Transgenic Plant Journal ©2009 Global Science Books

Agrobacterium tumefaciens-mediated Transformation of Broccoli (*Brassica oleracea* L. var. *italica*) Hypocotyls with *BO-ACO2* Gene

Jinfu Lü[1] • Feifei Qin[1,2,3] • Chengrong Wang[1] • Ran Wang[1*] • Gang Ma[1] • Yan Liu[1] • Hui-lian Xu[3]

[1] College of Horticulture, Qingdao Agricultural University, Qingdao 266109, China
[2] Peanut Research Institute, Shandong Academy of Agricultural Sciences, Qingdao 266100, China
[3] International Nature Farming Research Center, Hata, Nagano 390-1401, Japan

Corresponding author: * **rwang@qau.edu.cn**

ABSTRACT

Ethylene metabolism is associated with postharvest senescence in fruits and vegetables. It is important to elucidate the mechanisms of ethylene production, especially at the molecular level. Therefore, vectors carrying the sense or antisense *BO-ACO2* gene and a neomycin phosphotransferase II (*NPT-II*) gene have been constructed according to Homology-Dependent Gene Silencing (HDGS). In addition, in the present research, a genetic transformation system of broccoli (*Brassica oleracea* L. var. *italica*) by *Agrobacterium tumefaciens* was established. Factors that were known to influence genetic transformation were evaluated to optimize the transformation system. Hypocotyls from 10-day-old seedlings were used for transformation. The transformation protocol was divided into 3 periods including pre-cultivation, co-cultivation and keeping-cultivation, and the most appropriate time for each period was 2 days, 72 hours and 20 days, respectively. The critical determinant was 0.2 (OD_{600}) for *Agrobacterium* density and 5 min for infection. The kanamycin (Kan) selection pressures for bud differentiation and root induction were 30 and 15 mg L^{-1}, respectively. Carbenicillin disodium salt (Carb) at 300 mg L^{-1} was used to suppress bacteria reproduction. In conclusion, using the optimized protocol, seven positive transgenic plants were successfully obtained and the *BO-ACO2* and *NPT-II* genes detected by PCR were mobilized into broccoli. The transgenic plants produced from this work will be important in elucidating the effects of the introduced *BO-ACO2* gene on ethylene biosynthesis and post-harvest senescence in transgenic broccoli.

Keywords: antibiotic selection, ethylene, post-harvest senescence, transformation protocol
Abbreviations: **ACC**, 1-aminocyclopropane-1-carboxylate acid; **6-BA**, 6-benzyladenine; ***BO-ACO1***, 1-aminocyclopropane-1-carboxylate acid oxidase 1 gene; ***BO-ACO2***, 1-aminocyclopropane-1-carboxylate acid oxidase 2 gene; **Carb**, carbenicillin disodium salt; **Cef**, cefotaxime sodium; **CTAB**, cetyltrimethylammonium bromide; **HDGS**, homology-dependent gene silencing; **IBA**, indole-3-butyric acid; **Kan**, kanamycin; **NAA**, 1-naphthalene acetic acid; ***NPT-II***, neomycin phosphotransferase II; **YEP**, yeast extract peptone medium

