

## Development of *Agrobacterium*-Mediated Transformation of Pear (*Pyrus communis* L.) with Cotyledon Explants and Production of Transgenic Pears Using ACC Oxidase cDNA

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### Abstract

To develop a transformation system for pear cultivars (*Pyrus communis* L.), we investigated the ability of cotyledon explants produced from seeds of mature fruit to regenerate, and monitored transgene expression during the early stages of the transformation procedure. The greatest shoot regeneration was induced on MS medium, supplemented with 2  $\mu$ M NAA and 30  $\mu$ M TDZ for 'La France' cotyledons (40%), and supplemented with 8  $\mu$ M NAA and 30  $\mu$ M TDZ for 'Bartlett' cotyledons (75%). Of the three cytokinins (TDZ, BA and Zeatin) examined, TDZ was the most effective for adventitious bud formation. To investigate the transfer of genes into pear cotyledon explants, we used a modified GUS gene that is expressed in plant tissues exclusively. Gene transfer was measured via transient expression of the GUS gene. The greatest frequency of gene transfer (82.7%) occurred when the explants were co-cultivated in the dark for 7 days after being inoculated with *Agrobacterium tumefaciens* strain EHA101 without pre-culture. A practical transformation was also conducted, in which carnation cDNA encoding an ACC oxidase (*DC-ACO*) was induced into cotyledon explants of two pear cultivars. Four transgenic 'Bartlett' seedlings and two 'La France' seedlings were obtained. Simultaneously, three lines of nontransformed control plant that had the same genome as the transgenic plants were obtained by inducing regeneration of a noninoculated cotyledon derived from the same seed as an inoculated cotyledon. PCR and Southern blot analyses confirmed the integration of the transgene in the genomes of all the transgenic lines.

**Key words:** ACC oxidase, adventitious bud, cotyledon explants, *Pyrus communis* L., regeneration, transformation.

### Abbreviations

ACC, 1-aminocyclopropane-1-carboxylate; BA, 6-benzylaminopurine; CaMV35S, 35S RNA of cauliflower mosaic virus; Cf, cefotaxime; GUS,  $\beta$ -glucuronidase; *hpt*, hygromycin phosphotransferase gene; IBA, indol-3-butyric acid; Km, kanamycin; NAA, 1-naphthaleneacetic acid; *nptII*, neomycin phosphotransferase II gene; TDZ, Thidiazuron; X-Gluc, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide.

### Introduction

Genetic transformation techniques are becoming one of the most important means of breeding crops, including most woody fruits. To date, some of the major fruit species, including apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.), and citrus, have been successfully transformed in attempts to improve pest and disease resistance, to manipulate fruit ripening, and to alter tree architecture. However, the genotypes that can be reliably transformed are quite limited, and for most of the economically

important fruit cultivars, genetic transformation is still in the developmental stage. The main problem with *Agrobacterium*-mediated transformation is its low efficiency of gene transfer (James and Dandekar, 1991), which needs to be improved. The first successful gene transfer in the pear was in 1996 (Mourgues *et al.*, 1996), lagging behind transformation in the apple (James *et al.*, 1989), and to date only a few commercial transgenes (*attacin E*, *rol C*, and *rol B*) have been introduced into pear: 'Passe Crassane' (Reynoird *et al.*, 1999), 'Beurre Bosc' (Bell *et al.*, 1999), and *Pyrus* root stocks (Zhu and Welander, 2000). Since pears are generally consumed when fresh in Japan, both flavor and shelf life are considered important, and their short shelf life severely limits their distribution and consumption. Our ultimate goal is to delay fruit softening and improve shelf life by manipulating ethylene biosynthesis, using genetic engineering techniques.

There are two essential requirements for *Agrobacterium*-mediated transformation: (1) stable introduction of a desired gene into the plant genome, which depends on the virulence of *Agrobacterium* in the target plant species and the inoculation conditions; and (2) regeneration of a fertile plant from the transformed cells (van Wordragen and Dons, 1992; Fisk and Dandekar, 1993; Schuerman and Dandekar, 1993). The latter requirement is the most important, because good regeneration ability is usually considered a crucial prerequisite for successful gene transfer. Although plant regeneration

has been achieved in several pear cultivars using leaf explants (Chevreau *et al.*, 1989; Predieri *et al.*, 1989; Leblay *et al.*, 1991) or cotyledon explants (Browning *et al.*, 1987), regeneration conditions for a given genotype must be optimized for further transformation work because the regeneration frequency is very genotype-dependent.

