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Development of an *Agrobacterium*-mediated transformation method for pear (*Pyrus communis* L.) with leaf-section and axillary shoot-meristem explants

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Abstract We have developed a new *Agrobacterium*-mediated transformation method for the low-frequency-regenerating pear (*Pyrus communis* L.) cvs. Silver bell and La France. Leaf sections derived from in vitro shoots were initially used for the transformation procedure. Under optimum transformation conditions, which included culture and selection on 30 mg/l kanamycin (Km) combined with 500 mg/l sulbenicillin, a 3.2% transformation efficiency was obtained for cv. Silver bell, but no transformants of La France were obtained because of the very low regeneration frequency. Axillary shoot meristems were then examined as potential explants for La France. Selection in 5 mg/l Km and 375 mg/l carbenicillin resulted in transformed shoots being produced at an efficiency of 4.8%, and the apparent white Km-sensitive shoots were not formed during a 2-year subculture on micropropagation medium containing 50 mg/l Km. Therefore, transformations using axillary shoot meristems may be an alternative method for pear cultivars recalcitrant to regeneration from leaf sections.

Keywords Axillary shoot meristem · Leaf section · *Pyrus communis* L. · Regeneration · Transformation

Abbreviations *BA:* 6-Benzylaminopurine · *CaMV 35S:* 35S RNA of cauliflower mosaic virus · *Cb:* Carbenicillin · *Cf:* Cefotaxime · *GUS:* β-Glucuronidase · *hpt:* Hygromycin phosphotransferase gene · *Hyg:* Hygromycin B · *Km:* Kanamycin · *NAA:* α-Naphthaleneacetic acid · *nptII:* Neomycin phosphotransferase II gene · *Sb:* Sulbenicillin · *TDZ:* Thidiazuron

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Introduction

Many problems are associated with the traditional cross-breeding of fruit trees such as pear; these problems include a long juvenile period, high levels of genetic heterozygosity, and the need for a large area to screen ideal cultivars. Genetic transformation provides an alternative means for elucidating gene function and for making targeted, single-trait improvements in clonally propagated crops.

A number of genes have recently been isolated from fruit trees and analyzed following their introduction into model plants such as *Arabidopsis thaliana*, *Lycopersicon esculentum*, or *Nicotiana tabacum*. However, such model plants are unsuitable for examining genes for fruit features such as fruit development, maturation, and pathogen resistance that have been isolated from fruit trees. For successful analysis, these genes must be introduced into the fruit trees from which they were isolated. To date, genetic transformation methods have been established for some of the major fruit species, such as apple (*Malus* × *domestica* Borkh; De Bondt et al. 1996), grapefruit (*Citrus paradisi*; Luth and Moore 1999), and grapevine (*Vitis vinifera*; Yamamoto et al. 2000). With respect to pear (*Pyrus communis* L.), transformation has been achieved with leaf-section explants, but its efficiency is cultivar-dependent. Breeders have been successful at transferring markers or desirable genes in cvs. Burre Bosc (Bell et al. 1999), Conference, Doyenne du Comice, and Passe-Crassane (Mourgues et al. 1996). Nevertheless, it is still difficult to transform certain other pear cultivars, such as La France and Silver bell, because of the low regeneration frequency of the leaf-section explants. In Japan, La France and Silver bell are the major cultivars used in commercial pear production. Although La France has been transformed using cotyledon explants

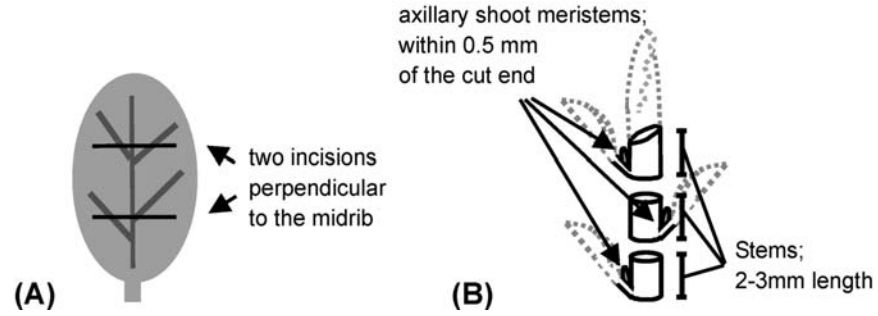

Fig. 1 Schematic representation of the procedure adopted for making the explants. **a** Leaf-section explants, **b** axillary shoot-meristem explants

