

Rooting and Other Characteristics of a Transgenic Walnut Hybrid (*Juglans hindsii* × *J. regia*) Rootstock Expressing *rolABC*

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ABSTRACT. Walnuts (*Juglans* spp.) are difficult-to-root woody plants. The *rolABC* genes (*rolA* + *rolB* + *rolC*), derived from the bacteria *Agrobacterium rhizogenes*, have been shown to increase the rooting potential of other difficult-to-root woody plants. We inserted the *rolABC* genes into somatic embryos of a ‘Paradox’ hybrid (*J. hindsii* × *J. regia*) clone PX1 using the *A. tumefaciens* gene transfer system. A transgenic sub-clone, designated PX1 *rolABC* 2-2 was selected and compared to the untransformed clone for a variety of phenotypic characteristics, including rooting potential. Transformed and untransformed shoots were budded onto seedling *J. regia* rootstock in the greenhouse and established in the field. Transformed trees displayed reduced internode length, an increase in lateral branching, and wrinkled leaves. In another test, a commercial persian walnut cultivar *J. regia* ‘Chandler’ was grafted onto rooted cuttings of both the untransformed and transformed plants. The presence of the *rolABC* genes in the rootstock had no visible effects on the grafted scion. Several of these trees were excavated from the field and the root systems of each genotype were examined for root number, diameter, and biomass. Trees with the *rolABC* rootstock had significantly more small diameter roots compared to the controls and less recovered biomass. Tests of the rooting potential of leafy semi-hardwood cuttings for two years resulted in 14% to 59% rooting of the transformed cuttings compared to 51% to 81% rooting of the control. Both transformed hardwood cuttings and microshoots in tissue culture also rooted significantly less (52% and 29% respectively) than untransformed hardwood cuttings and tissue cultured shoots (82% and 54% respectively). Thus, although the *rolABC* genes induced a shorter internode length and a more fibrous root system (typical of *rol*-transformed plants), they were not useful for increasing the rooting potential, and as rootstock they did not affect the phenotype of the scion.

‘Paradox’ walnut, an interspecific hybrid between black walnut (*Juglans hindsii* Jepson ex R.E. Smith) and persian/english walnut (*J. regia* L.), is used extensively as a rootstock for persian walnut cultivars in California (McGranahan and Catlin, 1987). Commercial vegetative propagation of walnut rootstocks is uncommon because of difficulties in rooting (Lynn and Hartmann, 1957; McKenna, 1997; Reil et al., 1998; Vahdati, 1996). The interest in rooting cuttings of ‘Paradox’ has recently increased due to the identification of rootstock selections that are potentially resistant or tolerant of destructive diseases (McGranahan et al., 1998).

Transformation with the *rol* genes is of interest in many woody species due to their effects on plant development, particularly rooting (Rugini and Mariotti, 1992). *Rol* genes were originally derived from a T-DNA located on large root-inducing (Ri) plasmids present in all virulent strains of *Agrobacterium rhizogenes* (Costantino et al., 1981). Among the 18 different open reading frames (ORFs) identified on this T-DNA, only four loci, *rol* (*rolA*, *rolB*, *rolC* and *rolD*), noticeably affect other attributes of plant growth and development (Slightom et al., 1986). These *rol* genes have been reported to change the following characteristics in transformed plants: *rolA* causes wrinkled leaves, condensed inflorescences, increased stigma

size and larger flowers; *rolB* increases rooting potential which may result from increased auxin sensitivity of the tissues, alters leaf morphology and increases flower size; *rolC* reduces internode length, produces flowering abnormalities, and increases branching; and, *rolD* causes dwarfing and early flowering (Rugini et al., 1997; Schmullig et al., 1988; Scorza et al., 1994).

The interest in genetic transformation of plants with *rol* genes as a potential means to enhance rooting competence is based on experiments that showed that *Agrobacterium rhizogenes* when applied to the base of micro and conventional stem cuttings increased adventitious rooting for several species (Caboni et al., 1996; Damiano and Monticelli, 1998; Rugini and Mariotti, 1992; Tzfira et al., 1998). Caboni et al. (1996) showed that *A. rhizogenes* applied to the base of microcuttings of persian walnut induced 58% rooting on hormone-free medium and 62.9% rooting in the presence of indolebutyric acid (IBA) while those treated with IBA alone did not root. In addition, it has been observed in several species that plants that have been genetically transformed with constructs containing single or multiple *rol* genes exhibit enhanced adventitious root initiation (DiCola et al., 1996; Rugini et al., 1991; van der Salm et al., 1996, 1997; Welander et al., 1998).