INTRODUCTION

Ethylene metabolism is associated with both ripening and postharvest senescence in fruits and vegetables. In order to develop technologies for longer shelf life of fresh horticultural products, it is important to elucidate the mechanisms of ethylene production in postharvest, especially at the molecular level. In research, one material often used is broccoli (*Brassica oleracea* L. var. *italica*), which is a member of the cruciferous cabbage family. Broccoli is harvested at the fast-growing stage when the flowering heads are immature. Post-harvest senescence of broccoli is characterized by floret chlorophyll loss resulting in yellowing of sepals. It is suggested that endogenous ethylene production plays an important role in floret chlorophyll loss. Application of ethylene biosynthesis inhibitors has proved able to delay chlorophyll loss (Kasai *et al.* 1996; Kato *et al.* 2002). Pogson *et al.* (1995a) isolated two cDNA clones, *ACC oxidase 1* (*BO-ACO1*) and *ACC oxidase 2* (*BO-ACO2*) from a *Brassica oleracea* L. var. *italita* cDNA library. It is reported that *BO-ACO2* mainly affects ethylene production and climacteric ethylene in broccoli florets (Pogson *et al.* 1995b; Wang *et al.* 2001). Reports on *Agrobacterium*-mediated transformation in broccoli have proved that it is possible to inhibit post-harvest senescence and extend the shelf life (Henzi *et al.* 1999a; Chen *et al.* 2001; Gapper *et al.* 2002; Xu *et al.* 2003; Chen *et al.* 2004; Higgins *et al.* 2006). However, the transgenes used in previous studies are nearly all from other crops. In broccoli, no studies on transformation of the *BO-ACO2* gene have been reported. In order to elucidate the mechanism of ethylene production in florets, especially the effect of *BO-ACO2* on ethylene production, vectors carrying the sense or antisense *BO-ACO2* gene have been constructed according to Homology-Dependent Gene Silencing (HDGS). In the present study, we established a transformation system for broccoli and discussed the transformation parameters that influenced transformation efficiency.

MATERIALS AND METHODS

Plant materials

Broccoli (*Brassica oleracea* L. var. *italica* 'Shenli') was used in this transformation experiment. Seeds, surface-sterilized in 0.1% $HgCl_2$ for 2 min and 75% ethanol for 5 min, were inoculated on solid MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.7% (w/v) agar for germination. The incubation conditions for *in vitro* culture were controlled at 25 ± 1°C and 1600 μmol m^{-2} s^{-1} with a 16 h photoperiod. Hypocotyls detached from the 10-day-old germinated seedlings were used as original explants.

Received: 26 December, 2008. Accepted: 10 April, 2009.

Original Research Paper

Bacterial strains and binary plasmid vectors

Agrobacterium tumefaciens strain LB4404 harbouring the binary vector pROKII was used. pROKII contains the *BO-ACO2* gene from broccoli in a sense or an antisense orientation driven by a CaMV 35 S promoter. The vector contains the chimeric gene (NOS-*NPT-II*-NOS) for plant kanamycin (Kan)-resistance selection (**Fig. 1**).

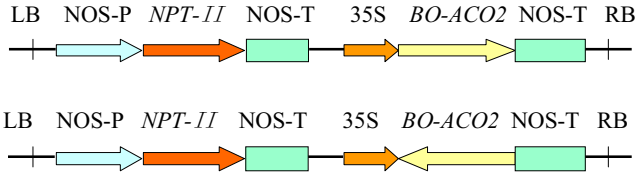

Fig. 1 Schematic representation of binary plasmid vector pROKII (upper, with sense *BO-ACO2* gene; lower, with antisense *BO-ACO2* gene).

Kanamycin sensitivity and bacteriostat definition

As explants are very sensitive to antibiotics and bacteriostats, the optimal concentrations that can not only select the Kan-resistant shoots but also suppress excessive *Agrobacterium* reproduction need to be determined before transformation. For this experiment, Kan was used as antibiotic, and Cefotaxime sodium (Cef) and Carbenicillin disodium salt (Carb) were used as bacteriostats. Concentrations were tested, one at a time, in a sequential order. Kan (0, 10, 20, 30, 40 and 50 mg L^{-1}) and Cef or Carb (100, 200, 300, 400 and 500 mg L^{-1}) were supplemented to bud induction medium (MS + 4.0 mg L^{-1} 6-benzyladenine (6-BA) + 0.3 mg L^{-1} indole-3-butyric acid (IBA), pH 5.8), respectively. After incubation for 30 days, the bud differentiation rate was recorded as follows: Bud differentiation rate = Number of differentiated explants/ Total number of inoculated explants. Kan (0, 5, 10, 15, 20, 25 and 30 mg L^{-1}) was added into rooting medium (MS + 0.05 mg L^{-1} 1-naphthalene acetic acid (NAA), pH 5.8). After incubation for 15 days, rooting rate was recorded as follows: Rooting rate = Number of rooted shoots/Total shoots.

