfer 1984), appears in these plants. Changes in root systems include increased branching and plagiotropic behaviour. These genes are thus useful in creating new genotypes and phenotypes and in attempting to understand how development is regulated (Tepfer 1989).

In a previous paper, we used *A. rhizogenes* to overcome a rooting deficiency in apple rootstocks (Lambert and Tepfer 1991). Chimeric plants were produced having root-inducing (Ri) T-DNA in their roots, but not in their aerial parts; they had extensive, highly branched root systems. We proposed (Tepfer 1983) that Ri T-DNA might be used to improve the rooting ability of rootstocks or to create root systems that are better adapted to environmental stress, e.g., drought. To this end, it was necessary to produce the abundant root phenotype in the absence of bacterial contamination, i.e., a rootstock entirely transformed by Ri T-DNA, and to propagate it by root cuttings.

In the following report we describe such a transformed apple rootstock, having an altered ability to form roots. We show that both the Ri TL-DNA (root-inducing, left-hand, transferred DNA) and TR-DNA (right-hand, transferred DNA) are present, and we discuss possible uses of this new genotype in apple improvement.

Materials and methods

Plant micropropagation

The rootstock clone M26 was used throughout. In-vitro shoots were obtained through micropropagation as described by Jones et al. (1977). These were maintained on the MS medium described by Murashige and Skoog (1962), supplemented with 0.5 mg/l 6-benzylaminopurine (BAP), 0,1 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l gibberellic acid (GA3), and were rooted on the same medium with macro-nutrients and sucrose reduced by one half and supplemented with 0.2 mg/l IBA.

Inoculation conditions

Agrobacterium rhizogenes strain A4 (Moore et al. 1979), was cultivated overnight as 28 °C, as described by Petit et al. (1983),

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and used for inoculation at approximately 10^5 cells/ml. The inoculation method was as described elsewhere (Lambert and Tepfer 1991). Briefly, 1-month-old in-vitro micro-cuttings were pricked at their base, dipped in the bacterial suspension and transferred to the above rooting medium. Three days later explants were transferred to the same medium, but containing the antibiotic cefotaxime (Roussel Laboratories) at 500 mg/l to inhibit bacterial growth.

Culture of transformed roots, plant regeneration and maintenance

Transformed roots were grown in liquid medium, containing the macro-nutrients of Tendille and Lecerf (1974), with nitrogen as KNO_3, the micro-nutrients of Murashige and Skoog (1962), the vitamins of Morel and Martin (1955), and sucrose (20 g/l).

Plant regeneration was based on the method developed by Druart (1980) for *Pyrus*. Shoot production was obtained in vitro by regeneration from roots still attached to the stem on which they had been induced. Conditions were different for regeneration from normal and transformed roots. Shoots were produced in the control using half-strength MS medium (Murashige and Skoog 1962) with 1 mg/l BAP and 0.2 mg/l IBA. In contrast, transformed roots regenerated shoots in 5 mg/l BAP. Both micropropagation and rooting of transformed shoots were obtained in MS medium (Murashige and Skoog 1962) containing 0.5 mg/l BAP as the sole hormone supplement. Control regenerants were rooted in half strength MS medium with 0.2 mg/l IBA.

DNA extraction and molecular hybridization

Plant DNA was isolated according to Dellaporta et al. (1983), digested with restriction endonucleases (5 units/μg) and electrophoresed (10 μg/sample) in Tris-borate horizontal agarose-gels. Zeta probe blotting membranes were used for transfer and hybridization, as recommended by the supplier (Bio-Rad). Probes were pLJ1 and pLJ85 (Jouanin 1984), covering respectively the TL- and TR-DNAs, with pLJ1 extending approximately 11 kb to the left of the TL-DNA and pLJ85 approximately 12 kb to the left of the TR-DNA. Labeling was with ^{32}P-dCTP by nick translation (Amersham kit). Hybridized blots were autoradiographed with an Amersham MP film and two intensifying screens.