The primary objective of this work was to determine the effect of *rolABC* genes on walnut rootability. Several rooting trials were conducted using semi-hardwood cuttings, hardwood cuttings and tissue cultured shoots of a transformed and untransformed ‘Paradox’ clone. Because expressing these three genes in the plants could alter other traits such as vigor, lateral branching and root architecture, shoot and root characteristics of the transgenic walnut trees were also examined.

Material and Methods

VECTOR CONSTRUCTION AND TRANSFORMATION CONFIRMATION. A

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vector was constructed containing the *rolABC* genes from *Agrobacterium rhizogenes* strain A4 by using subclones of the TR-DNA of pRiA4. The *rolABC* region was obtained as a 4.3-kb *EcoRI* fragment. This fragment was purified by gel electrophoresis and subcloned into pBluescript, creating a plasmid designated pDN152 (containing the *rolABC* genes regulated by their native promoters). In addition, we subcloned a gene cassette containing an intron containing the GUS gene (35SGUS-INT) into pBluescript creating plasmid pDN352 (containing 35SGUS-INT). The GUS gene from pDN352 and the *rolABC* fragment from pDN152 were introduced into the binary vector pBIN19 to create the new vector pDN3514, the details of which have been described by Negri (1992).

For Southern blots, DNA was isolated from 100 mg of young leaf tissue using the Qiagen DNeasy Plant Kit (Valencia, Calif.). About 4 µg of each DNA sample was digested with the restriction endonuclease *HindIII* as described by the manufacturer (Gibco BRL). Digested fragments of walnut genomic DNA were separated via electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane by Southern blotting (Sambrook et al., 1989), and UV cross-linked. The coding sequence of *rolABC* was obtained by digestion of pDn152 with *EcoRI* and isolating the 4.3-kb fragment. This fragment was labeled with digoxigenin-d UTP using Roche Molecular Biochemicals Genius Kit (Indianapolis, Ind.) and used as a probe.

Blots were prehybridized for 4 h at 65 °C and hybridized for 16 to 24 h at the same temperature to the labeled probe. The blots were washed at 65 °C in 0.1× SSC, 0.1% SDS. The hybridization was detected by chemiluminescent detection as described by Boehringer Mannheim. Images of the hybridization were obtained by exposure of the blots to X-ray film for 2 to 5 h at room temperature.

RNA for RT-PCR was isolated from 100 mg of tissue (embryos or young leaves) using Qiagen RNeasy Plant Mini Kit (Valencia). RT-PCR was carried out according to Promega (Madison, Wis.) using their Access RT-PCR system and total RNA as template. The sequences for the primers were derived from Slightom et al. (1986).

PLANT MATERIAL. Immature open-pollinated seed of *J. hindsii* ‘Rawlins’ were collected in June, surface sterilized, and the excised cotyledons were cultured to produce somatic embryos (Tulecke and McGranahan, 1985). Repetitively embryogenic cultures were maintained and individual embryos were germinated to identify hybrids. Hybrids between *J. hindsii* and *J. regia* were identified based on bud position (Jay-Allemand et al., 1990). Somatic embryos from one of these hybrid clones, designated ‘PX1’, were inoculated with engineered *Agrobacterium tumefaciens* as described by McGranahan et al., 1990, and a transgenic subclone, designated ‘PX1 *rolABC* (2-2)’ was retrieved. Shoot cultures of the control (PX1) and the transgenic line were developed and buds of the in vitro shoots were budded onto seedling *J. regia* rootstock in the greenhouse. The budded plants were transplanted to the field the following year (1996). Three trees of each were maintained for descriptive purposes, and one to three other trees of each served as a source of cuttings. Those designated for cuttings were severely pruned annually. The remaining trees were left unpruned in 1999 and 2000 so that the phenotype could be described in 2001.