In this study, we measured the frequency of regeneration from cotyledon explants of two pear cultivars, 'La France' and 'Bartlett', which are the most popular cultivars in Japan, on several media, and investigated the factors that influence gene transfer to cotyledon explants during the early steps of the transformation procedure. Furthermore, we developed an easy and efficient transformation method using pear cotyledon explants; using this method, we successfully obtained transgenic pears that expressed the carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene.

## Materials and Methods

### Explant materials

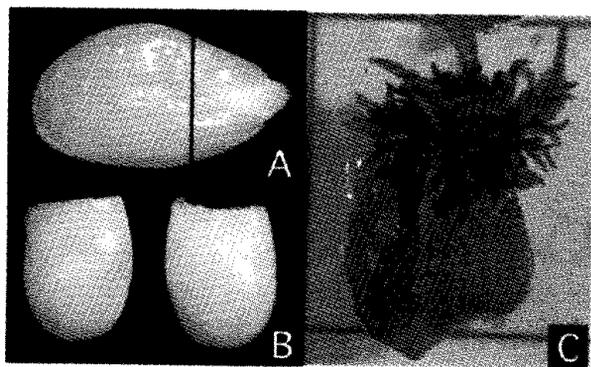
Mature pears (*Pyrus communis* L. cv. 'Bartlett' and 'La France') were harvested at commercial maturity from the orchard of the Yamagata Prefectural Horticultural Experimental Station, Japan, and stored at 5 °C. After three months, seeds from these fruits were used. The seeds were sterilized with 70% ethanol for 2 minutes, followed by 0.5% sodium hypochlorite solution containing 0.1% Tween 20 for 15 minutes, and then rinsed three times with sterile distilled water. The sterilized seeds were peeled, the proximal one-third of the embryo was excised on a clean bench, and the cotyledons were used as explant material (Fig. 1A, B).

### Adventitious bud formation

To determine the optimum basal medium and plant growth regulators, cotyledon explants were cultured on MS medium (Murashige and Skoog, 1962) or MS (1/2N), in which the nitrates were reduced to half strength. Both media were supplemented with 0.5  $\mu$ M 1-naphthaleneacetic acid (NAA) together with 20  $\mu$ M of either 6-benzylaminopurine (BA), Thidiazuron (TDZ), or zeatin. Control medium was prepared without cytokinin (Table 1).

To optimize the growth regulator concentration, explants were cultured on MS medium supplemented with 0, 0.5, 2, or 8  $\mu$ M NAA combined with 0, 3, 10, or 30  $\mu$ M TDZ in all possible combinations.

To determine whether kanamycin (Km) and cefotaxime (Cf) are suitable for use in the selection of



**Fig. 1** Induction of adventitious buds from pear cotyledons. Mature fruit of 'La France' and 'Bartlett' were harvested and stored at 5 °C for 3 months. Seeds from the fruit were sterilized with 70% ethanol for 2 min and with 0.5% sodium hypochlorite solution containing 0.1% Tween 20 for 15 min, and were then peeled on a clean bench. (A). A peeled seed. The vertical bar shows the position of the excision. (B). Cotyledon explants with the proximal one-third of the embryo removed. (C). Multiple adventitious shoot formation from a pear cotyledon explant.

**Table 1.** Effect of basal salt composition and cytokinins on adventitious bud formations from pear cotyledon explants. All media contained 0.5  $\mu$ M NAA

Basal medium	Cytokinin 20 $\mu$ M	Explants forming adventitious buds (%)	
		Cultivar	
		'La France'	'Bartlett'
MS	None	0	0
	BA	10	22
	TDZ	38	50
	Zeatin	30	0
MS (1/2 N)	None	0	0
	BA	20	14
	TDZ	30	25
	Zeatin	10	33

*nptII*-transgenic cells and for sterilizing *Agrobacterium*, explants were cultured on the optimum medium determined in the above experiments, supplemented with either 50 mg l<sup>-1</sup> Km or 300 mg l<sup>-1</sup> Cf.

All media used in the above experiments contained 0.8% (w/v) agar and 3% (w/v) sucrose and were adjusted to pH 5.8. The cultures were maintained at 25 °C under a 16-h photoperiod with a light intensity of 68  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (via cool white fluorescent light). After 30 days of culture, we estimated the percentage of explants forming adventitious buds and conditions of callus growth. Twenty explants were used in each treatment, and each experiment was repeated three times.