Preparation of engineering bacteria

The *Agrobacterium* strain was cultured in liquid YEP medium (10 g yeast extract, 10 g Bacto peptone, 5 g NaCl per liter, pH adjusted to 7.0) at 28°C, shaken steadily at 120 r min^{-1} until OD_{600} reached 0.5-0.8. After the suspension was centrifuged and the cells were collected, the *Agrobacterium* cells were re-suspended in an equal volume of liquid MS medium with the density of bacteria diluted OD_{600} into 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6.

Plant transformation

In order to optimize the transformation system, the plant transformation parameters were tested in the following order: duration of pre-culture (0, 1, 2, 3 and 4 d), infection time (3, 5, 8, 10 and 15 min), then duration of co-cultivation (0, 24, 48, 72 and 96 h). Hypocotyls were pre-cultured on pre-cultivation medium (MS + 4.0 mg L^{-1} 6-BA + 0.3 mg L^{-1} IBA, pH 5.8). After infection with *Agrobacterium*, explants were inoculated on co-cultivation medium (MS medium) paved with a piece of filter paper in the dark at 26°C. The explants were then washed by sterilized water and liquid MS medium (both supplemented with 300 mg L^{-1} Carb) and then transferred to keeping medium (MS + 4.0 mg L^{-1} 6-BA + 0.3 mg L^{-1} IBA + 300 mg L^{-1} Carb, pH 5.8) or selection medium (MS + 4.0 mg L^{-1} 6-BA + 0.3 mg L^{-1} IBA + 300 mg L^{-1} Carb + 30 mg L^{-1} Kan, pH 5.8). The explants were sub-cultured every 10 days. Shoots 1 cm in length were transferred to rooting selection medium (MS + 0.1 mg L^{-1} NAA + 300 mg L^{-1} Carb + 15 mg L^{-1} Kan, pH 5.8) for further selection. The transformation experiment was repeated six times with 50 hypocotyls used for initial explants at each time.

PCR amplification

Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method outlined by Wang and Fang (2002). PCR was conducted using specific primers to identify the *NPT-II* chimeric gene construct (Forward: 5′-CGA ATG CGA CGG ATC CGG TAC CG -3′; Reverse: 5′-CAC CAT GAT ATT CGG CAA GC-3′). Bacterial plasmid was used as the positive control and DNA from non-transformed plants as a negative control. The 50 µl PCR reaction contained 4 µl dNTP (10 nM), 1 µl of each primer (50 µM), 6 µl 10 × PCR buffer, 0.5 µl *Taq* polymerase (5 U μl^{-1}), 35.5 µl H_2O and 2 µl template DNA. Reaction conditions were characterized by pre-denaturalization at 94°C for 5 min, denaturalization at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 90 s, for 34 cycles, and re-elongation at 72°C for 7 min. PCR products were visualized under a DNA/ Protein Gel Graph Analyzing System (Hema GSG-2000) after electrophoresis in 0.8% agarose (GENE TECH, Co. Ld., Shanghai) gel.

RESULTS AND DISCUSSION

Kanamycin sensitivity

Correct antibiotic selection pressure is key to successful transformation. Hypocotyls were very sensitive to Kan (**Table 1**). However, the differentiation rate decreased from 96 to 54% once only 5 mg L^{-1} Kan was appended into the medium. No buds differentiated at 30 mg L^{-1} with all explants whitening and dying soon (**Fig. 2**). The antibiotic selective pressure is suggested by some researchers to be the complete lethal concentration under which all the negative individuals can not survive. While some researchers opposed this view and considered the suitable pressure should be the concentration at which most but not all bud