Results

Axenic, transformed root cultures

Roots induced by *A. rhizogenes* on cuttings were excised and cultured in vitro, as organ clones, after disinfection in hormone-free liquid medium. They exhibited the transformed phenotype observed in other species, producing numerous, plagiotropic lateral roots that were adapted to in-vitro growth (Fig. 1). Non-transformed controls did not grow. The extent of phenotypic change varied from clone to clone, and in general those that were the most branched exhibited the best growth. Transformed roots synthesized the two opines, agropine and mannopine, considered to be markers for the presence of the Ri TR-DNA (data not shown).

Regenerated plants

Regeneration was from roots still attached to the mother plant. Shoots appeared from normal roots 2–4 months after several subculutres on regeneration medium (Fig. 2a). They were subsequently excised and rooted as for the mother plants. Regeneration was more difficult in the case of transformed roots; however, buds regenerated from a single root induced by *A. rhizogenes* (Fig. 2b) in medium containing 5.0 mg/l BAP. Growth of the regen-

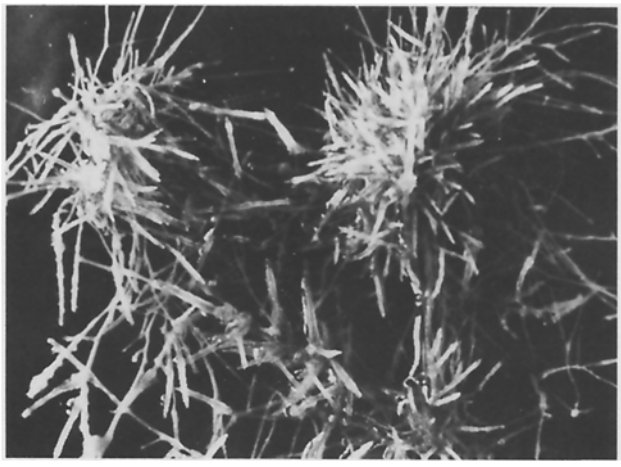


Fig. 1. One-month-old culture of transformed apple roots, showing their ability to grow in vitro and the typical, highly branched, phenotype

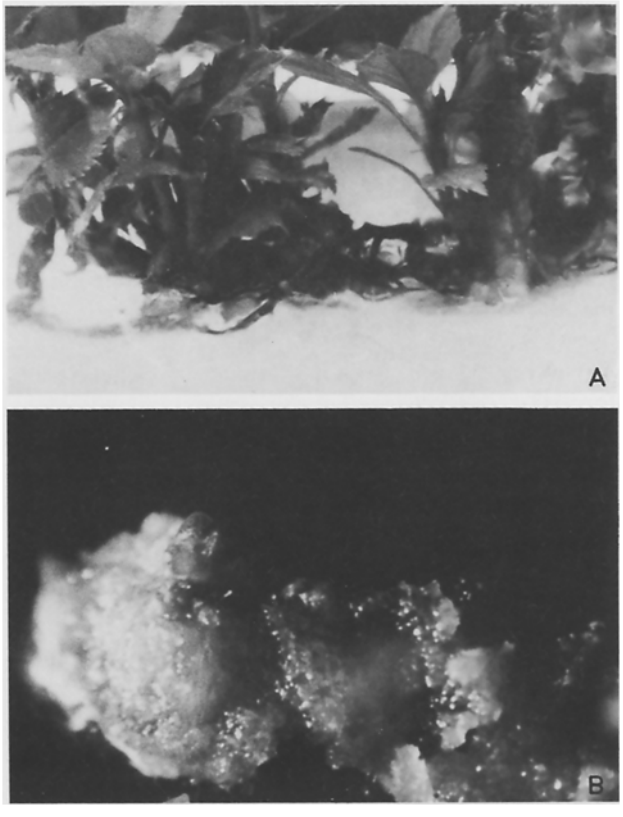
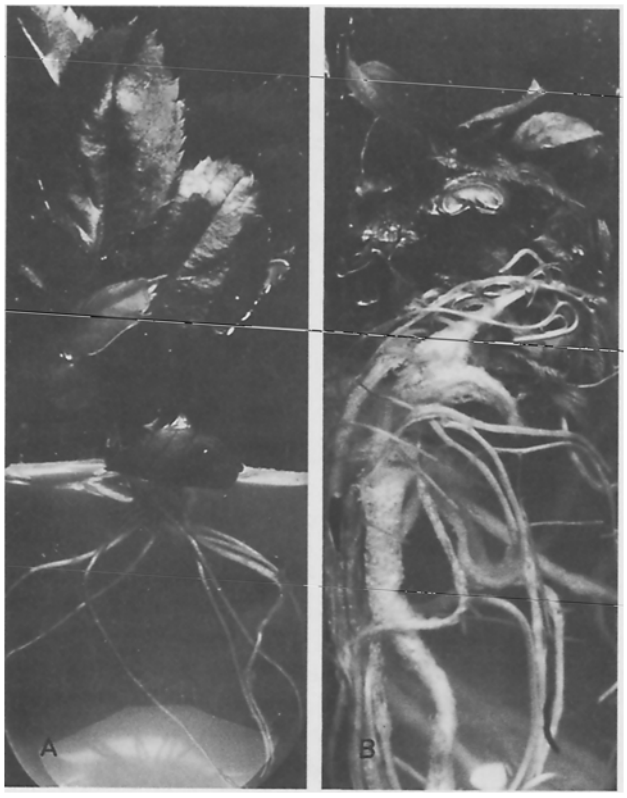