#### Investigation of gene transfer efficiency at an early stage

The effects of explant pre-cultivation, *Agrobacterium* strain, and duration and conditions of co-cultivation on the efficiency of early gene transfer were investigated by examining the transient expression of the GUS gene. Three strains of *Agrobacterium* (EHA101, LBA4404, and C58C1) harboring a p35S-GUS-OCS vector (Janssen and Gardner, 1989) were used to inoculate pear cotyledon explants. The plasmid vector p35S-GUS-OCS contained a kanamycin-resistance gene (*nptII*) under control of the CaMV 35S promoter, and a modified GUS gene that expresses GUS in plant cells but not in bacterial cells (because the prokaryotic ribosome binding site sequence upstream from its coding sequence has been replaced with a plant transcription sequence). This modified GUS gene was linked with the CaMV 35S promoter in the 5' region and

with the termination sequence of the octopine-synthase gene at its 3' end. The *Agrobacterium* strains were cultured in 523 medium (Kado *et al.*, 1972) for 24 h at 28 °C, centrifuged at 3,000 rpm for 15 minutes, and resuspended in low-pH (5.2) virulence-induction medium, consisting of MS with 20 mg l<sup>-1</sup> sucrose plus 0.1 mM acetosyringone and 1 mM proline (James *et al.*, 1993) for a final OD<sub>420</sub> of 0.5. The bacteria were then incubated at 25 °C for 5 h to induce bacterial virulence.

Cotyledon explants were either pre-cultivated in the dark for 4 days at 25 °C or not pre-cultivated at all. The explants were then inoculated by immersing them in the bacterial suspension for 15 min. Then they were blotted briefly on sterilized filter paper and placed on the co-cultivation medium. Co-cultivation was carried out for 3, 5, or 7 days at 25 °C, either in the dark or under a 16-h light/day. The explants were then transferred to disinfection medium containing 300 mg l<sup>-1</sup> Cf. After one week, expression of the GUS gene by the explants was determined by the method of Jefferson *et al.* (1987) with several modifications. The explants were incubated overnight at 37 °C in a 1 mg ml<sup>-1</sup> solution of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-Gluc) in 50 mM sodium phosphate (pH 7.0). The explants were then washed with 70% ethanol. The efficiency of early gene transfer was defined as the percentage of explants in which over 80% of the area of the cut ends was stained blue. The media used in pre-cultivation, co-cultivation, and disinfection are described in the Results section.

#### Transformation of pear cotyledons with the ACC oxidase gene

The plasmid vector pMLH2113-DCACO (OR+) (Fig. 4B; Mitsuhashi *et al.*, 1996; Kosugi *et al.*, 2000), containing carnation cDNA encoding ACC oxidase (DC-ACO; kindly provided by Dr. Shigeru Satoh of Tohoku University, Japan), was used for practical transformation based on the transformation system described above. To obtain isogenic transformed and nontransformed plants simultaneously, two pieces of cotyledon from the same seed were treated individually: one piece was inoculated with *Agrobacterium* to produce a transgenic plant, and the other was directly induced to regenerate to obtain a nontransformed control plant. The cotyledon explants from each seed were numbered to distinguish the two pieces of cotyledon from the same seed.

To obtain transformants, the explants were inoculated, co-cultivated, and disinfected using the optimum treatment determined in the above experiments. The inoculated explants were then

transferred to selection medium, which comprised the optimum medium plus 50 mg l<sup>-1</sup> Km and 300 mg l<sup>-1</sup> Cf. The explants were cultured in 16-h light/day conditions at 25 °C, and were transferred to fresh medium every two weeks. Adventitious buds that formed from the inoculated explants were removed and transferred to shoot proliferation medium, which consisted of MS with 0.5 mg l<sup>-1</sup> BA plus 50 mg l<sup>-1</sup> Km and 300 mg l<sup>-1</sup> Cf. The shoots were maintained by sub-culturing the axillary buds in medium that was changed every 6 weeks. To obtain nontransformed control shoots, the other piece of the cotyledon explant was treated in the same manner, but was not inoculated with *Agrobacterium*, and the medium used contained no antibiotics.

After several subcultures, shoots from 3 to 5 cm high (transformants and nontransformed control) were transferred to MS (1/2N) medium supplemented with 5 μM IBA (indol-3-butyric acid) to induce rooting. These shoots were cultured at 25 °C in the dark for the first two weeks, and then transferred to a 16-h light/8-h dark cycle at 25 °C. The rooted shoots were planted in plastic packs of an autoclaved perlite:vermiculite mixture (1:1) wetted with 1/1,000 HYPONEX® (HYPONEX, Japan), and grown under the same conditions. After 1.5 to 2 months, the cover of each pack was gradually removed and the pack was irrigated with HYPONEX® to prevent the plantlet from wilting (this process is referred to as *acclimation*). When the cover had been completely removed and new shoots had grown, the plantlets were transplanted to pots filled with soil and grown in a P1-level greenhouse under natural light at 25 °C.

