Transgenic Apple (*Malus* × *domestica*) Shoot Showing Low Browning Potential

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Transgenic apple shoots were prepared from leaf disks by using *Agrobacterium tumefaciens* carrying the kanamycin (KM) resistance gene and antisense polyphenol oxidase (PPO) DNA. Four transgenic apple lines that grew on the medium containing 50 μg/mL KM were obtained. They contained the KM resistance gene and grew stably on the medium for >3 years. Two transgenic shoot lines containing antisense PPO DNA in which PPO activity was repressed showed a lower browning potential than a control shoot.

Keywords: *Apple (Malus × domestica); enzymatic browning; polyphenol oxidase; antisense; transgenic plant; Agrobacterium transformation*

INTRODUCTION

Polyphenol oxidase (PPO; EC 1.10.3.1), carrying two Cu^{2+} ions in the active site, catalyzes the oxidation of phenolic compounds to corresponding quinones (Mayer and Harel, 1979). In plants, a variety of polyphenols such as chlorogenic acid and (−)-epicatechin are oxidized by PPO to reactive quinones, which then polymerize to form brown pigments. This phenomenon is known as enzymatic browning, and its regulation is important for the processing and preservation of agricultural products.

The apple (*Malus* × *domestica*) is one of the most popular fruits, and browning is commonly observed when apples are consumed. Control of the enzymatic browning of this fruit is thus an important topic for juice processing and circulation of cut apples. In previous studies, we isolated chlorogenic acid oxidase as the main PPO in mature apples (Murata et al., 1992) and studied it immunochemically (Murata et al., 1993). Chlorogenic acid, which is the major polyphenol in mature apples (Murata et al., 1995b), was the best substrate for the enzyme. The optimal pH of the enzyme is ∼4, which is close to the pH of apple flesh. The enzyme is unstable at pH 4. Therefore, apple PPO is active immediately after cutting or wounding and the activity is quickly lost (Murata et al., 1992). Western blot analysis has shown that various cultivars of apple have the same PPO. The localization of PPO is mainly around the core of the fruit, the location corresponding to intense browning around the core (Murata et al., 1993). Apple PPO is mainly located in plastids as are other plant PPOs (Murata et al., 1997). The potential of apple browning decreases during maturation, because both PPO activity and polyphenol content decrease during this phase (Murata et al., 1995c). PPO was also expressed in a cell culture of apple, the expression being induced by the addition of Macerozyme or apple cell wall digests (Murata et al., 1995a). A PPO cDNA has been recently cloned from apple peel by Boss et al. (1995), showing that the PPO mRNA accumulated in immature fruits and wounded tissues. Our group also cloned two genomic DNAs encoding apple PPO by Polymerase Chain Reaction (PCR) (Haruta et al., 1998). We showed that PPOs from Rosaceae fruit plants are very similar by both molecular and immunological criteria (Haruta et al., 1999).

Our goal is to regulate the expression of PPO and the enzymatic browning of apple. Antisense methods are a useful strategy for decreasing enzyme expression (Martinez and Whitaker, 1995). Here we report the preparation of apple shoots in which the expression of PPO is down-regulated using antisense methods. Bachem et al. (1994) have produced transgenic potatoes that do not turn brown, through antisense inhibition of PPO translation. Plants lacking PPO activity might be useful not only for the food industry but also for studies of the metabolism of polyphenols and the function of PPO. In this study we have prepared transgenic apple shoots by using *Agrobacterium tumefaciens* carrying the sense or antisense PPO DNA and show that a transgenic apple shoot in which PPO expression is reduced has a lower browning potential.

EXPERIMENTAL METHODS

Preparation of Expression Vectors. Apple PPO DNA containing a *Bam*HI site at both ends was amplified from PPO7, which had been isolated from DNA of cultivar (cv.) Fuji (Haruta et al., 1998), by PCR. After the PCR product was digested with *Bam*HI, this fragment was ligated into pBI121 (Toyoboseki Ltd., Osaka, Japan) and introduced into *Escherichia coli* strain HB101. The fragment was inserted downstream of cauliflower mosaic virus (CaMV) 35S RNA promoter (Figure 1). pBI121 has the neomycin phosphotransferase (NPT) II gene for kanamycin (KM) resistance.