(Gao et al. 2002), the transformants do not have the same genetic background as La France. Consequently, methods using cotyledons are not a practical source for improving fruit trees, including La France.

In the study reported here, we improved *Agrobacterium*-mediated transformation methods for two economically important cultivars, Silver bell and La France, using leaf sections or axillary shoot meristems as explants, respectively.

Materials and methods

Explant materials

Leaf sections and axillary shoot meristems excised from 4-week-old in vitro shoots of pears (*Pyrus communis* L. cvs. Silver bell or La France) were used as explants in this study. Clonal shoots were cultured in micropropagation medium consisting of MS basal salts and vitamins (Murashige and Skoog 1962), 1.0 mg/l BA, 30 g/l sucrose, and 0.8% agar at pH 5.8. The cultures were grown at 25°C under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent lighting at an intensity of 68 μmol m^2 per second. Leaves of Silver bell were excised and wounded by making two incisions perpendicular to the midrib, taking care not to cut through the leaf edges (Fig. 1a). Axillary shoot meristems of La France, 2–3 mm in length, were excised from the stems, taking care to include the axillary meristems within 0.5 mm of the cut end (Fig. 1b).

Agrobacterium strain, vector, and inoculation

Agrobacterium tumefaciens strain EHA101 (Hood et al. 1986) containing pIG121-Hm was used. pIG121-Hm is a binary vector that contains the *npt*II gene for kanamycin resistance, the *hpt* gene for hygromycin (Hyg) resistance, and the *GUS* gene—which contain an intron at the 5′ end of the coding sequence—in the T-DNA region (Hiei et al. 1994). The *GUS* and *hpt* genes were connected to the *35S* promoter of cauliflower mosaic virus (Ohta et al. 1990). *A. tumefaciens* was grown for 24 h at 28°C in YEP medium (10 g/l Bacto peptone, 10 g/l Bacto yeast extract, 5 g/l NaCl, and 2 m*M* $MgCl_2$, pH 7.2) containing 50 mg/l Km and 50 mg/l Hyg. The culture was diluted to 1/10 with inoculation liquid medium containing 1/10 NN basal salts and vitamins (Nitsch and Nitsch 1969), 63.0 g/l sucrose, 36.0 g/l glucose, 0.02% pluronic F68 (Sigma, St. Louis, Mo.), and 100 μM acetosyringone at pH 5.2. Pluronic F68 and glucose were added to the inoculation liquid medium because they have a positive effect on *Agrobacterium* inoculation (Cheng et al. 1997). Leaf-section and axillary shoot-meristem explants were soaked in the *Agrobacterium* inoculation liquid for 5–10 min, placed on a sterilized paper towel to remove the remaining liquid, and transferred to a piece of filter paper placed on co-culture medium (NN basal salts and vitamins, 5.0 mg/l TDZ, 0.2 mg/l NAA, 3.0% sucrose, 0.4% gelrite, pH 5.2).

Transformation using leaf sections as explants

After inoculation, the leaf sections were cultured for 5 days in the dark at 25°C on co-culture medium. The leaf sections were used to determine the efficiency of the antibiotic selection of transformants or the elimination of *Agrobacterium*.

For selection purposes, the leaf sections were transferred, following co-culture, to regeneration medium (NN basal salts and vitamins, 5.0 mg/l TDZ, 0.2 mg/l NAA, 3.0% sucrose, 0.8% phytoagar, pH 5.8) supplemented with 500 mg/l cf and cultured for 1 week. The leaf sections were then cultured on regeneration medium supplemented with 25, 30, 40, or 50 mg/l Km and 500 mg/l Cf.