#### PCR analysis and Southern blot analysis

For PCR analysis, genomic DNA was isolated from the leaves of shoot cultures by the SDS method (Edwards *et al.*, 1991). The primers used to amplify the three genes were as follows: for *nptII* (654-bp product): 5'-GCTTGGGTGGAGAGGC-TATT-3' and 5'-CCCGTGATATTGCTGAAGAG-3'; for the full-length ORF of *DC-ACO* cDNA (966-bp product): 5'-ATGGCAAACATTGTCAACTT-3' and 5'-TCAAGCAGTTGGAATGGGAC-3'; and for *hpt* (500-bp product): 5'-ATGAAA-AAGCCTGAACTCACCGCGA-3' and 5'-TCC-ATCACAGTTTGCCAGTGATACA-3'. PCR amplification was conducted using total reaction volumes of 25 μl, and 30 cycles of 45 sec at 94 °C, 1 min at 60 °C, and 2 min at 72 °C.

For genomic blot analysis, total DNA was extracted according to the modified CTAB method for deciduous fruit trees (Yamamoto *et al.*, 2001). Five

micrograms of genomic DNA were digested with *HindIII*. The digest was separated on a 0.8% agarose gel and blotted onto a positively charged nylon membrane (Roche Diagnostics GmbH, Germany). The filter was probed with DIG (Boehringer Mannheim, Germany)-labeled coding regions of the *nptII*, *DC-ACO*, and *hpt* genes.

## Results and Discussion

### *Effect of basal medium and plant growth regulators on adventitious bud formation*

To determine a suitable auxin for regeneration of pear cotyledon explants, preliminary experiments were carried out in which explants were cultured on MS medium supplemented with either 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA (3 mg l<sup>-1</sup> or 6 mg l<sup>-1</sup>) combined with BA, TDZ, or zeatin (2 mg l<sup>-1</sup>). The calli formed from the cut ends in 2,4-D media were loose and colorless and did not undergo organogenesis, whereas hard globular calli and adventitious buds were formed in NAA media (data not shown). Browning *et al.* (1987) reported the successful induction of shoot and root regeneration from 'Bartlett' cotyledon explants, and concluded that the highest frequency of shoot organogenesis was obtained on medium supplemented with 5 mg l<sup>-1</sup> BA and 0.1 mg l<sup>-1</sup> NAA. Replacing NAA with 0.1 mg l<sup>-1</sup> 3-indoleacrylic acid (IAA) increased the frequency of root regeneration at the expense of shoot production. The addition of 0.1 mg l<sup>-1</sup> 2,4-D to the medium completely prevented shoot and root production, stimulating vigorous callus development (Browning *et al.*, 1987). Similar studies, in which NAA, IBA, and 2,4-D were examined, reported that NAA was the most effective auxin for shoot regeneration from leaf tissue in pear (Chevreau *et al.*, 1989). Therefore, we chose to use NAA as the auxin in subsequent experiments.

The cotyledon explants swelled and became green within one week of initiating culture; in some cases, greenish calli developed from the cut ends. The earliest adventitious bud formation occurred 10 to 15 days after starting the culture. Almost all the adventitious buds formed on the cut surface, and most formed by indirect organogenesis, in which regeneration occurred on callus tissues and development usually continued in clusters (**Fig. 1C**). Although the calli were small or undistinguished in some cases, the tissues around the cut ends were usually swollen and callused. Kouider *et al.* (1984) studied adventitious shoot formation in apple cotyledons subjected to different excision treatments. They reported that an adventitious shoot formed when the embryonic axis was excised and the

cotyledons were grown *in vitro*, and that adventitious shoot formation occurred only at the proximal cut end of the cotyledon. Adventitious shoot formation and development was optimal with excision of the proximal one-third of the embryo. We excised pear cotyledons in the same manner and obtained similar results.

For 'La France' and 'Bartlett' pear cotyledons, MS basal medium seems to induce adventitious bud formation better than MS (1/2N) medium (Table 1). No shoot regeneration was observed with MS containing 0.5  $\mu\text{M}$  NAA and no cytokinin, but many adventitious roots formed (data not shown). TDZ was the best cytokinin of the three tested; 38% and 50% of the cotyledon explants from 'La France' and 'Bartlett' formed adventitious buds, respectively, in MS supplemented with 0.5  $\mu\text{M}$  NAA and 20  $\mu\text{M}$  TDZ (Table 1).