Plasmids were prepared from several colonies of KM-resistant *E. coli* according to standard methods (Sambrook et al., 1989), and the orientation of the PPO gene was determined

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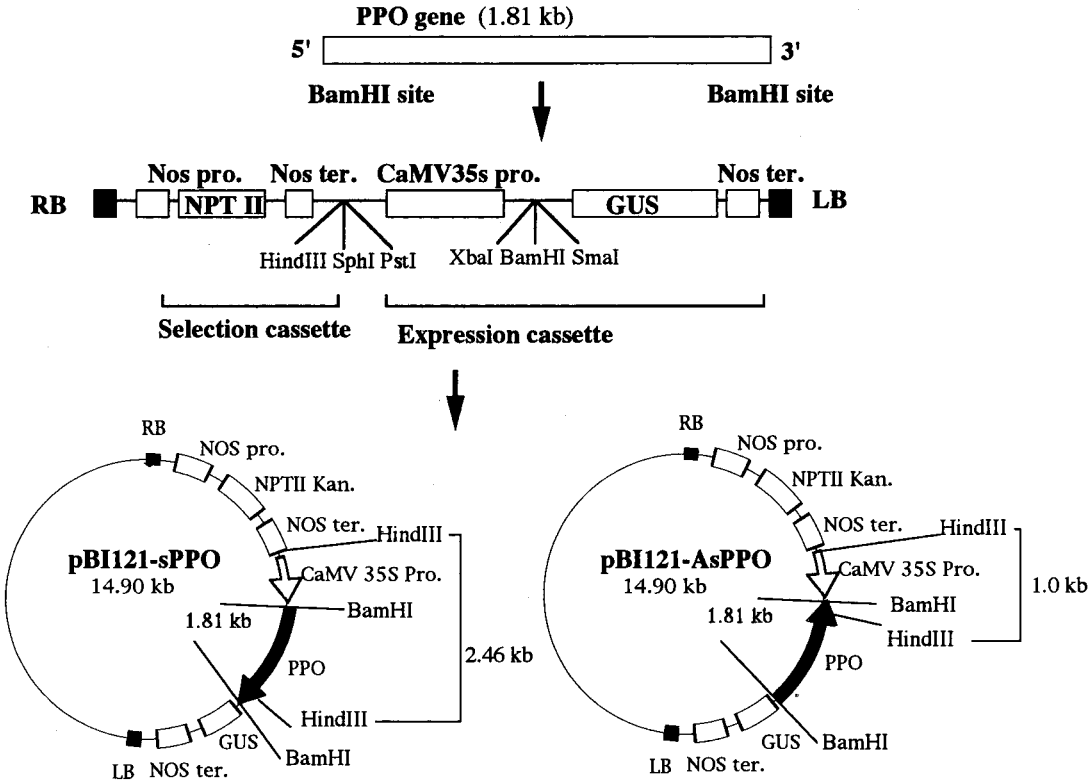

Figure 1. Construction of expression vectors. An apple PPO DNA was ligated into pBI121 at the *Bam*HI site downstream of the CaMV 35S promoter. The vector containing PPO DNA of sense orientation or antisense orientation was designated pBI121-sPPO or pBI121-AsPPO, respectively.

by digesting the plasmids with *Hin*dIII. Plasmids containing either sense or antisense apple PPO DNA were designated pBI121-sPPO or pBI121-AsPPO, respectively.

Introduction of Vector into *A. tumefaciens*. pBI121-sPPO or pBI121-AsPPO was introduced into *A. tumefaciens* LBA 4404 (Hoekema et al., 1983) by the triparental method (Ditta et al., 1980) using pBK2013 as a helper plasmid. *E. coli* HB101/pBK2013 and *E. coli* HB101/pBI121-sPPO or /pBI121-AsPPO were incubated overnight in LB medium containing 50 μg/mL KM at 37 °C. *A. tumefaciens* LBA4404 was incubated overnight in LB medium containing 0.3 mg/mL streptomycin and 0.1 mg/mL rifampicin at 27 °C. These three bacterial strains were mixed on LB agar and incubated overnight at 27 °C. After harvesting of bacteria with 5 mL of 10 mM $MgSO_4$, the bacteria were incubated overnight in LB medium containing 50 μg/mL KM, 0.3 mg/mL streptomycin, and 0.1 mg/mL rifampicin at 27 °C. Several colonies were selected, plasmids were prepared, and the presence of pBI121-sPPO or pBI121-AsPPO was verified.