To eliminate *Agrobacterium*, we used four combinations of antibiotics combinations: (1) 500 mg/l sulbenicillin (Sb500), (2) 250 mg/l Sb+250 mg/l carbenicillin (Sb250+Cb250), (3) 375 mg/l Cb (Cb375), and (4) 500 mg/l Sb was used for the first month, then 500 mg/l Cb (Sb500-to-Cb500). Following co-culture, the leaf sections were cultured on regeneration medium supplemented with the above-mentioned combinations of antibiotics for 1 week (i.e., combinations 1–4. The leaf sections were then transferred to regeneration medium supplemented with 30 mg/l Km and the same four combinations of antibiotics. The cultures were grown at 25°C in the dark for 1 month and then transferred to a 16/8-h (light/dark) photoperiod. The cultures were transferred to fresh medium every 4 weeks. Following selection for 2 months, the transient transformation efficiency was calculated using the number of green calli and GUS-positive calli assayed, as described by Jefferson et al. (1987). The Km-resistant calli were subcultured in regeneration medium, and the regenerated green shoots were excised and transferred to micropropagation medium supplemented with 50 mg/l Km and 500 mg/l Sb.

Transformation using axillary shoot-meristem explants

The axillary shoot meristems inoculated with *Agrobacterium* were cultured on co-culture medium for 5 days in the dark at 25°C. The axillary shoot meristems were used to determine the efficiency of Km selection or the elimination of *Agrobacterium*.

For selection, the explants were transferred, following co-culture, to regeneration medium supplemented with 500 mg/l Cf and 5, 10, or 25 mg/l Km.

For elimination, the explants were transferred, after co-culture, to, and cultured on, regeneration medium supplemented with the same antibiotics used for the leaf sections described above, except that 5 mg/l Km was used. The cultures were grown at 25°C under a 16/8-h (light/dark) photoperiod. After a 2-month period of growth in this selection culture, the green shoots were excised and transferred to micropropagation medium containing 50 mg/l Km and 500 mg/l Sb. The transgenic shoots were maintained on 50 mg/l Km by transferring the axillary bud to fresh medium every 4 weeks.

Table 1 Comparison of the efficiency of the antibiotic kanamycin for transformant selection in cv. Silver Bell 2 months after co-cultivation

Km (mg/l)	Number of explants	Number of selected green calli	Number of GUS-positive calli	Survival rates (%)[a]	Rates of GUS-positive (%)[b]	Transient transformation efficiency (%)[c]
25	150	48	3	32.0	6.3	2.0
30	131	97	16	74.0	16.5	12.2
40	148	70	3	47.3	4.3	2.0
50	96	45	6	46.9	13.3	6.3

[a] Number of selected green calli/Number of explants
[b] Number of GUS-positive calli/Number of selected calli
[c] Number of GUS-positive calli/Number of explants

Confirmation of transformation

Genomic DNA was extracted from the leaves of Km-resistant shoots using the modified CTAB protocol (Yamamoto et al. 2000). A pair of primers for amplifying part of the *GUS* gene was designed: the forward and backward sequences were 5′-GCA ACG TCT GGT ATC AGC GC-3′ and 5′-ACG GTT TGT GGT TAA TCA GG-3′, respectively. A pair of primers for amplifying part of the *npt*II gene was designed: the forward and backward sequences were 5′-GCT TGG GTG GAG AGG CTA TT-3′ and 5′-CTC TTC AGC AAT ATC ACG GG-3′, respectively. PCR analyses were performed in a 20-μl reaction volumes containing 10 ng of genomic DNA, 1 μM each primer, 200 μM dNTPs, and 0.5 U Ex *Taq* polymerase (TaKaRa, Japan) in 1× Ex *Taq* polymerase buffer using a DNA thermal cycler (Perkin Elmer, Foster City, Calif.). Amplification was carried out using 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final 6 min at 72°C. The amplified DNA fragments were electrophoresed on 0.8% agarose gels and detected using ethidium bromide staining.