We tested four concentrations of TDZ and NAA to optimize the concentrations of each growth regulator: 0, 0.5, 2, and 8  $\mu\text{M}$  NAA and 0, 3, 10, and 30  $\mu\text{M}$  TDZ (16 combinations in total). Callus formation from cotyledons of the two pears was positively related to the NAA concentration, but the NAA requirements for callus initiation differed between

cotyledons of the two cultivars. Callus formation was promoted by 0.5 to 2  $\mu\text{M}$  NAA in 'La France' cotyledons, whereas 'Bartlett' cotyledons formed calli in the absence of auxin, although this required a high TDZ concentration (Table 2). The effects of NAA and TDZ on adventitious bud formation also differed between cotyledons of the two cultivars. Auxin and cytokinin were necessary for bud regeneration in 'La France' cotyledons, whereas regenerated buds were observed in the absence of NAA in 'Bartlett' cotyledons, although cytokinin was necessary (Table 2). The greatest percentage of adventitious bud formation was obtained by combining 2  $\mu\text{M}$  NAA and 30  $\mu\text{M}$  TDZ for 'La France' cotyledons (40% formation), and 8  $\mu\text{M}$  NAA and 30  $\mu\text{M}$  TDZ for 'Bartlett' cotyledons (75% formation). In contrast, adventitious roots formed from explants of the two cultivars only in medium containing more than 2  $\mu\text{M}$  NAA, regardless of the presence of TDZ (Table 2), indicating that auxin alone is an indispensable growth regulator for root regeneration from pear cotyledons. Although high concentrations of NAA (2 and 8  $\mu\text{M}$ ) and TDZ (30  $\mu\text{M}$ ) were advantageous for bud regeneration, the buds were more frequently irreg-

**Table 2.** Effect of NAA and TDZ concentration on callus, adventitious bud and root formations from pear cotyledon explants. MS was used for basal medium.

Plant growth regulator ( $\mu\text{M}$ )		Condition of explants with callus <sup>1)</sup>		Explants forming adventitious buds (%)		Explants forming adventitious Roots (%)	
NAA	TDZ	'La France'	'Bartlett'	'La France'	'Bartlett'	'La France'	'Bartlett'
0	0	-	-	0	0	0	0
	3	+	+	0	0	0	0
	10	+	+	0	0	0	0
	30	+	++	0	20	0	0
0.5	0	+	+	0	0	0	0
	3	++	++	15	0	0	0
	10	+	++	10	28	0	0
	30	+	++	24	30	0	0
2	0	+	++	0	0	20	10
	3	++	+++	10	70	0	10
	10	++	+++	35	65	5	0
	30	++	+++	40	68	10	5
8	0	++	+++	0	0	43	30
	3	+++	++++	30	44	30	39
	10	+++	++++	15	50	0	45
	30	++++	++++	35	75	25	10

<sup>1)</sup> - : explants did not change in size, and no callus occurred;

+ : explants grew a little but not swelling, rare callus occurred at cut surface;

++ : explants grew larger, a little and compact callus occurred at cut surface;

+++ : explants were overall swelling, more and hard globular callus occurred around cut surface;

++++ : explants were overall swelling and callused, a lot of callus occurred around cut surface

ular, and these irregular buds did not develop into normal shoots. Therefore, we used MS medium supplemented with 1  $\mu$ M NAA and 20  $\mu$ M TDZ to investigate gene transfer efficiency and to conduct practical transformation experiments.

In tests of the effects of antibiotics in selecting transformants and sterilizing bacteria, 300 mg l<sup>-1</sup> Cf did not affect adventitious bud formation or regeneration frequency, whereas none of the cotyledon explants of either pear cultivar survived 30 days in medium containing 50 mg l<sup>-1</sup> Km (data not shown).

