Transformation of ‘Beurre Bosc’ Pear with the rolC Gene

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Abstract. ‘Beurre Bosc’ pear (Pyrus communis L.) was transformed with Agrobacterium tumefaciens (E.F. Smith & Townsend) Conn strain EHA101 containing the binary vector pGA-GUSGF into which the rolC gene had been inserted. Leaf explants from in vitro shoot tip cultures were wounded, Agrobacterium-inoculated, and cultured on kanamycin selection medium. Regenerating shoots were transferred to proliferation medium without antibiotics. Three clones tested positive for GUS and nptII enzyme activity. Transformation with the rolC gene was confirmed by DNA, RNA, and protein blot analyses. The number of copies of the rolC transgene varied from one to three. Plantlets of the three transgenic clones were acclimated and transferred to the greenhouse. Preliminary observations of phenotype indicate that the rolC gene reduced height, number of nodes, and leaf area of transgenic ‘Beurre Bosc’.

Genetic transformation using Agrobacterium tumefaciens and engineered binary vectors provides a means of making targeted single trait improvements in clonally propagated crops. Production of pear (Pyrus communis L.) in the United States is dominated by five cultivars; improvements in any of these genotypes could have a great impact on the pear industry. Successful transformation of pear has recently been reported (Merkulov et al., 1998; Mourgues et al., 1996), using marker and reporter genes, but no genes of economic importance.

Production efficiency of deciduous tree fruits has been dramatically improved through the use of dwarfing rootstocks to reduce tree size (Rom and Carlson, 1987; Tukey, 1964). Additional efficiencies have been associated with alterations in growth habit, particularly the introduction of semidwarf or spur-type mutants of apple (Malus xdomestica Borkh.) cultivars. Reduced tree stature in pears has been achieved through the use of quince (Cydonia oblonga L.) as a dwarfing rootstock, and more recently, by several semidwarfing ‘Old Home’ × ‘Farmingdale’ clonal rootstocks (Lombard and Westwood, 1987). However, quince rootstocks are not well adapted to most of the major pear production regions of North America, and the degree of size reduction of the ‘Old Home’ × ‘Farmingdale’ rootstocks is inferior to quince. Genetically dwarfed scion cultivars have not been commercially introduced.

The rolC gene from Agrobacterium rhizogenes (Riker et al.) Conn, the plant pathogenic bacterium which causes hairy root disease, has been used to transform such species as tobacco (Nilsson et al., 1993; Schmulling et al., 1988; Scorza et al., 1994), potato (Fladung, 1990), tomato (van Altvorst et al., 1992), aspen (Nilsson et al., 1997), and rose (van der Salm, et al., 1992). Growth habit and number of nodes, and leaf area of transgenic ‘Beurre Bosc’.

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Materials and Methods

Source of pear leaf explants. Shoot proliferation cultures of ‘Beurre Bosc’ and ‘Bartlett’ pear cultivars were established in vitro from cultures (CPYR 1165.001 and CPYR 0038.003, respectively) obtained from the National Plant Germplasm Repository in Corvallis, Ore. Axillary shoots were multiplied in a pear shoot proliferation medium (PSM) containing Murashige and Skoog (1962) basal salts and vitamins (Sigma Chemical Co., St. Louis, Mo.), 30 g·L⁻¹ sucrose, 5 µM benzyl adenine (BA), 0.6 µM gibberellic acid (GA3), 0.5 µM indole butyric acid (IBA), and 6 g·L⁻¹ noble agar (US Biochemical, Cleveland, Ohio). The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were grown at 24 ± 1 °C with a 16-h photoperiod provided by an equal mixture of warm-white fluorescent and Vita-lite full spectrum fluorescent (Duro Test Corp., Bergen, N.J.) lamps, with an intensity of 45 to 55 µmol·m⁻²·s⁻¹. Young expanding leaves from the actively growing shoot tips were excised from proliferation cultures 4 to 6 weeks after their last transfer, and used for transformation.