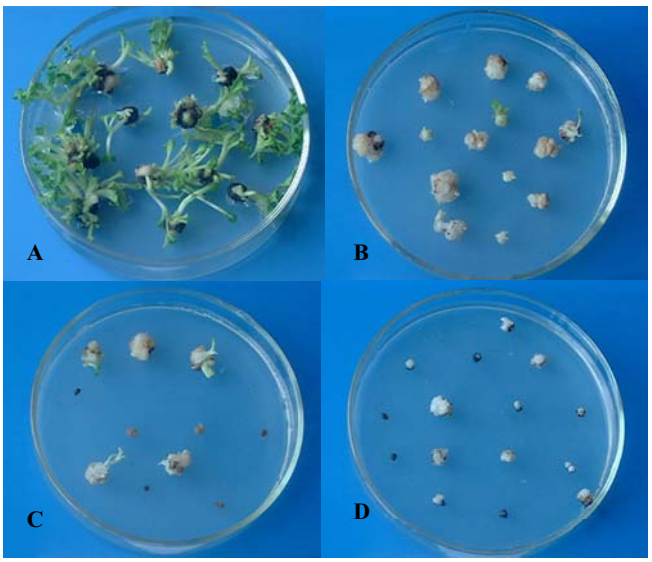

Fig. 2 Hypocotyls incubated in the medium appended with Kan. A, Kan 0 mg L^{-1}; **B**, 10 mg L^{-1}; **C**, 15 mg L^{-1} and **D**, 20 mg L^{-1}.

Table 1 Effect of Kan on bud differentiation (%) of hypocotyls and rooting (%) in broccoli.[a]

Concentration (mg L^{-1})	0	5	10	15	20	25	30	35	40	45	50
Bud differentiation	96.2 ± 1.5	54.5 ± 1.0	23.9 ± 1.8	16.6 ± 0.4	10.4 ± 0.7	6.1 ± 0.3	0.0	0.0	0.0	0.0	0.0
Rooting rate	100.0 ± 0.0	40.2 ± 2.2	10.3 ± 0.6	0.0	0.0	0.0	0.0	--	--	--	--

[a] 50 hypocotyls were inoculated in each treatment for bud induction, and 30 shoots for rooting induction. Each treatment was performed three times. Data was analyzed by ANOVA. All values are given in percentage, mean ± S.D.

Table 2 Effect of Carb and Cef on adventitious bud induction (%) of hypocotyls in broccoli.[a]

Bacteriostat	100 (mg L^{-1})	200 (mg L^{-1})	300 (mg L^{-1})	400 (mg L^{-1})	500 (mg L^{-1})
Carb	78.5 ± 2.4	56.1 ± 1.6	46.6 ± 1.9	24.2 ± 1.1	14.1 ± 0.9
Cef	37.9 ± 1.7	32.3 ± 1.7	21.8 ± 0.6	12.4 ± 0.3	6.5 ± 0.7

[a] Each treatment was inoculated with 50 hypocotyls. Data were analyzed by ANOVA. All values are given in percentage, mean ± S.D.

differentiation are inhibited (Lin *et al*. 2001; Wang and Fang 2002). In most studies, low concentrations of Kan were used, but successful cases of up to 50 mg L^{-1} Kan were also reported in vegetable *Brassica* cultivars including cabbage, cauliflower, Chinese cabbage and broccoli (Christey *et al*. 1997; Cao *et al*. 1999). In our experiment, the appropriate concentration of Kan which could select antibiotic shoots and which did not affect bud differentiation from hypocotyls was 30 mg L^{-1}.

Kan negatively affected the rooting time and position and consequently decreased rooting rate of *in vitro* shoots (**Table 1**). The inhibition increased as Kan concentration increased. Under Kan-free conditions, shoots started to root 5 days after inoculation, with the roots forming from the cut end of shoots, and the rooting rate soon reached 100%. After supplemented with Kan, rooting of shoots was slowly inhibited on day 11, after when rooting decreased sharply. The part from which roots formed was just above the medium but not at the cut end. At 15 mg L^{-1} no roots formed at all. Under all the conditions with supplemented Kan, the poorly rooted shoots whitened until death. Therefore, the selective concentration of Kan for rooting was determined as 15 mg L^{-1}.