Fig. 2 A, B. Shoot buds regenerated from normal roots (**A**), and transformed roots (**B**)



erated buds was inhibited in this medium, but resumed after repeated transfer on medium containing 0.5 mg/l BAP as the sole hormone. Rooting in this medium attained 100%. (Under identical conditions, controls were unable to form roots.) Furthermore, transfer of the putatively transformed shoots to the rooting medium used for the controls (containing 0.2 mg/l auxin) caused necrosis.

Not only was the rhizogenic potential of shoots regenerated from transformed roots different from that of shoots regenerated from normal roots, but the root systems of the former were more developed, and they produced more shoots than the controls (Fig. 3).

Transformation was assessed by measuring opines and through molecular hybridization. As expected in tissues containing the TR-DNA of *A. rhizogenes* strain A4, both mannopine and agropine were found in the leaves of the three regenerants (data not shown). The TL- and TR-DNAs are indeed both present in these leaves, in a configuration similar to that reported for other species (Fig. 4). In the transformed apple genome the TL-DNA probe hybridized to *Eco*R1 restriction fragments that co-migrated with pRi *Eco*R1 fragments 15, 36, 37ab and

Fig. 3A, B. Control M26 apple plant rooted with auxin (**A**), compared with a transgenic plant rooted with cytokinin (**B**)