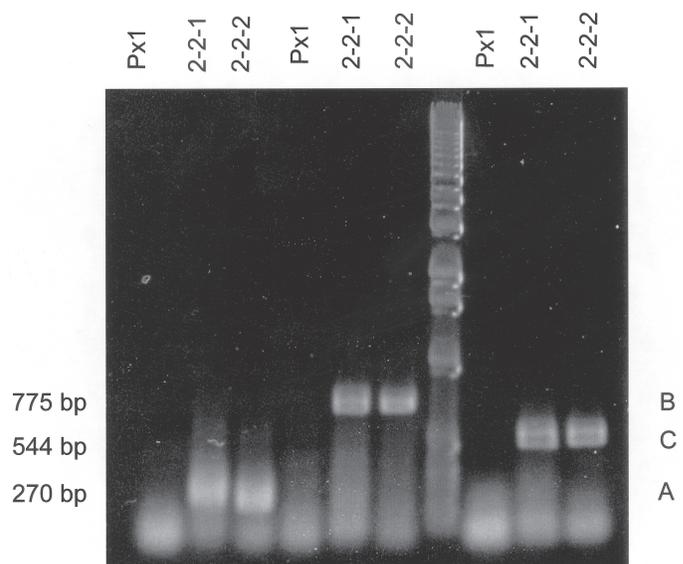
Rooted cuttings of both transformed and untransformed PX1 were produced in 1997 and grown in the field in 1998 at Burchell Nursery Inc, Oakdale, Calif. Trees were dug in the winter, planted at the University of California, Davis, in 1999 and grafted with

scions of the *J. regia* cultivar Chandler in 2000. These trees were used to describe the effects of the *rolABC* genes present in the rootstock on both the transgenic root system and the nontransgenic scion.

ROOTING STUDIES. Leafy semi-hardwood cuttings were collected early in the morning from the severely pruned stock plants in 1997 and 1998. Three-node cuttings of current-year shoots with a stem diameter of 10 to 15 mm and 10 to 20 cm in length were made. In 1997, cuttings were collected 27 Aug. and three replications of 36 cuttings of both the untransformed and transformed genotypes were treated with the auxin potassium indole-3-butyric acid, (K-IBA; Sigma Chemical Corp, St. Louis, Mo.) at an optimized concentration of 8,000 mg·L⁻¹ (Lynn and Hartmann, 1957; McKenna, 1997) and applied as a 30-s quick dip. Cuttings were stuck in individual solid-block-media sponges (Growtech Inc., San Juan Bautista, Calif.) and arranged on a mist bench in a completely randomized design. Cuttings were misted with deionized water for 15 s every 4 min during daylight hours. Bottom heat was supplied by heating mats at 24 ± 5 °C.

The time-course for rooting semi-hardwood cuttings was investigated in 1997 by observing cuttings for rooting beginning 19 d after sticking (DAS) and then about every 3 d afterwards until day 56. A cutting was scored as rooted when at least one adventitious root protruded through the sponge. Three experiments, arranged as completely randomized designs with three replications of each rootstock were conducted beginning on 19 Aug., 27 Aug., and 4 Sept. 1997. The time-course for rooting was determined by averaging the mean rooting percentage of each rootstock within a range of 2 to 4 DAS across the three experiments. Thus, DAS values are an average of the two to four values among each of the three experiments and the standard deviations presented are pooled among the three experiments. In 1998, the same experiment was repeated only on July 11th with an additional water-only control treatment.

Hardwood cuttings, 12 to 18 cm long, containing at least three nodes were taken from dormant 1-year-old shoots in February 2001. A range of auxin (K-IBA) concentrations was tested (in mg·L⁻¹): 8,000, 4,000, 2,000, and 0 (water control). For each treatment, there were four replications of fifteen cuttings. Cut-



Rol RT PCR of Walnut Leaves

Fig. 1. RT-PCR analysis of *rolABC* gene expression in walnut leaves.

tings were stuck in sponges and placed outdoors in a lathhouse on heating mats set at 30 ± 2 °C. Rooting percent was determined 60 DAS by scoring all cuttings with at least one visible adventitious root as rooted. Each cutting was then assigned to one of three quality classes based on the number of roots per cutting.

Tissue-cultured shoots of transformed and untransformed plants were tested for rooting in vitro. Shoots were derived from the somatic embryo lines described above that had been established in 1996, and maintained in culture following the methods of McGranahan et al. (1988). Thirty-two shoots, 3 to 4 cm in length, of each genotype were treated for rooting following the methods of Jay-Allemand et al. (1992).