Results and discussion

Transformation using leaf sections as explants

We initially investigated the regeneration frequency from leaf explants of two pear cultivars, La France and Silver bell, using MS-based medium. However, these frequencies were much lower than those of other major cultivars for which transformation systems have been reported—Conference: 97%, Passe-Crassane: 77%, Doyenne du Comice: 71%; Leblay et al. 1991). Hennayake et al. (2003) reported distinct improvements in the regeneration frequency from cvs. La France (10.7%) and Silver bell (35.4%) when a NN-based medium was used. Although this frequency was still lower than those obtained for the cultivars tested by Leblay et al. (1991), we attempted to develop a improved transformation method for the low-regeneration cultivars La France and Silver bell based on NN-based medium in lieu of the methods reported for high-regeneration cultivars (Mourgues et al. 1996; Bell et al. 1999).

To improve the transformation method using leaf explants of La France and Silver bell, we investigated the optimum concentrations and combinations of antibiotics for the selection and for the elimination of *Agrobacterium*. *A. tumefaciens* strain EHA101 was used because Gao et al. (2002) reported that it was the most efficient strain for pear transformation using cotyledons of La France.

Different concentrations of Hyg (1.0–5.0 mg/l) and Km (25, 30, 40, and 50 mg/l) were examined as selection agents. In this experiment, 500 mg/L Cf was used to eliminate *Agrobacterium*, as described by Lebedev and Dolgov (2000). With 1.0 mg/l Hyg, all of the regenerated shoots were escapes, while 5.0 mg/l Hyg was too strong for explant survival (data not shown). Therefore, since the suitable range of Hyg concentration was very narrow, we rejected Hyg as a means for selecting transformants.

A concentration of 30 mg/l Km produced the highest rates of callus survival and the highest number of GUS-positive calli (Table 1). When the Km concentration was below 30 mg/l, mostly non-transformed, white calli grew, resulting in a 2.0% transient transformation efficiency (Table 1). When the Km concentration was 40 mg/l or 50 mg/l, the transformation efficiency was lower than that with 30 mg/l Km (Table 1) and the growth of the transformed calli was retarded, which inhibited regeneration (data not shown). These results suggested that a concentration of 25 mg/l Km was insufficient for selecting transformants, whereas 40 mg/l or 50 mg/l Km was too strong for callus survival.

Km has also been used as the selection antibiotic in the transformation of other pear cultivars (Mourgues et al. 1996; Bell et al. 1999). Mourgues et al. (1996) used 100 mg/l Km to transform Conference, Passe Crassane, and Doyenne du Comice. In our experiment, the transformation efficiency decreased at Km concentrations exceeding 30 mg/l, suggesting that high Km concentrations inhibit the regeneration of transformants of low-regeneration frequency cultivars such as Silver bell (Table 1). In addition, it took at least 3 months to obtain transformants with Silver bell. We assumed that this longer period might also affect the transformation efficiency in low-regeneration frequency cultivars. Therefore, it may be important to determine the antibiotic concentration that extends the culture period of the low-frequency cultivars.

To investigate the efficiency of antibiotics for eliminating *Agrobacterium*, we first examined Cf and timentin, both of which have been used in pear transformation (Bell et al. 1999; Lebedev and Dolgov 2000). However, the results were unsatisfactory when these were tested on cvs. Silver bell and La France because the *Agrobacterium*

Table 2 Comparison of transformation efficiencies of cv. Silver bell by specific concentrations and combinations of antibiotics 8 months after co-cultivation following *Agrobacterium* elimination[a]

Elimination antibiotics (mg/l)[b]	Number of explants	Number of green calli	Number of transformants	Transformation efficiency (%)[c]
Sb500	125	24	4	3.2
Sb250+Cb250	125	17	3	2.4
Cb375	125	41	0	0.0
Sb500-to-Cb 500	125	18	3	2.4