Bacteriostat effect

A bacteriostat not only plays a role in stopping or inhibiting *Agrobacterium* from reproducing but also shows a biological effect on plant cells (Teixeira da Silva and Fukai 2001; Wojtania *et al*. 2005). The appropriate bacteriostat is a prerequisite in a genetic transformation experiment and inhibits *Agrobacterium* growth but shows little effect on bud regeneration. In this study, both Carb and Cef showed adverse effects on bud induction and differentiation of hypocotyl explants (**Table 2**). However, at the same concentration, hypocotyls were more susceptible to Cef than to Carb. It was hard for buds to differentiate and consequently bud induction rate sharply decreased in the presence of Cef. Compared to Cef, Carb showed less negative effect on hypocotyls. The reason might be that Carb had broken down to give physiologically active levels of the auxin phenylacetic acid, which offered a mechanism for the stimulation of growth (Lin *et al*. 1992). However, after exposure to a high concentration of Carb, shoot formation was also seriously affected and ultimately resulted in death. In order to inhibit excessive *Agrobacterium* growth after infection and reduce the amount of damage to explants as much as possible, Carb was selected as the appropriate bacteriostat, the optimum concentration being 300 mg L^{-1}.

Duration of pre-culture

Hypocotyls without pre-culture or pre-culturing for a long time after infection by *Agrobacterium* showed low bud differentiation (**Fig. 3**). One-day pre-culture increased bud differentiation rate from 2.5 to 15% by infection of the sense expression vector and from 1.67 to 12.5% by infection of the antisense expression vector. Bud differentiation rate peaked after 2 days' pre-culture. Extending pre-culture beyond 2 days was not beneficial for bud differentiation. Especially after infection by the antisense expression vector, hypocotyls became very brown and soon died. This indicated that pre-culture was not only advantageous in integration of the exogenous gene but also played a role in explant differentiation, consequently enhancing the transient expression and stable integration of the exogenous gene. Therefore, the optimum duration of pre-culture was 2 days.

Density of bacteria and infection time

Adherence of *Agrobacterium* to the cell surface of explants is a prerequisite to T-DNA transfer and integration (Karami 2008). The infection time reported in most studies (Sparrow *et al*. 2007; Vinterhalter *et al*. 2007) was 3-5 min, but the density of *Agrobacterium* used for transformation was diverse due to the diversity of variety, genotype, physiological condition and position as well as the sensitivity of explants to *Agrobacterium*. *Agrobacterium* growing to the log stage (0.3-0.5 at OD_{600}) or the diluted density of *Agrobacterium* cell suspension (Wang and Fang 2002) was generally used for transformation. In this experiment, an appropriate bacterial density and infection time were 0.2 (OD_{600}) and 5 min, respectively, at which adventitious bud differentiation rate was highest after transformation by both sense and antisense *BO-ACO2* expression vectors (**Fig. 4**). High density ($OD_{600} \geq 0.4$) or long infection time (beyond 8 min) resulted in seriously browning cuts of explants and excessive *Agrobacterium* growth around explants.