Transformation of pear leaf explants. The disarmed A. tumefaciens strain EHA101 containing pGA-GUSFrolC (Fig. 1) was used for transformation of pear leaves (Scorza et al., 1994). The GUS gene is controlled by the CaMV 35S promoter and the nptII gene is under the control of the nopaline synthase promoter. The rolC gene (ORF-12 in A. rhizogenes) is controlled by its native promoter. Cultures of EHA101 were grown overnight, pelleted at 2,940 g, and resuspended in Agrobacterium virulence induction medium consisting of filter sterilized liquid Chevreau and Leblay (CL) basal salts and organics (Chevreau and Leblay, 1993), 10 µM thidiazuron (TDZ), 1 µM IBA, and 100 µM acetosyringone (AS). The bacterial cultures were incubated for 4 h on a shaker (100 rpm) at 20 °C to induce the expression of virulence genes of Agrobacterium (James et al., 1993). After induction, the cultures were diluted to 0.1 and 0.5 OD (at 600 nm). An OD600 of 0.1 corresponds to 10⁷ cfu/mL.
Sterile scalpel blades dipped in the bacterial suspension (0.1 or 0.5 OD₆₀₀ₐₕₐₜₜ) were used to make transverse cuts across the midrib of each leaf. The number of cuts per leaf varied from 3 to 10, depending on the size of the leaf. Fifty leaves of each cultivar were inoculated with the 0.1 OD₆₀₀ₐₕₐₜₜ inoculum and 75 leaves of ‘Beurre Bosc’ were inoculated with the 0.5 OD₆₀₀ₐₕₐₜₜ inoculum. In addition to inoculation of freshly harvested leaves, 50 leaves of each of the two cultivars were wounded and precultured for 1 week in the dark on shoot induction medium (SIM) consisting of CL macronutrients, micro-nutrients, and organics, plus 10 μM TDZ and 1μM IBA, and solidified with 2.5 g·L⁻¹ Phytagel (Sigma Chemical Co., St. Louis, Mo.) in 100 × 15 mm covered petri plates. These leaves were then inoculated by dipping for 20 min in the bacterial suspension. After inoculation, all leaves were transferred abaxial side down onto cocultivation medium consisting of SIM and 100 μM AS. Leaf explants inoculated with 0.5 OD₆₀₀ₐₕₐₜₜ inoculum were cocultivated for 3 d, while leaves inoculated with 0.1 OD₆₀₀ₐₕₐₜₜ inoculum were cocultivated for 4 d. Thirty un inoculated leaves from shoot proliferation cultures of each cultivar were also wounded and cultured on the same cocultivation medium.

The leaf explants were then transferred to antibiotic selection plates containing SIM, 80 mg·L⁻¹ kanamycin, and 300 mg·L⁻¹ timentin (Smith Klein Beecham, Philadelphia, Pa.), and incubated in the dark. After 4 weeks, the cultures were transferred onto shoot expression medium (SEM), which consisted of SIM and 300 mg·L⁻¹ timentin; IBA and kanamycin were deleted. Cultures were transferred to fresh SEM every 4 weeks, and returned to the dark. When adventitious shoots appeared, they were excised and transferred to fresh SEM, and cultured in the light under the same conditions as that stated for the shoot proliferation cultures. When the shoots had produced two to three leaves, they were transferred to PSM containing 300 mg·L⁻¹ timentin for shoot proliferation. Preliminary experiments indicated that this concentration of timentin did not adversely affect adventitious regeneration. The transformation procedure used in this study differed from that used by Mourgues et al. (1996) and Merkulov et al. (1998) in the vector used, our use of a virulence induction medium containing acetylsyringone to resuspend the Agrobacterium culture, our use of timentin instead of ticarcillin and/or cefotaxime to control Agrobacterium after cocultivation, our use of IBA instead of NAA as the auxin used in the shoot induction medium, and our shorter length of time on kanamycin selection medium. Merkulov et al. (1998) used Agrobacterium tumefaciens strain A821.

Histochemical GUS assays. Calli, leaves, and stem tissue from the regenerated shoots were assayed for β-glucuronidase activity following the procedure of Jefferson (1987). GUS-positive clones were multiplied on PSM medium.

Molecular confirmation of transformation. The GUS-positive clones were subjected to molecular analyses including polymerase chain reaction (PCR) to detect the presence of the gene, and DNA, RNA, and protein blots to confirm the integration and expression of rolC in the pear genome. Pear genomic DNA was extracted from in vitro putative pear transformants by the CTAB procedure of Doyle and Doyle (1990), as modified by Callahan et al. (1993). PCR for rolC was done as described by Scorza et al. (1994). DNA blots were probed using the nonradioactive Genius digoxigenin (DIG) labeling and detection kit (Boehringer Manheim Corp., Indianapolis, Ind.). Ten micrograms of DNA of each GUS-positive pear clone were used for DNA blotting. EcoRI and HindIII digests were used to determine the integration of the rolC insert into the pear genome and the number of insertion events (Scorza et al., 1994). The DNA blots were also probed with the nptII gene probe, the primer sequence of which was kindly provided by J.L. Slightom, and as described by Fitch et al. (1992). The oligonucleotide primers for a 514-bp internal fragment of the rolC gene were described by Scorza et al. (1994). The rolC primers and nptII probes for DNA blotting were generated by PCR using DIG dUTP in the reaction mix as described by the manufacturer.