SHOOT AND ROOT CHARACTERISTICS. Shoot characteristics (length of 1-year shoots, number of nodes per shoot, and number of lateral branches per shoot) were measured on four randomly selected 1-year-old shoots from each of three trees grafted with scions of either PX1 or PX1 *rolABC*. Shoots similar in canopy position on both genotypes were selected. Length of 1-year old shoots was recorded. The number of nodes and lateral branches were counted on 1 m shoot segments, 25 cm above the base of the shoot. Similar measurements were taken on the ‘Chandler’ scions grafted on the control and transgenic rootstock.

Root systems of trees originating from rooted cuttings of PX1 and PX1 *rolABC* and grafted with scions of *J. regia* ‘Chandler’ were examined. Three trees of each type were excavated with a backhoe for determination of root biomass and root architecture. Following excavation, root systems were washed and photographed. Then, all recovered roots 20 cm below the crowns were dissected, assigned a diameter class (1 to 5), and the dry weight of the roots within each class was determined.

Results and Discussion

MOLECULAR ANALYSIS. Southern analysis of the transformed lines showed the expected internal 1.8-kb fragment that hybridized with the *rolABC* probe and a single border fragment indicating a single insertion event. The control PX1 showed no hybridization with the *rolABC* probe. RT-PCR analysis of 1 µg of total RNA from embryos indicated the presence of *rolA* (270 bp), *rolB* (775 bp) and *rolC* (544 bp). In leaves, the same result was obtained with 100 ng of total RNA (Fig. 1).

SHOOT AND ROOT CHARACTERISTICS. Field-grown *rolABC* and PX1 control trees, both grafted on *J. regia* rootstock, reached the same height (Fig. 2), but clearly differed in canopy phenotype. The transgenic trees had denser and bushier foliage, with wrinkled and slightly curled leaves. They also had more nodes, shorter internodes, and more branches per meter (Table 1). Shoot elongation was delayed during the normal spring growth period in the transgenic trees but continued later into the summer. By fall, trees had roughly equivalent growth but the transgenic trees were delayed entering fall dormancy. Their leaves were still green after the control’s had turned yellow, and leaf abscission also occurred later. Delayed dormancy in the transgenic trees resulted in branch tip die-back in an early frost year (2001). Although internodes were shortened, a dwarfed phenotype that would be of interest as a rootstock was not conferred by the *rolABC* genes.

Examination of ‘Chandler’ scions grafted on the *rolABC* and PX1 rootstock showed no difference in number of nodes (15 to 17) per meter of shoot measured from 25 cm above the graft union (data not shown). Trunk circumference and leaf appearance were also similar on both rootstocks and typical of ‘Chandler’. These findings indicate that the transgenic rootstock had no discernible

effect on the scion. To our knowledge this is the first report of the lack of influence of a *rolABC* transformed rootstock on a nontransformed scion cultivar.

The root architecture of the transgenic rootstock, however, was clearly different from that of the control (Fig. 2). Roots of the *rolABC* rootstock were fibrous with few roots over 15 mm diameter. Consequently, although there was a significantly greater number of roots recovered from the transgenic rootstock, the root dry weight was significantly less than that of the PX1 control