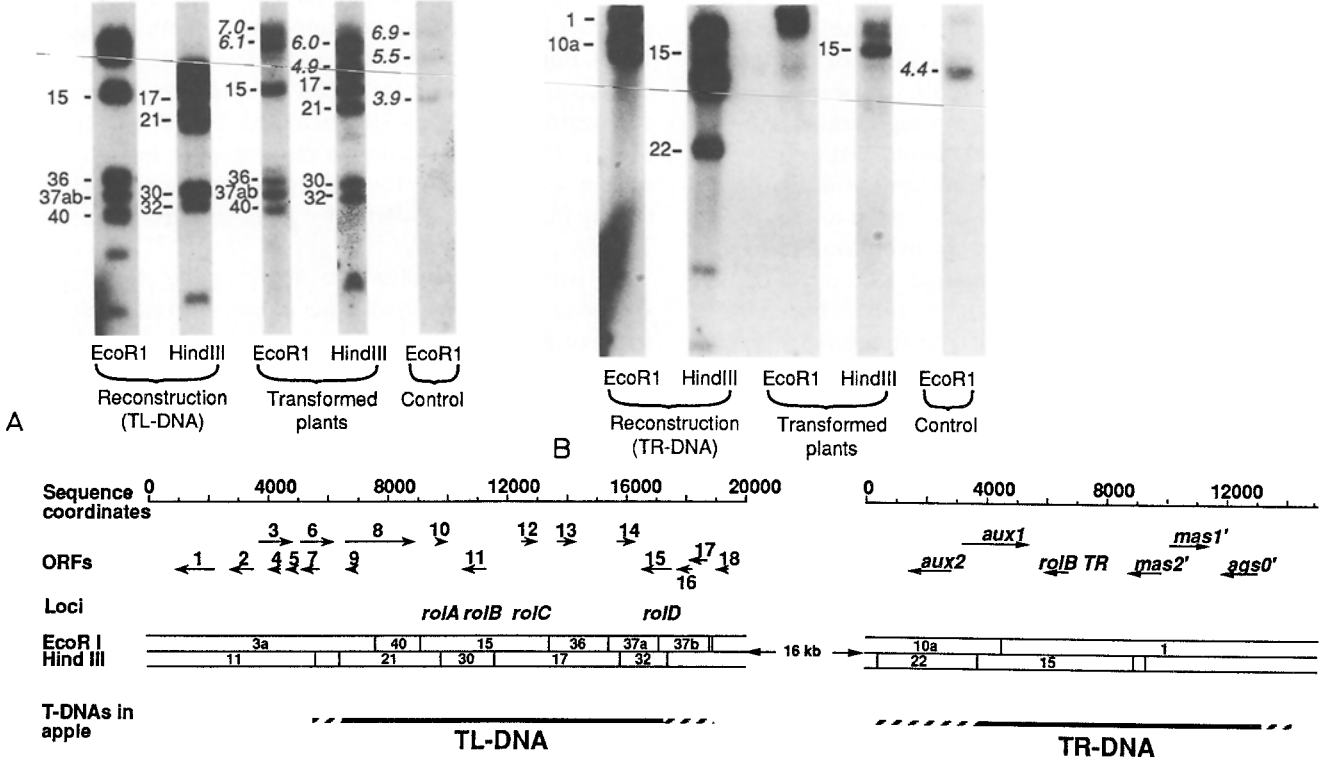


Fig. 4. Hybridization of apple DNA digests with probes pLJ1 (**A**) and pLJ85 (**B**) and a restriction map of the T region of pRIA4 (**C**). Fragment numbers are given adjacent to identified fragments using the nomenclature of Jouanin (1984), other fragments (e.g., putative junctions between T-DNA and plant DNA) are identified by size in kb (*in italics*). Parts of the autoradiogram appear over-exposed in order to clearly show hybridization to fragments in the control. Fragment numbers were assigned using an autoradiogram exposed for a shorter time

40. Hybridization was detected by transformed apple *Hin*dIII fragments co-migrating with pRi *Hin*dIII fragments 17, 21 a, 30 a and 32. We conclude that ORFs 8–15 (including *rol A, B and C*) are present. Two putative junction fragments, not co-migrating with fragments from the reconstruction, and thus presumably containing both plant DNA and T-DNA, were observed at 7.0 and 6.1 kb, using *Eco*R1, and at 4.9 and 6 kb, using *Hin*dIII. In control DNA weak hybridization occurred to three *Eco*R1 fragments at 6.9, 5.5, 3.9 kb.

Hybridization with the TR-DNA probe, revealed *Eco*R1 fragment 1 and *Hin*dIII fragment 15 as internal. The former carries opine synthesis functions and an ORF similar to *rolB* from the Ri TL-DNA (Fig. 4) (Bouchez and Camilleri 1990). The TR-DNA auxin synthesis genes were not detected. Strong hybridization occurred between pLJ85 and a 4.4 kb *Eco*R1 restriction fragment in the control.