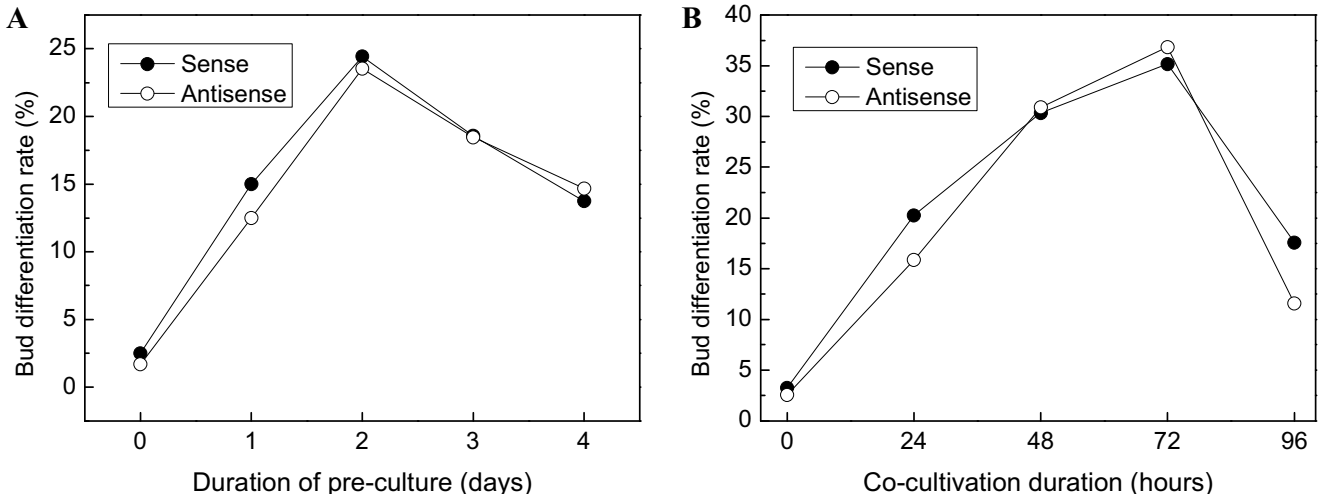

Fig. 3 Effects of pre-culture duration (A) and co-cultivation duration (B) on bud differentiation of hypocotyls after infection by sense or antisense expression vector.

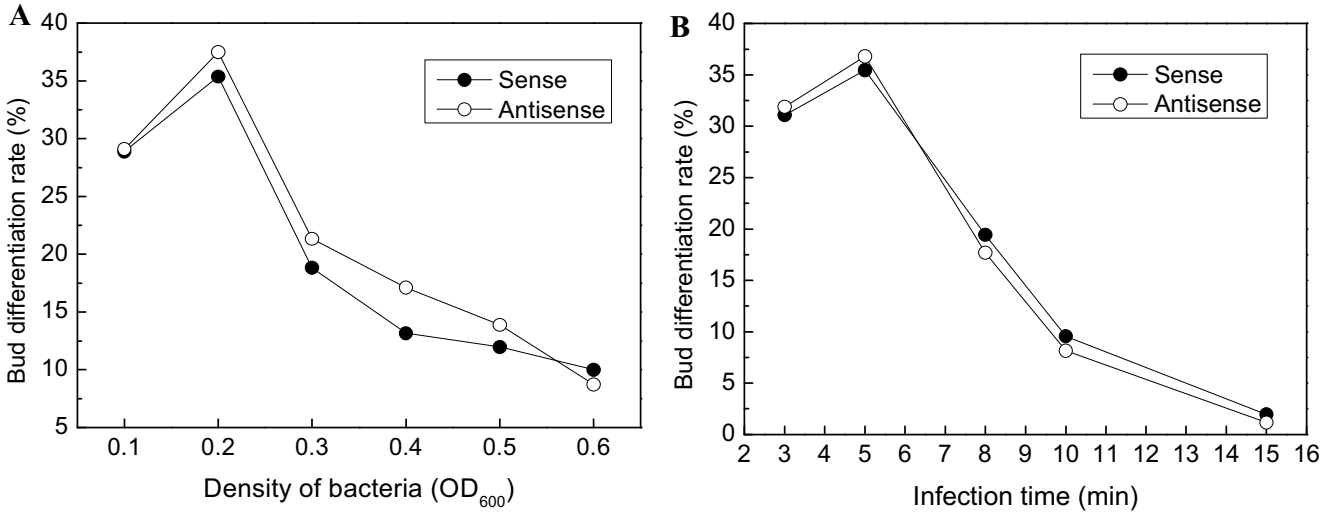

Fig. 4 Effects of density of bacteria (A) and infection time (B) on bud differentiation of hypocotyl after infection by sense or antisense expression vector.