[a] 30 mg/l Km was used as selection antibiotics
[b] Sb500, 500 mg/l Sb; Sb250+Cb250, 250 mg/l Sb+250 mg/l Cb; Cb375, 375 mg/l Cb; Sb500-to-Cb500, 500 mg/l Sb was used for the first month, then 500 mg/l Cb
[c] Number of transformants/Number of explants

propagated, even after several subcultures, and the leaf explants died (data not shown). Therefore, we tested Sb and Cb for their efficiency in eliminating *Agrobacterium.* The results of these experiments, after an 8-month selection culture for Silver bell, are shown in Table 2. With Sb500, 24 green calli were obtained from 125 explants, and four transgenic shoots were regenerated from the green calli (Table 2). In contrast, although most green calli were produced with Cb375, no transformed shoots regenerated (Table 2). Under the other conditions, the transformation efficiencies were lower than with Sb500.

Although all of the antibiotic combinations inhibited the growth of *Agrobacterium* in all of the cv. Silver bell cultures, more time was required to regenerate shoots in medium containing Cb than in medium containing Sb (data not shown). In addition, the leaf explants in medium containing Cb were more likely to form calli than those in medium containing Sb. Because Cb shows auxin-like activity, owing to its structural similarity to auxin (Lin et al. 1995), it may have affected callus formation and regeneration of the leaf explants. Consequently, we concluded that the Sb500 condition was the optimal one to use for Silver bell transformation.

The results for cv. La France transformation using leaf explants were very different, and no Km-resistant shoots were obtained, although some transformed calli appeared (data not shown). Furthermore, no notable differences in gene transfer efficiency, detectable as transient expression of the *GUS* gene, were found between La France (60.6%) and Silver bell (58.6%) immediately after co-cultivation (data not shown). Therefore, we postulated that the failure of gene transfer caused the low regeneration frequency of La France leaf explants.

Transformation using axillary shoot-meristem explants

Since there was little regeneration from La France leaf explants, we failed to transform this cultivar using the standard method. Therefore, we attempted to develop a new method that was independent of regeneration.

When the axillary shoot meristems were cultured on regeneration medium, they formed multiple shoots (Fig. 2a). We believed that this characteristic could be used for transformation. Therefore, the inoculation efficiency with *Agrobacterium* was investigated using axillary shoot meristems. We inoculated stems, including the axillary meristem, to within 0.5 mm of the cut end with *A. tumefaciens* strain EHA101 containing the binary vector pIG121-Hm (Hiei et al. 1994) and co-cultured them for 5 days in the dark at 25°C. Most of the axillary meristems showed GUS activity following the cultivation period (Fig. 2b). On the basis of these results, it appeared that the axillary shoot meristem may be the optimal material for La France transformation. Therefore, we conducted further investigations to determine the optimal concentration and/or combination of antibiotics for transformant selection and the elimination of *Agrobacterium*. We first tested three concentrations of Km (5, 10, and 25 mg/l) and the four combinations of Sb and Cb used in the transformation of the leaf-section explants.

One month after co-cultivation, the selection efficiency was highest in medium containing 5 mg/l Km, based on GUS expression (Table 3), while all of the explants died in 25 mg/l Km. Therefore, we used 5 mg/l Km as the selection condition in subsequent experiments.

The results of the investigation of the antibiotics used to eliminate *Agrobacterium* are shown in Table 4. The highest transformation efficiency was obtained in the Cb375 condition—three transformants were obtained from 63 explants (4.8% efficiency). With Sb500, no transformed shoots were obtained, and in the Sb250+Cb250 and Sb500-to-Cb500 conditions, the transformation efficiencies were both only 1.6% (Table 4). Since transformants were obtained when Cb was used, we believe that the auxin-like activity of Cb benefits the growth of transformed shoot meristems when the axillary meristem is used as explants.