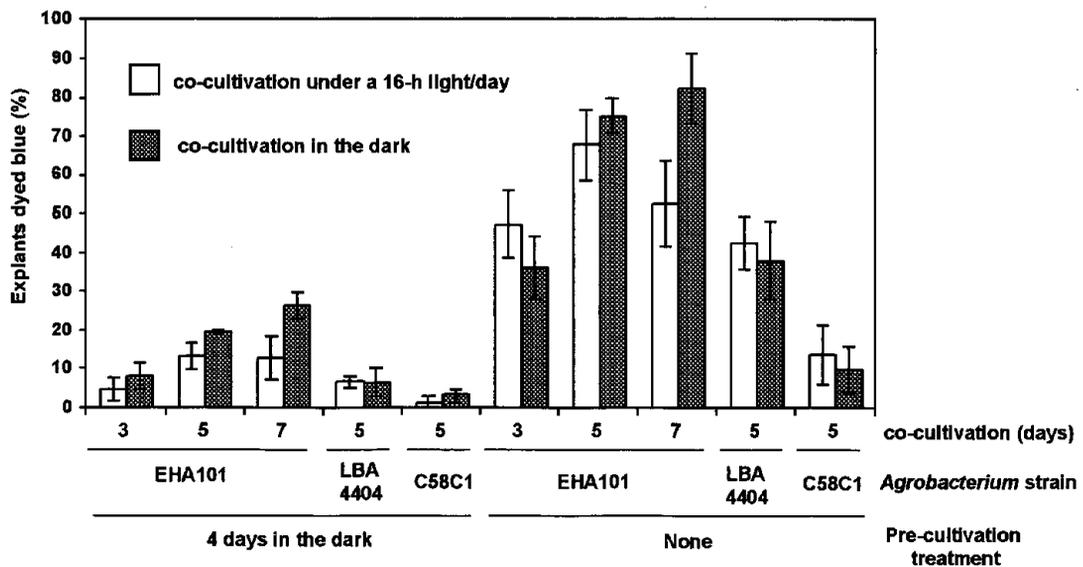
*Effects of pre-cultivation treatment of explants, Agrobacterium strain, and co-cultivation on gene transfer efficiency*

The ability to transfer genes into cotyledons of the two pear cultivars in the early stages of transformation was affected by the pre-cultivation treatment of explants, *Agrobacterium* strain, and duration and conditions of co-cultivation. **Fig. 2** shows the percentage of 'Bartlett' cotyledon explants in which transient expression of the GUS gene was detected in each treatment after pre-cultivation, inoculation with *Agrobacterium*, co-cultivation, and one-week disinfection with Cf. Because the GUS gene used in this study has been modified to create a eukaryotic translational initi-

ator while simultaneously destroying the consensus bacterial ribosome-binding site, its activity is negligible in *Agrobacterium* but good in plant cells (Janssen and Gardner, 1989; Raineri *et al.*, 1990). Therefore, we were able to eliminate contamination due to activity of GUS derived from *Agrobacterium*. For the 'Bartlett' cotyledons, *Agrobacterium* EHA101 was the most effective of the three strains tested. Pre-cultivation of explants had a notably negative effect on gene transfer, and co-cultivation in the dark was more advantageous than 16-h light/day. Similar results were obtained with 'La France' cotyledons (data not shown). For 'Bartlett' cotyledons, the highest frequency of transient expression of GUS activity (82.7%) was obtained when explants were not cultured before being inoculated with *Agrobacterium* strain EHA101, and when co-cultivation was carried out for seven days in the dark. Although seven days of co-cultivation maximized early transformation efficiency, the damage to explants was relatively serious and bacterial propagation inhibited regeneration. Therefore, we used five days of co-cultivation for the practical transformation.

*Transformation of pear with the ACC oxidase gene*

Based on the results described above, a test of



**Fig. 2** Effect of pre-cultivation treatment of explants, *Agrobacterium* strains, and co-cultivation on transient expression of GUS gene. 'Bartlett' cotyledons were subjected to either pre-cultivation for 4 days in the dark or no pre-cultivation treatment. Then, the explants were inoculated with EHA101, LBA4404, or C58C1 harboring a p35S-GUS-OCS vector by immersing them in bacteria culture for 15 min. The inoculated explants were placed on co-cultivation medium and cultured for 3, 5, or 7 days in the dark or under 16-h light/day. After co-cultivation, the explants were transferred to disinfection medium containing 300 mg l<sup>-1</sup> Cf and cultured for one week. Then, the explants were used to examine transient expression of the GUS gene. Twenty explants were used for each treatment and three replicates were conducted. The vertical bars indicate the standard error (n=3).

practical transformation was conducted, using 72 'La France' seeds and 96 'Bartlett' seeds. As described in the Methods section, one piece of cotyledon explant from each seed was inoculated with EHA101 harboring pMLH2113-*DCACO* (OR+) plasmid vector containing *DC-ACO* cDNA in the sense orientation. After inoculation, the cotyledon explants were placed on co-cultivation medium, which consisted of MS medium supplemented with 1  $\mu$ M NAA and 20  $\mu$ M TDZ (pH 5.2). Co-cultivation was carried out for 5 days at 25 °C in the dark. After co-cultivation, the explants were transferred to disinfection medium containing 300 mg l<sup>-1</sup> Cf and cultured in a 16-h-light photoperiod for seven days. They were then transferred to the same MS medium, this time containing 50 mg l<sup>-1</sup> Km, to select transformants.