Total RNA was extracted from in vitro shoots using Purescript RNA isolation kit (Genta System Inc., Minneapolis, Minn.) as modified for woody plant tissue (Robert Webb, USDA–ARS, Kearneysville, W. Va., personal communication). Fifty micrograms total RNA from each transgenic pear clone and untransformed controls were used for RNA blots. The RNA was separated in a 1.4% formaldehyde agarose gel, transferred onto a Nytran membrane (Schleicher and Schuell, Keene, N.H.) by overnight capillary transfer using 20× SSPE (3 M sodium chloride, 0.2 M monobasic sodium phosphate, and 20 mM EDTA), and UV cross-linked. The rolC DNA fragment probe was generated using PCR, separated on 1.0% agarose gel, and eluted with a Boehringer Mannheim agarose gel extraction kit. The probe was labeled with [32P]CTP (NEN, Boston, Mass.) using RTS RadPrime DNA labeling system (BRL Life Technologies, Gaithersburg, Md.). After 2 h of prehybridization, the RNA blot was hybridized overnight at 42 °C with Hybrisol 1 solution (Oncor, Gaithersburg, Md.). After high stringency washing as suggested by the manufacturer, the blot was exposed to X-ray film with two intensifier screens at ~80 °C. In addition, the blot was exposed for two hours to a phosphor screen (Eastman Kodak Co., Rochester, N.Y.), and the phosphor image was scanned with a molecular imaging system (Storm 860; Molecular Dynamics, Sunnyvale, Calif.).

Extraction and immunoblotting of the rolC protein from in vitro grown transgenic pear clones were as described by Arora et al. (1992), with the following modifications. Total proteins were extracted by homogenizing in vitro rooted plantlets in the extraction buffer. After precipitating the proteins in 10% trichloroacetic acid at 0 °C for 30 min, and centrifuging for 15 min at 15,000 g, the pellets were resuspended in 0.1% L-methionine, 50 mM DTT, 4% SDS, and 0.01% bromophenol blue. Samples of 100 μg total protein were separated in 12% (w/v) sodium dodecyl sulfate polyacrylamide Ready Gels (Bio-Rad, Hercules, Calif.), and electroblotted to an immobilized-P membrane (Millipore Corp., Danvers, Mass.).

Primary antibody was anti-rolC rabbit IgG that had been purified by affinity chromatography as described by Estruch et al. (1991). The final concentration was 4 μg·mL⁻¹. Secondary antibody was anti-rabbit goat IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). The chromogenic substrate used was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/
shoot length and number of nodes on each plant were recorded. At placed at 4°C to 4°C, stocks were cut 2 to 4 cm above the inserted buds, and the plants were greenhouses for 7 months. One week following budding, the root- and regenerant controls after the plants had been growing in the plants of F22 and F23 transgenic clones, and the shoot proliferation stocks were 0.7 to 0.9 cm in diameter. Buds were excised from single self-rooted and grown at 28°C for budbreak. In June 1998 the plants were moved to the greenhouse. Total shoot length was measured in millimeters, and the number of nodes were counted in March 1998, and mean internode length per plant was computed by dividing shoot length by number of nodes. The data were subjected to 1/2 MS salts, MS vitamins, 20 g·L⁻¹ sucrose and 6 g·L⁻¹ agar, with pH adjusted to 5.6. Rooting cultures were incubated under light. Root initiation occurred within a week. After 2 to 3 weeks in the rooting medium, rooted plants were transferred to Magenta vessels containing an autoclaved potting mixture (4 perlite : 1 vermiculite), wetted with liquid MS salts and 5 g·L⁻¹ RootShield (Bio Works, Inc., Fresno, Calif.). One or 2 weeks later, the plants were transferred to 3-inch square peat pots containing the potting mix. Acclimatization of rooted plants in the greenhouse was achieved by covering the plants with inverted Magenta vessels and irrigating with water to pH 6 to 7) and planted in rooting medium containing 1/2 MS salts, MS vitamins, 20 g·L⁻¹ sucrose and 6 g·L⁻¹ agar, with pH adjusted to 5.6. Rooting cultures were incubated under light. Root initiation occurred within a week. After 2 to 3 weeks in the rooting medium, rooted plants were transferred to Magenta vessels containing an autoclaved potting mixture (4 perlite : 1 vermiculite), wetted with liquid MS salts and 5 g·L⁻¹ RootShield (Bio Works, Inc., Fresno, Calif.). One or 2 weeks later, the plants were transferred to 3-inch square peat pots containing the potting mix. Acclimatization of rooted plants in the greenhouse was achieved by covering the plants with inverted Magenta vessels and irrigating with MS salts containing Rootshield to prevent root and collar rots. The plants were maintained under light at 24°C. Three to four weeks later, the vessels containing plants were transferred to the greenhouse, and the plants were sprayed with an aqueous solution (1:20 v/v) of AntiStress NBT) (Bio-Rad, Hercules, Calif.).