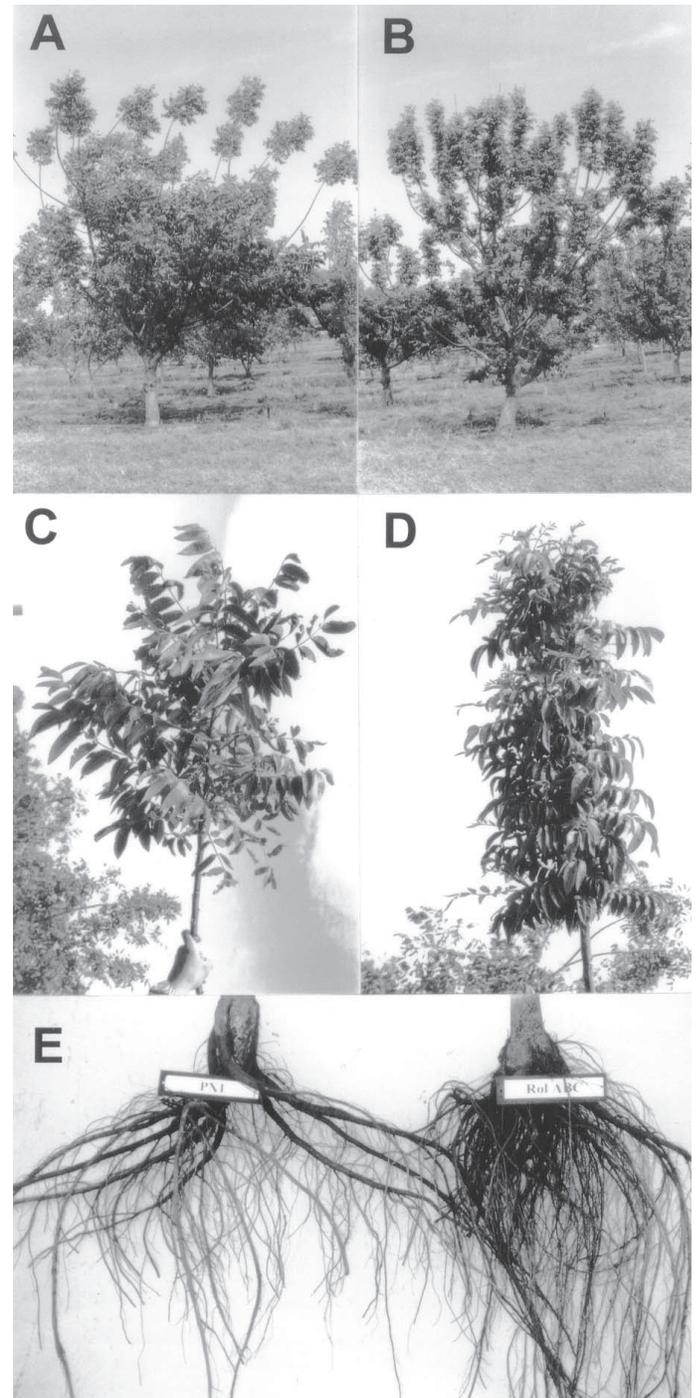


Fig. 2. Morphological characteristics of Paradox (PX1) untransformed control [A, C, and E (left)] and trees transformed with *rolABC* genes (B, D, and F); (A and B) 5-year-old trees in the field; (C and D) 1-year-old shoots illustrating compressed internode length of *rolABC* trees; (E) 4-year-old field-grown root systems showing greater number of small-diameter roots on the *rolABC* trees.

Table 1. Characteristics of shoots and roots of own-rooted *rolABC* 'Paradox' walnut trees compared to the untransformed PX1 control.

Genotype	Shoot system ^z				Root system ^y					Total no. of roots	Root dry wt (g)
	Leaf surface	Length of 1-year shoots (cm)	No. of lateral branches (M ⁻¹)	No. of nodes (M ⁻¹)	No. of roots/class (mm)						
					0.1-1	1-5	5-15	15-30	>30		
PX1 (control)	Smooth	168 ± 11	17 ± 4	32 ± 4	10	9	7	6	2	34	488 ± 65
<i>rolABC</i>	Wrinkled	157 ± 7	29 ± 3	52 ± 1	51	34	22	3	0	110	229 ± 77

^zShoot characteristics were determined on 5-year-old grafted field grown trees that had not been pruned for 2 years. Values are averages based on 4 shoots measured on each of 3 trees ± SD.

^yRoot characteristics are based on 4-year-old field grown cuttings. Values are the average of 3 trees ± SD.

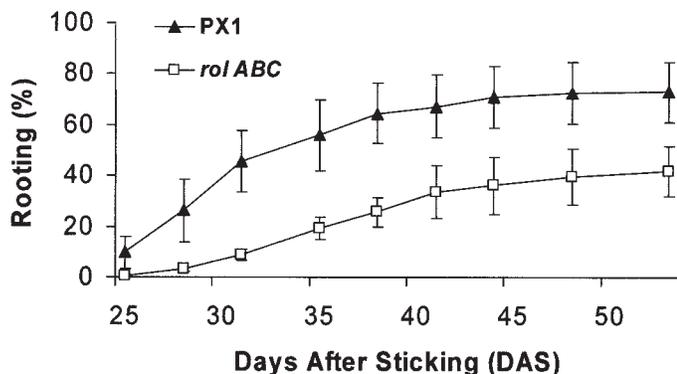


Fig. 3. Rooting percent of untransformed (PX1) and transformed (*rolABC*) Paradox semi-hardwood cuttings over time in 1997. Values represent the average of three experiments ± pooled SD.

Table 2. Rooting of untransformed and transformed 'Paradox' walnut semi-hardwood cuttings treated with or without auxin.