Apple Tissue Culture. Aseptic apple shoots (*Malus* × *domestica* cv. Orin and cv. Fuji) were maintained on standard medium [Murashige−Skoog (MS) medium (pH 5.8) containing 1 mg/mL benzyladenine, 0.1 mg/mL indolebutyric acid, and 0.8% agar] at 24 °C with 16 h of light daily in a plant incubator (FLI-301N, Tokyo Rikakikai Co., Tokyo, Japan). Shoot tips (2−3 cm in length) were dissected from a shoot, and the cultures were maintained by subculturing every 4−5 weeks.

Transformation. Transgenic apple shoots were induced from leaf disks according to the method of Horsch et al. (1985) using the binary vector system (Bevan, 1984). Explants used for transformation experiments were fully expanded 4−5-week-old leaves. Leaves were excised and cut into two to three pieces at right angles to the midrib. These leaf explants were used in all transformation work. After *A. tumefaciens* carrying pBI121, pBI121-sPPO, or pBI121-AsPPO was incubated overnight in LB medium containing 50 μg/mL KM, the bacteria were washed with MS medium and resuspended in MS medium. Leaf explants were dipped into the suspension for 30 min at room temperature. Explants were blotted onto a sterile filter paper before they were plated on the cocultivation medium [MS medium containing 15 μM thidiazuron, 5 μM naphthaleneacetic acid (NAA), 100 μM acetosyringone (James et al., 1993), and 0.2% Pytagel (Sigma, St. Louis, MO)] and incubated for 1 week at 24 °C in the dark. Explants were then transferred to selection medium 1 (MS medium containing 15 μM thidiazuron, 5 μM NAA, 0.2% Pytagel, and 25 μg/mL KM) and subcultured every 2 weeks in the dark. When adventitious buds were observed, explants were transferred to selection medium 2 (MS medium containing 22 μM benzyladenine, 5 μM NAA, 0.2% Pytagel, 250 μg/mL carbenicillin, and 25 μg/mL KM) and subcultured every 2 weeks with 12 h light daily. When green shoots were formed, shoots were transferred to the standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin and subcultured every 4−5 weeks.

Detection of NPT-II Gene by PCR. Chromosomal DNA was isolated from apple shoots using the crude nuclear method (Walbot and Warren, 1988). Two oligonucleotide primers (the sense primer, 5′-AGACAATCGGCTGCTCTGAT, positions 81−100; and the antisense primer, 5′-CGCCAAGCTCTTCAGCATA, positions 700−681) were prepared according to the sequence of NPT-II (Hamill et al., 1991) and used for PCR (30 cycles of 94 °C for 1 min, 55 °C for 1 min, 70 °Cfor 1.5 min). One hundred nanograms of DNA was used for each template.

Southern Blot Analysis. Fifteen micrograms of apple shoot DNA was digested with *Bam*HI, separated on 0.8% agarose gels, and then transferred to nylon Hybond-N membranes (Amersham). A full-length fragment of an apple PPO gene (PPO7; Haruta et al., 1998) was labeled with the DIG-High Prime system (Boehringer Mannheim). Hybridization was performed at 68 °C with DIG-labeled PPO7, with detection performed according to the kit manufacturer's instructions (DIG Luminescent Detection Kit, Boehringer Mannheim).