Confirmation of transformation

We confirmed gene integration of the transformants obtained from the leaf sections of Silver bell and from the axillary shoot meristems of La France. While the non-transformed control lacked GUS activity (Fig. 2c), the leaves of Km-resistant shoots derived from axillary meristem explants of La France did show GUS activity (Fig. 2d). PCR analysis using the primer sets for *npt*II (Fig. 3a) and *GUS* (Fig. 3b) yielded the expected products in the GUS-positive transformants obtained from Silver bell leaf sections and La France axillary shoot meristems.

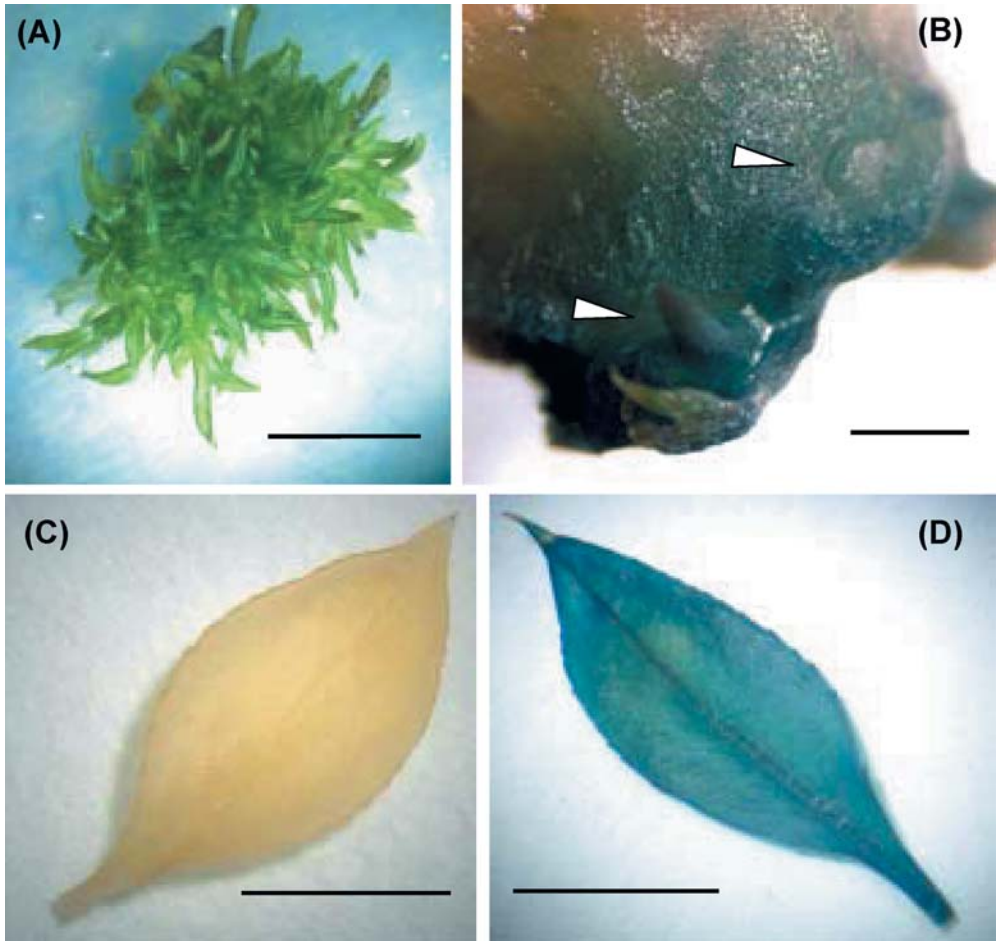

Fig. 2 Transformation of axillary shoot-meristem explants. **a** Multiple shoots derived from axillary shoot meristem on regeneration medium. **b** GUS activity in axillary meristem explants (*arrow heads*). **c**,**d** Histochemical identification of *GUS* gene expression. The leaf tissue of the non-transformed control showed no GUS activity (**c**), while the transgenic shoot showed GUS activity (**d**). *Bars*: 5 mm

Table 3 Selection efficiency of the antibiotic kanamycin 1 month after co-cultivation with cv. La France explants