Genotype	Auxin (mg·L ⁻¹)	Rooting (%)	No. rooted/ no. stuck
PX1	8,000	81	87/108
	0	26	22/86
<i>RolABC</i>	8,000	14	15/108
	0	6	5/99

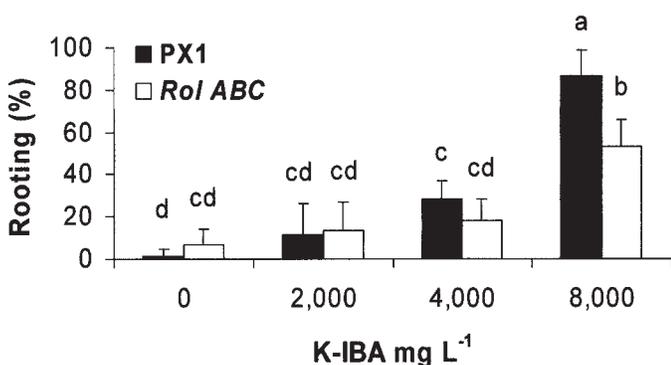


Fig. 4. Rooting of Paradox control (PX1) and transformed Paradox (*rolABC*) hardwood cuttings treated with different auxin (K-IBA) concentrations. Values are the average of 4 replications of 15 cuttings ± SE stuck in early February 2001.

Table 3. Rooting of tissue-cultured untransformed and transformed 'Paradox' walnut shoots in vitro.

Genotype	Rooting (%)	No. of roots/ rooted cuttings	Root diam (mm)	Longest root length ^z (mm)	Avg root length (mm)
PX1	54	4.3 ± 3.7	1.8 ± 0.6	40 ± 21	29 ± 15
<i>rolABC</i>	29	2.0 ± 1.1	2.0 ± 0.8	67 ± 35	49 ± 36

^zValues are based on average of 32 shoots ± SD.

rootstock (Table 1). The characteristics of these 'Paradox' trees transformed with *rolABC* are consistent with the characteristics of other species transformed with single or multiple *rol* genes (Rugini et al., 1992; Schmullig et al., 1988; Scorza et al., 1994; Welander et al., 1998).

ROOTING LEAFY SEMI-HARDWOOD CUTTINGS. As shown in Fig. 2, walnut semi-hardwood cuttings started to root 25 DAS, and rooting percentage plateaued by ≈45 DAS. At 56 DAS, 73% (±20) of the control PX1 cuttings had rooted compared to only 42% (±17) of transformed *rolABC* cuttings. In 1998, nontransformed and transformed cuttings rooted 81% and 14% respectively (Table 2). In the water-only control treatment PX1 cuttings rooted better than transformed ones.

ROOTING DORMANT HARDWOOD CUTTINGS. Increasing K-IBA concentrations increased rooting percentage in dormant hardwood cuttings from both transformed and nontransformed clones but the response was greater in the nontransformed clone. The greatest difference was seen at 8000 mg·L⁻¹ IBA (Fig. 4). These results do not support the hypothesis that *rol* genes increase the sensitivity to auxin for root initiation but instead suggest that *rolABC* transformed 'Paradox' cuttings have a reduced ability to respond to auxin for rooting.

ROOTING TISSUE CULTURE SHOOTS. Shoots of the PX1 control clone rooted more frequently and with greater root number than shoots from the *rolABC*-transformed clone (Table 3). Roots had about the same diameter in both clones, but roots were longer in transformed shoots (Fig. 5). Although *rolABC* shoots had longer roots, the roots had a looser connection to their shoots and none of the rooted *rolABC* shoots survived transplanting and acclimatization in the greenhouse.

These results, in which transformation with *rolABC* has a negative influence on rooting percentage of 'Paradox' cuttings, is in contrast to research results from several other species (Rugini and Mariotti, 1992; Rugini et al., 1991; Van der Salm et al., 1996, 1997). The reason for the observed negative effect of *rolABC* on rooting of 'Paradox' cuttings is not known. Additional studies to determine the relationship between *rolABC* expression levels and auxin metabolism could help to clarify this issue.

In conclusion, although there were distinct differences in growth habit of shoots and roots due to insertion of *rolABC*, the rooting ability of this 'Paradox' clone was not improved by insertion of these genes and the transgenic 'Paradox' rootstock



Fig 5. In-vitro rooting of Paradox control (PX1) and transformed Paradox (*rolABC*) microcuttings.

did not affect the phenotypic characteristics of the persian walnut scion, suggesting that the alterations in the shoot morphology were not graft transmissible.

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