Analysis of PPO in Transgenic Apple Shoots. Leaves were frozen in liquid nitrogen and pulverized with a pestle and mortar. PPO was extracted with 2 volumes of 10 mM potassium phosphate buffer (pH 7.2) containing 10% PolyclarAT and 3% Triton X-100. Enzyme activity was measured in MacIlvain buffer (pH 4.0) using chlorogenic acid as the

Table 1. Summary of Transformation Experiments

		number of			
cultivar	vector	explants[a]	explants arising buds[b]	regenerated shoots[c]	KM resistant shoots[d]
Fuji	pBI121	1230 (100)	0 (0.0)	0 (0.0)	0 (0.0)
Fuji	pBI121-sPPO	1359 (100)	5 (0.37)	1 (0.07)	0 (0.0)
Fuji	pBI121-AsPPO	1714 (100)	0 (0.0)	0 (0.0)	0 (0.0)
Orin	pBI121	1619 (100)	87 (5.4)	28 (1.7)	2 (0.1)
Orin	pBI121-sPPO	1422 (100)	60 (4.2)	21 (1.5)	0 (0.0)
Orin	pBI121-AsPPO	2072 (100)	93 (4.5)	28 (1.4)	2 (0.1)

[a] Percentage of explants is given in parentheses. [b] Transferred to selection medium 2 containing 25 μg/mL KM and 250 μg/mL carbenicillin. [c] Transferred to standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin. [d] Maintained for >2 years on the standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin.

substrate previously described (Murata et al., 1992). A decrease in 0.1 absorbance unit at 320 nm was defined as 1 unit. The enzyme extract was applied to SDS-PAGE according to the method of Laemmli et al. (1970) without heating. The gel was incubated with a mixture of 10 mM chlorogenic acid and 10 mM (−)-epicatechin for staining of PPO activity (Murata et al., 1992). Western blotting analysis was done as previously described (Murata et al., 1995c).

Detection of β-Glucuronidase (GUS) Activity. GUS activity was histochemically detected in transgenic plants carrying pBI121. Leaf pieces were dipped in 10 mM 2-(*N*-morpholino)ethanesulfonic acid buffer containing 0.3% formaldehyde and 0.3 M mannitol for 45 min for fixation. After washed with 50 mM phosphate buffer (pH 7.2), leaves were dipped in 50 mM phosphate buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) and 20% methanol. Tissue was degassed, incubated for 2 h, and washed with 70% methanol, before it was observed under the microscope.

Analysis of Phenolics and Browning Potency. Leaves of apple shoots were homogenized in 10 mM phosphate buffer (pH 7.2) and incubated for 1 h at room temperature. Phenolics before and after incubation were analyzed according to the method of a previous paper (Murata et al., 1995c). Total phenolics and chlorogenic acid were determined according to the Folin−Denis method and HPLC, respectively. Each measurement was taken twice for two shoots.

RESULTS AND DISCUSSION

Construction of Expression Vector. pBI121 is a plasmid derived from pBIN19 (Bevan, 1984), in which two maker genes (NPT-II and GUS) are inserted between the nopaline synthase gene (Nos) promoter and the Nos terminator and between the CaMV 35S RNA promoter and the Nos terminator, respectively. The sense or antisense apple PPO DNA was inserted at the *Bam*HI site downstream of the CaMV 35S promoter (Figure 1). A fragment of 1.0 or 2.46 kb was detected when plasmid DNA was cut with *Hin*dIII, which corresponds to the correct sense or antisense orientation, respectively. In pBI121-sPPO and pBI121-AsPPO, mRNA transcripts downstream of the CaMV 35S promoter are sense PPO connected with sense GUS and antisense PPO connected with sense GUS, respectively.

Transformation of Apple Shoots. In this study, the regeneration ratio of cv. Orin was several times higher than that of cv. Fuji (data not shown). Regeneration of apple shoot from leaf explants has been reported by several groups including James et al. (1988), Fasolo et al. (1989), Predieri et al. (1989), and Korban (1992). They also showed significant differences for regeneration frequencies among genotypes.