Co-cultivation and keeping-cultivation

The duration of co-cultivation was another key factor affecting transformation frequency. The experiment failed completely when hypocotyls were plated on Kan-resistant selective medium without co-cultivation after infection (**Fig. 3**). This indicated that the exogenous gene did not integrate into the plant genome. The transfer and integration of T-DNA occurs during the period of co-cultivation after *Agrobacterium* adheres to the cell surface of explants (Wang and Fang 2002). A 72-h co-cultivation period was a better option over 24 or 48 h. Beyond 72 h, *Agrobacterium* excessive growth or some other factors inhibited bud differentiation.

As mentioned in the above paragraphs, hypocotyls from sterilized seedlings of broccoli were quite sensitive to *Agrobacterium* and Kan. Moreover, Carb plays some inhibitory role in bud regeneration. Therefore, the selection regime had to be performed separately. As reported by Metz *et al*. (1995), delayed application of a selective agent leads to excessive escapes, while too early selection adversely affects shoot regeneration (Van Wordragen and Dons 1992). In the present study, keeping-cultivation was used to improve transformation efficiency before Kan selection. Hypocotyls without infection by *Agrobacterium* began to differentiate buds 15 days after inoculation, while the infected hypocotyls started bud differentiation on the 20th day. Therefore, the best duration for keeping-cultivation was 20 days.

Molecular analysis of transgenic broccoli plants

The transformation experiment using *A. tumefaciens* produced green putative transgenic shoots from hypocotyl explants 3-4 weeks following co-cultivation. The shoots that were still maintained on Kan medium were subjected to PCR analyses, and PCR products of expected size were obtained (**Fig. 5**). Based on these results, 7 transgenic lines of broccoli were obtained using this hypocotyl transformation system, including two sense *BO-ACO2* lines and five antisense *BO-ACO2* lines. PCR with *NPT-II* gene-specific primers amplified 760 bp fragments from genomic DNA of Kan-resistant shoots, indicating the presence of the *BO-ACO2* transgene in the regenerated plants as the Kan-resistant gene *NPT-II* and the target gene *BO-ACO2* were constructed to be tightly linked in T-DNA region (Libiakova *et al*. 2001).

In conclusion, a protocol was successfully developed for *A. tumefaciens*-mediated transformation of a single broccoli cultivar 'Shenli' using hypocotyl explants. Seven transgenic broccoli lines harbouring the antisense *BO-ACO2* or sense *BO-ACO2* gene were successfully produced.

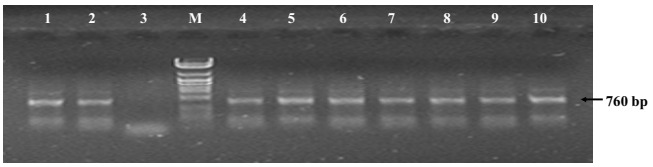

Fig. 5 PCR amplification for *NPT-II* in 7 individual transgenic lines transformed with sense or antisense *BO-ACO2* construct. Lane 1, positive control (pROKII-*BO-ACO2*); lane 2, empty plasmid pROK; lane 3, negative control (non-transformant); lanes 4 and 5, individual transgenic lines with sense *BO-ACO2*; lanes 6-10, individual transgenic lines with antisense *BO-ACO2*; M, DNA Marker *Eco*R 471, 8126, 6555, 3676, 2606, 2134, 1951, 1611, 1420, 1284, 985, 894, 590, 511, 433, 398, 345, 308 bp.

Optimal conditions should include 2 days of pre-culture, 72 h of co-cultivation, and 20 days keeping-cultivation period and the following selection culture containing 30 mg L^{-1} Kan. Although only 7 individual shoots were obtained using the transformation system, the protocol proved to be repeatable and feasible for the transfer of agronomically important genes. The transgenic plants produced from this work are important for our future work in which the effects of the introduced *BO-ACO2* gene will be elucidated on ethylene biosynthesis and post-harvest senescence in transgenic broccoli.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 30370976), the Natural Science Foundation of Shandong Province, China (No.Y2002D04), and the Natural Science Foundation of Qingdao, China (No. 06-2-2-15-jch).

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