Km (mg/l)	Number of explants	Number of selected green shoots	Number of GUS-positive shoots	Rate of survivors (%)[a]	Rate of GUS-positive (%)[b]	Transient transformation efficiency (%)[c]
5	103	49	20	47.6	40.8	19.4
10	104	22	7	21.2	31.8	6.7
25	105	0	–	0.0	–	–

[a] Number of selected green shoots/Number of explants
[b] Number of GUS-positive shoots/Number of selected green shoot
[c] Number of GUS-positive shoots/Number of explants

Table 4 Comparison of transformation efficiencies of cv. La France following *Agrobacterium* elimination[a] by specific concentrations and combinations of antibiotics 4 months after co-cultivation

Elimination antibiotics (mg/l)	Number of explants	Number of transformants	Transformation efficiency (%)[b]
Sb500	63	0	0.0
Cb250+Sb250	63	1	1.6
Cb375	63	3	4.8
Sb500-to-Cb500[c]	63	1	1.6

[a] 5 mg/l Km was used for the first month after co-cultivation, then 50 mg/l km
[b] Number of transformants/Number of explants
[c] 500 mg/l Sb was used for the first month, then 500 mg/l Cb

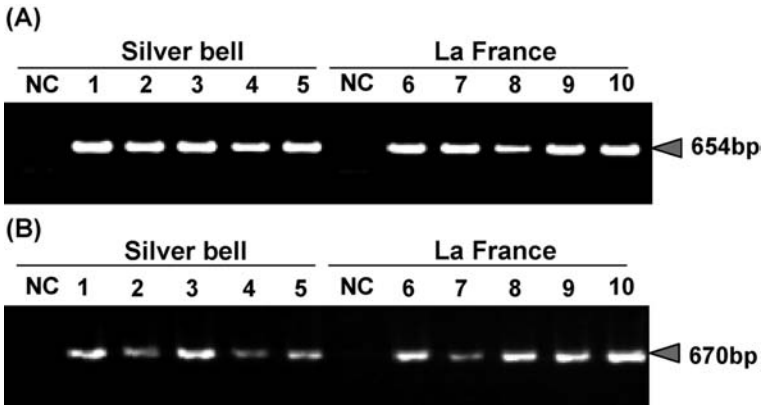

Fig. 3 PCR analysis of the *npt*II (**a**) and *GUS* (**b**) genes. *NC* Non-transformed control; *lanes*: *1–5* independent transformants derived from leaf-section explants of Silver bell, *6–10* independent transformants derived from axillary shoot-meristem explants of La France

No bands were detected in the non-transformed control (Fig. 3, lane NC). These results indicated that Km-resistant plants from both cultivars were truly transformants and not escapes.

Comparison of leaf-section explants and axillary shoot-meristem explants

Using leaf explants, we succeeded in transferring the reporter gene to Silver bell with a 3.2% transformation efficiency, we failed to transform La France because of its lower regeneration frequency relative to cultivars such as Conference. Consequently, we decided that in order to develop a transformation method for La France, we would have to increase the regeneration frequency and/or develop a protocol that was independent of regeneration. In the study reported here, we developed a new transformation method using axillary shoot meristems of La France as explants and obtained a 4.8% transformation efficiency. This is the first report of pear transformation using axillary shoot meristems as explants. In addition, an advantage of this transformation method is that it is independent of regeneration. Therefore, it may be possible to apply this method to other fruit tree species that are difficult to regenerate from leaf-section explants.

The risk that a transformant obtained from an axillary shoot meristem could be a chimera should be considered. During a 2-year subculture, we did not observe any Km-sensitive shoots at any time following the propagation of clonal shoots of transformants under a selection regime of 50 mg/l Km. Therefore, we suggest that the risk of chimeras is negligible.

We are currently investigating the use of this transformation method in the analysis of genes expressed in the *Rosaceae*. This method should help us to obtain pear plants with commercially valuable traits, such as an extended shelf life, resistance to pathogens, and enhanced flavor.

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