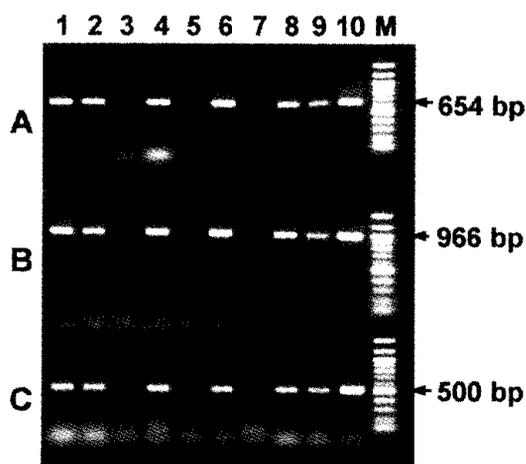
In the first three weeks after inoculation, seven lines of adventitious bud were obtained from individual explants: two from 'La France' cotyledons [named La (3)-19 and La (3)-21] and five from 'Bartlett' cotyledons [named B (5)-3, B (5)-4, B (5)-5, B (9)-8, and B (13)-10]. These adventitious buds were transferred to shoot proliferation medium containing 50 mg l<sup>-1</sup> Km and 300 mg l<sup>-1</sup> Cf, and all of them showed kanamycin resistance. Ultimately, seven vigorous lines of transformed shoots were obtained. Based on the inoculated explants, the transformation rates were 2.7% for 'La France'

cotyledons and 5.2% for 'Bartlett' cotyledons. Simultaneously, we obtained three lines of adventitious buds from the cotyledons corresponding to the transformants from the noninoculated controls; they were all from 'Bartlett' cotyledons [named BC (5)-4, BC (5)-5, and BC (9)-8]. No nontransformed control plants for La (3)-19, La (3)-21, B (5)-3, and B (13)-10 were obtained.

PCR analysis using the *nptII*, *DC-ACO*, and *hpt* primers yielded the expected products in all the transgenic lines (Fig. 3), except B (13)-10, which was contaminated during sub-culturing. No amplification products were recognized in the three isogenic nontransformed control lines (Fig. 3; Lanes 3, 5 and 7).

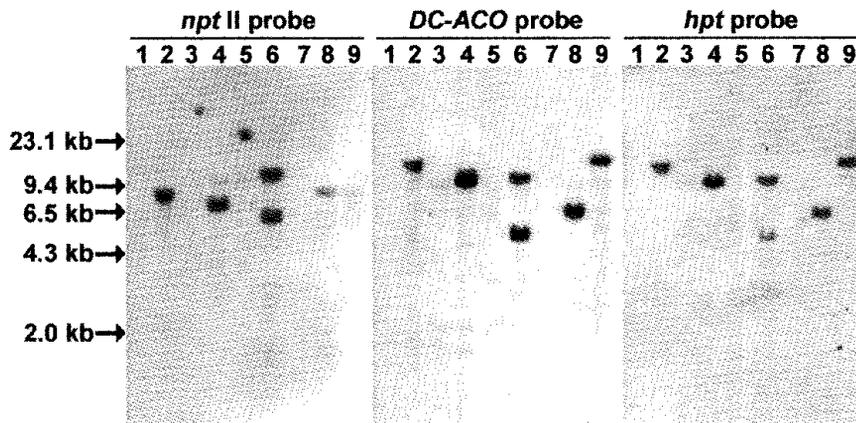
Stable genetic transformation was also confirmed by Southern blot analysis in three 'Bartlett' seedling lines [B (5)-4, B (5)-5, B (9)-8] and two 'La France' seedling lines. The number of copies of the T-DNA inserted into the genome could be estimated by hybridizing the same DNA blot with the *nptII*, *DC-ACO*, and *hpt* probes. The results showed that a single copy was integrated in the genomes of B (5)-4, B (5)-5, La (3)-19, and La (3)-21 (Fig. 4; Lanes 2, 4, 8, and 9), while two copies were integrated in B (9)-8 (Fig. 4; Lane 6). No signal was detected in the three control lines [BC (5)-4, BC (5)-5, and BC (9)-8] (Fig. 4; Lanes 1, 3 and 5).