Table 1 shows the results of transformation experiments. Figure 2 shows the stages in the development of transformed apple shoots. We could obtain no transgenic cv. Fuji from a total of 4303 (1230 + 1359 + 1714) explants. Bondt et al. (1996) reported that no transformants of Fuji, Braeburn, and Gala were obtained, although transformants of Elstar, Jonagold, and Golden Delicious were obtained at rates of 3.7, 1.5, and 0.5%, respectively. These authors stated that Fuji, Braeburn, and Gala are highly recalcitrant to transformation and that the transformation efficiency depends strongly on

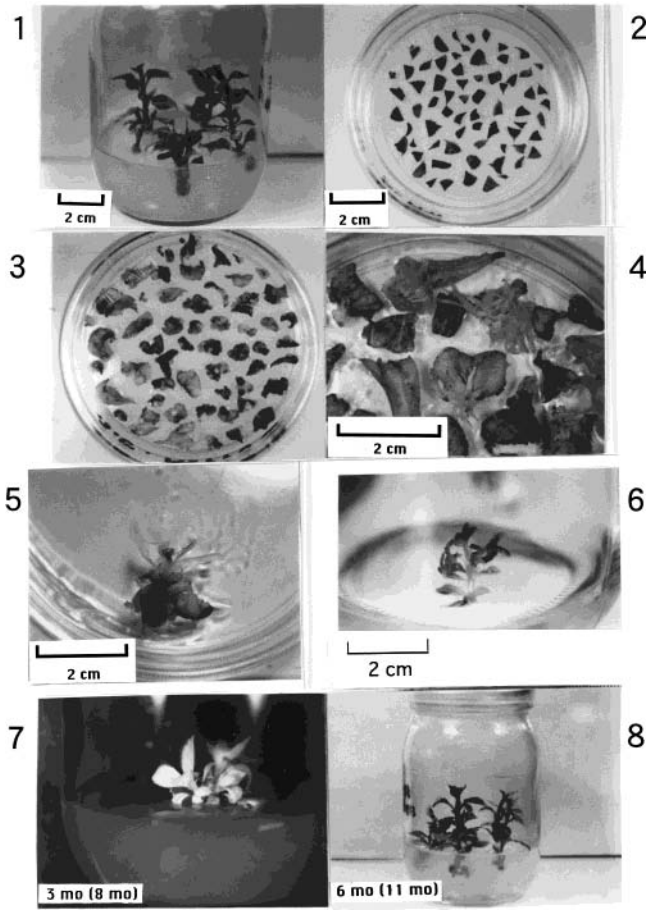

Figure 2. Development of transgenic apple shoots: 1, normal apple shoot (cv. Orin); 2, leaf disks cultivated on the cocultivation medium for 2 days after *Agrobacterium* treatment; 3, leaf disks cultivated on selection medium 1 containing 25 μg/mL KM for 1.5 months; 4, leaf disks cultivated on selection medium 1 containing 25 μg/mL KM for 3 months (adventitious buds appeared); 6, regenerated green adventitious bud incubated on selection medium 2 containing 25 μg/mL KM and 250 μg/mL carbenicillin for 1 week (total of 5 months of incubation after *Agrobacterium* treatment); 6, transgenic shoot incubated on the standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin for 1 month (total of 6 months of incubation after *Agrobacterium* treatment); 7, dead shoot incubated on standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin for 3 months; 8, transgenic shoot incubated on standard medium containing 50 μg/mL KM and 250 μg/mL carbenicillin for 6 months (total of 11 months of incubation after *Agrobacterium* treatment).

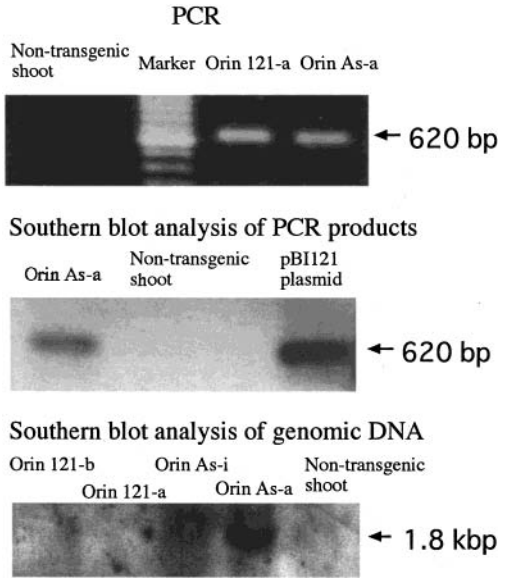

Figure 3. PCR and Southern blot analysis of nontransgenic and transgenic apple shoots. A fragment (620 bp) of NPT-II DNA was amplified by PCR (top) and was probed with pBI121 plasmid (middle). A *Bam*HI fragment of inserted PPO DNA (1.8 kbp) was detected by Southern blot hybridization (bottom).