Growth characteristics of transgenic plants. Both transgenic and untransformed plants were rooted in the laboratory growth room and acclimatized in the greenhouse. Shoots 2 to 4 cm long with three to seven leaves were used for rooting. The base of each shoot was dipped in 500 mg·L⁻¹ IBA (dissolved in 1 mL 1 n KOH and diluted with water to pH 6 to 7) and planted in rooting medium containing 1/2 MS salts, MS vitamins, 20 g·L⁻¹ sucrose and 6 g·L⁻¹ agar, with pH adjusted to 5.6. Rooting cultures were incubated under light. Root initiation occurred within a week. After 2 to 3 weeks in the rooting medium, rooted plants were transferred to Magenta vessels containing an autoclaved potting mixture (4 perlite : 1 vermiculite), wetted with liquid MS salts and 5 g·L⁻¹ RootShield (Bio Works, Inc., Fresno, Calif.). One or 2 weeks later, the plants were transferred to 3-inch square peat pots containing the potting mix. Acclimatization of rooted plants in the greenhouse was achieved by covering the plants with inverted Magenta vessels and irrigating with MS salts containing Rootshield to prevent root and collar rots.

Results and Discussion

Transformation. Following incubation on kanamycin selection medium, eighteen regenerated shoots of ‘Beurre Bosc’ and three regenerated shoots of ‘Bartlett’ were obtained from the fresh explants cultures inoculated with the 0.1 OD suspension. Seven regenerated shoots of ‘Beurre Bosc’ were obtained from the explants inoculated with the 0.5 OD suspension. No regenerants were obtained from explants that had been wounded and precultured before inoculation. Although preculture of explants has improved transformation efficiency in other plant species, such as flax (McHughen et al., 1989), it did not appear to be beneficial for pear transformation. No regeneration was obtained from uninoculated control explants cultured on kanamycin selection medium.

Histological and molecular assays. Positive GUS assays were obtained for two of the eighteen regenerated ‘Beurre Bosc’ plantlets inoculated with 0.1 OD inoculum and one of the seven ‘Beurre Bosc’ plantlets from the 0.5 OD inoculum. None of the three ‘Bartlett’ regenerants were GUS positive, suggesting that transformation had not occurred in this cultivar. Previous research has also shown that ‘Bartlett’ does not regenerate as easily as other pear cultivars (Leblay et al., 1991; R.L. Bell, unpublished data), which may explain the lack of GUS-positive ‘Bartlett’ transformants. Failure of integration of the GUS gene, as has been shown to occur in tobacco (Scorza et al., 1994), is another possible explanation.

PCR analysis detected the presence of the rolC gene in the three GUS-positive ‘Beurre Bosc’ clones (data not presented). DNA hybridization (Fig. 2) of the GUS-positive transgenic clones confirmed integration of the rolC gene into the ‘Beurre Bosc’ chromosomal DNA. DNA hybridization of the EcoRI digests indicated that the transgenic clone F23 had one copy of the rolC gene, while F22 had two copies, and F24 had three copies. The HindIII results shown that ‘Bartlett’ does not regenerate as easily as other pear cultivars (Leblay et al., 1991; R.L. Bell, unpublished data), which may explain the lack of GUS-positive ‘Bartlett’ transformants. Failure of integration of the GUS gene, as has been shown to occur in tobacco (Scorza et al., 1994), is another possible explanation.