Although transformation of several pear cultivars has been reported, including 'Conference' (Mourgues *et al.*, 1996), 'Beurre Bosc' (Bell *et al.*, 1999), and 'Passe Crassane' (Reynoird *et al.*, 1999), the transformation rates were generally 1% to 4%, with the exception of an impressive efficiency of up to 42% in 'Conference'. In most of these cases, obtaining transgenic shoots took at least 6 months, whereas we obtained transgenic shoots within 1.5 months. Our results demonstrate that transformants of woody fruit trees can be produced quickly using cotyledons when no specific cultivar is considered. Moreover, we developed a new transformation method, in which two pieces of cotyledon from the same seed are treated individually to produce transformed and nontransformed plants. Our method overcomes the disadvantage of transformants originating from different cotyledons and thus differing both genetically and physiologically, because individual cotyledons are genetically heterogeneous. As a result, we were able to strictly estimate the expression and function of the transgenes. We have already obtained transgenic plantlets that integrate sense *DC-ACO* cDNA and their isogenic nontransformed controls. The morphology of transformed and nontransformed plantlets did not obviously differ (Fig. 5).

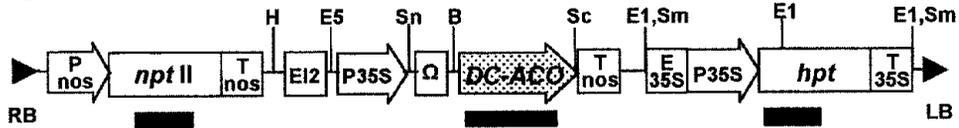


**Fig. 3** PCR analysis for the *nptII* (A), *DC-ACO* (B), and *hpt* (C) genes using leaves of *in vitro* shoots. Lane 1, B (5)-3 transgenic line; Lane 2, B (5)-4 transgenic line; Lane 3, BC (5)-4 nontransformed control; Lane 4, B (5)-5 transgenic line; Lane 5, BC (5)-5 nontransformed control; Lane 6, B (9)-8 transgenic line; Lane 7, BC (9)-8 nontransformed control; Lane 8 and 9, La (3)-19 and La (3)-21 transgenic lines; Lane 10, EHA101/pMLH2113-*DCACO* (OR+); Lane M, 100-bp DNA ladder marker.

A



B



**Fig. 4** Southern blot analysis of transformed pear shoots. (A) The DNA blot was hybridized with three probes, *nptII* (left), *DC-ACO* (middle), and *hpt* (right). Lane 1, BC (5)-4 nontransformed control; Lane 2, B (5)-4 transgenic line; Lane 3, BC (5)-5 nontransformed control; Lane 4, B (5)-5 transgenic line; Lane 5, BC (9)-8 nontransformed control; Lane 6, B (9)-8 transgenic line; Lane 7, blank; Lanes 8 and 9, La (3)-19 and La (3)-21 transgenic lines. (B) Physical map of the T-DNA region of pMLH2113-*DCACO*(OR+). The solid bars below the construction show the positions of the probes used for Southern blot analysis. (RB: right border; LB: left border; Pnos: promoter of nopaline synthase gene; *nptII*: neomycin phosphotransferase gene for kanamycin resistance; Tnos: terminator of nopaline synthase gene; E12: 5'-upstream sequence of the CaMV 35S promoter; P35S: CaMV 35S promoter;  $\Omega$ : 5'-untranslated sequence of TMV; *DC-ACO*: carnation cDNA encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase; E35S: enhancer of 35S promoter; *hpt*: gene for hygromycin resistance; T35S: polyadenylation signal of the CaMV 35S transcript. H, *HindIII*; E5, *EcoRV*; Sn, *SnaBI*; B, *BamHI*; Sc, *SacI*; E1, *EcoRI*; Sm, *SmaI*.)



**Fig. 5** Appearance of pear plants transformed with carnation cDNA encoding ACC oxidase. There are no obvious differences in the morphology of the isogenic transgenic and nontransformed control plants derived from the same seed. From the left: B (5)-5 transgenic plant; BC (5)-5 nontransformed control plant; B (9)-8 transgenic plant; BC (9)-8 nontransformed control plant.

Since our ultimate goal is to improve the shelf life of pears by manipulating ethylene biosynthesis, we induced an ACC oxidase into pear cotyledons. Kosugi *et al.* (2000) reported that the cut flowers from a carnation plant transformed with the same

vector, pMLH2113-*DCACO* (OR+), had twice the vase life of control flowers due to decreased ethylene production. Therefore, introducing this gene into pear plants is expected to prolong the shelf life of the fruit.

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