Discussion

We introduced Ri T-DNA into apple rootstocks with the aim of increasing their rhizogenicity. As yet we have produced only one transformed line, so the efficiency of the method used still needs to be established. The plants we produced were transformed according to two independent criteria: opine synthesis and molecular hybridization. Opines in the leaves could not be due to translocation from transformed roots; they were found in plants that had been rooted from regenerated shoots, thus none of the original, bacterially-induced, root tissue remained. Bacterial contamination cannot account for these results, because opines are not known to be synthesized in bacteria, and we observed hybridization to restriction fragments that are internal to the TL-DNA, while other fragments included in the probe, but outside the T-DNA, were not detected. The structure of the T-DNA inserted into the apple genome is similar to that observed in other species. We detected weak hybridization signals with the probe representing the TL-DNA. These were reported by other authors (Tepfer 1982; White et al. 1983) and in one case were shown to come from a duplicated fragment that is highly homologous to the region encoding the *rolB* and *rolC* loci (Furner et al. 1986). We were surprised to discover a strong hybridization signal with the Ri TR-DNA probe, and are currently determining its exact origins.

In general, genetic transformation of trees has been limited. Walnut (Mc Granahan et al. 1988), citrus (Hidaka et al. 1990), grapevine (Mullins 1990), apple (James et al. 1990) and plum (Mante et al. 1991) have been transformed with reporter genes encoding resistance to kanamycin and hygromycin and/or with β-glucuronidase (GUS).

Over 30 species of dicots have been genetically transformed with Ri T-DNA, producing a transformed phenotype (See Tepfer 1989 for review). Trees expressing Ri T-DNA have numerous shoots and plagiotropic root systems: these include Allocasuarina (Phelep et al. 1991), kiwi (Rugini et al. 1991) and poplar (Pythoud et al. 1987; Chriqui personal communication). In the apple trees described above, the organogenic response to hormones was clearly altered. Shoot formation required high cytokinin levels, and subsequent growth and rooting only took place in the presence of cytokinins at a lower concentration, which also inhibited root differentiation in the controls. On the other hand, the auxin concentration that promoted rooting in the controls caused necrosis in the transformed plants. Thus, the response to exogenous growth substances is altered in the transformants. This was not the case in *Allocasuarina*, where both transformed and control plants rooted on the same medium (Phelep et al. 1991).

Two physiological correlates of the phenotypic changes observed in plants transformed by Ri T-DNA have been reported: increased sensitivity to auxin (Spano et al. 1988; Shen et al. 1988; Maurel et al. 1991) and decreased accumulation of polyamines and their conjugates with the hydroxycinnamic acids (Burtin et al. 1991; Martin-Tanguy et al. 1990). The *rolB* gene is considered to be important for root induction, but the expression of *rolA* and *rolC* amplified this phenomenon (Schmulling et al. 1988). Both *rolB* and *C*, are thought to encode β-glucosidases, with specificity for auxin or cytokinin glucoside conjugates, respectively (Estruch et al. 1991 a, b). Moreover, *rolB* gene expression is responsive to auxin (Capone et al. 1991). It is thus likely that auxin and/or cytokinin metabolism is altered by genes encoded by the Ri T-DNA.

It would be premature to interpret the altered response to auxins and cytokinins in transformed apple in terms of Ri T-DNA gene function; indeed the responses to hormones we have observed seem paradoxical. Nor can one accurately compare the phenotypes of the normal and transformed apple plants, since they must be maintained on different media. Even after establishment in the soil, phenotypes can be described only after several years of growth. Nevertheless, under the present conditions (which are adjusted for each genotype) root production is more prolific in the transformed clone, while the wrinkled leaves characteristic of other plants containing Ri T-DNA were not observed. These results are consistent with our goal of altering rooting ability and the root system morphology in apple. Ultimately it would be useful to design root systems to overcome environmental constraints, such as lack of water.

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