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Budded plants were produced on ‘Bartlett’ seedling rootstock 0.7 to 0.9 cm in diameter. Buds were excised from single self-rooted plants of F22 and F23 transgenic clones, and the shoot proliferation and regenerant controls after the plants had been growing in the greenhouse for 7 months. One week following budding, the rootstocks were cut to 2 cm above the inserted buds, and the plants were placed at 4°C in the dark for 70 d to meet the dormancy requirement for budbreak. In June 1998 the plants were moved to the greenhouse and grown at 28°C under natural daylength. After 3 and 6 weeks, shoot length and number of nodes on each plant were recorded. At 6 weeks, the leaf area of three leaves from the middle of each shoot was measured using a portable area meter (LI-3000; LI-COR, Lincoln, Nebr.). Clone means were separated using the Waller-Duncan K ratio t test (K ratio = 100). The variance in leaf area among plants within clones was considered a random effect, and, therefore, was used as the error term in F tests of differences among clones.

Fig. 2. DNA blot of EcoRI digests, probed with a 514-bp internal fragment of rolC gene, of GUS-positive ‘Beurre Bosc’ pear clones F22, F23, and F24, plus an untransformed ‘Beurre Bosc’ clone (UT Bosc), following 1 h exposure.

Fig. 3. RNA blot, probed with a 514-bp internal fragment of the rolC gene, of transgenic ‘Beurre Bosc’ pear clones F22, F23, and F24, plus an untransformed clone of ‘Beurre Bosc’ (UT Bosc) as a negative control and a transgenic clone of tobacco (N. tab) containing two copies of the rolC gene as a positive control. The rolC transcript is ~540 bp in length (Nilsson and Olsson, 1997; Slightom et al., 1986).
Table 1. Mean height, number of nodes, and internode length of 6-month-old self-rooted F22, F23, and F24 transgenic clones, untransformed regenerants, and plants derived from shoot proliferation cultures of 'Beurre Bosc' pear.

| Clone       | Plants (no.) | Ht (cm) | Nodes (no.) | Internode length (cm) |
|-------------|--------------|---------|-------------|-----------------------|
| Proliferation | 10           | 36.0 a  | 23.5 a      | 1.6 a                 |
| Untransformed | 10           | 31.2 a  | 27.4 a      | 1.1 ab                |
| F24          | 10           | 9.9 b   | 13.4 b      | 0.8 b                 |
| F22          | 10           | 6.1 b   | 6.3 c       | 1.1 ab                |
| F23          | 5            | 3.8 b   | 5.9 c       | 0.8 b                 |

*Mean separation among clones by Waller-Duncan R ratio t test, K ratio = 100.

Table 2. Mean height, number of nodes, internode length, and leaf area of budding plants of two rolC transformed pear clones, F22 and F23, and untransformed regenerants, and plants derived from shoot proliferation cultures of 'Beurre Bosc' pear.

| Clone       | Plants (no.) | Ht (cm) | Nodes (no.) | Internode length (cm) | Ht (cm) | Nodes (no.) | Internode length (cm) | Leaf area (cm²) |
|-------------|--------------|---------|-------------|-----------------------|---------|-------------|-----------------------|----------------|
| Proliferation | 8            | 167.4 a  | 15.9 a      | 10.5 a                | 381.3 a | 33.6 a      | 11.2 a                | 6.6 a           |
| Untransformed | 3            | 109.7 b  | 15.7 a      | 6.9 b                 | 246.7 b | 26.0 b      | 9.0 b                 | 4.7 b           |
| F23          | 11           | 64.9 c   | 11.5 b      | 5.3 bc                | 72.7 c  | 21.9 bc     | 3.1 c                 | 2.7 c           |
| F22          | 14 (12)      | 38.8 c   | 9.8 b       | 3.5 c                 | 53.8 c  | 17.4 c      | 2.9 c                 | 2.4 c           |

*Mean of three leaves per plant.

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*Mean separation among clones by Waller-Duncan R ratio t test, K ratio = 100.

*At 6 weeks, only 12 plants were analyzed, due to broken grafts.
productivity is not adversely affected. Guivarc’h et al. (1996), using
immunolocalization, found that the expression of the rolC gene in
transgenic tobacco was restricted to companion cells of the phloem
strands, and to the initials of root protophloem cells. Their studies
also indicated a physical separation between the sites of expression
and actions of the rolC gene in transgenic plants, with meristem,
phloem, and leaf parenchyma cells affected by rolC expression,
presumably through an alteration in hormonal balance. Their findings
suggest that it is possible that rolC transgenic pears used as root-
stocks may have growth-altering effects on scions. The transgenic
clones will continue to be evaluated for differences in growth habit
to assess the potential of rolC for producing beneficial alterations in
growth and development in pear scion and rootstock cultivars.

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