the genotype. When 1619, 1422, and 2072 explants of cv. Orin were treated with *A. tumefaciens* carrying pBI121, pBI121-sPPO, and pBI121 AsPPO, respectively, 87 (5.4%), 60 (4.2%), and 93 (4.5%) adventitious buds were generated. After these adventitious buds were subcultured in the light, 28, 21, and 28 green tissues were transferred to the standard medium containing 50 μg/mL KM, respectively. After several subcultures, almost all shoots turned white or brown and died. Finally, two (Orin 121-a and Orin 121-b) and two (Orin As-a and Orin As-i) lines were obtained from explants treated with *A. tumefaciens* carrying pBI121 and pBI121-AsPPO, respectively. They have been maintained for >3 years on the standard medium containing 50 μg/mL KM. Apple transformation experiments were previously reported by several groups such as James et al. (1989), Norelli et al. (1994), Sriskandarajah et al. (1994), Yao et al. (1995), and Bondt et al. (1996). In addition to marker genes such as antibiotic resistance (James et al., 1989) and GUS (Yao et al., 1995; Bondt et al., 1996), attacin E, a lytic enzyme against plant pathogenic bacteria, was transformed into apple (Norelli et al., 1994). For example, Yao et al. (1995) used the commercial apple cultivar Royal Gala for transformation. KM-resistant transformants were obtained at the rate of 2.8%. The majority of KM-resistant shoots also expressed GUS activity. Norelli et al. (1994) used 648 leaf segments of Malling 26 apple rootstock for transformation and showed that a transformant expressing attacin E was more resistant to *Erwinia amylovora* than the nontransgenic control.

Genotype of Transgenic Apple Shoots. Genotypes of four transgenic apple shoot lines (Orin 121-a, Orin 121-b, Orin As-a, and Orin As-i) were examined. We expected that these four lines contained and expressed the KM-resistant gene because they were resistant to KM. When a portion of NPT-II gene was amplified by PCR, a fragment of the predicted size (620 bp) was detected in transgenic apple shoots, although no product was detected in nontransgenic shoot (Figure 3, top). This fragment was hybridized with a pBI121 probe by Southern hybridization (Figure 3, middle).

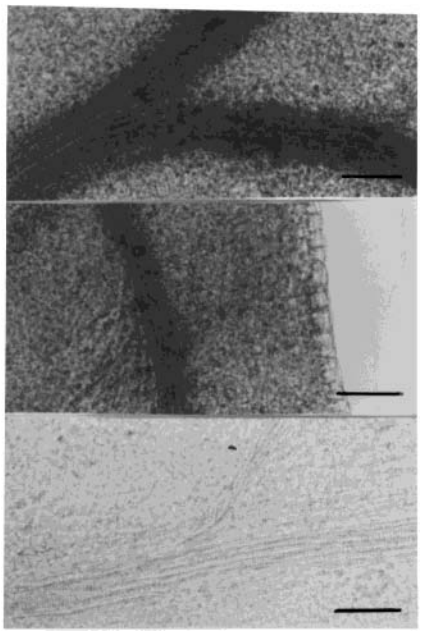

Figure 4. GUS expression in transgenic apple shoots. Transgenic (Orin 121-b, top, and Orin 121-a, middle) and nontransgenic shoots (bottom) were stained with X-Gluc. The place where GUS is expressed turned blue (top and middle). Scale bars correspond to 0.05 mm.

Total genomic DNA was extracted from transgenic and nontransgenic apple shoots, digested with *Bam*HI, and subjected to Southern blot analysis using DIG-labeled apple PPO gene as the probe. A band of 1.8 kb, which is the predicted size of an inserted PPO DNA, was detected in Orin As-a and Orin As-i, whereas the same band was not detected in nontransgenic shoots, Orin 121-a and Orin 121-b (Figure 3, bottom). Because Orin 121-a and Orin 121-b were transformants obtained from explants treated with *A. tumefaciens* carrying pBI121, they did not have an external PPO DNA. These results show that an external PPO DNA is integrated into genomic DNA of Orin As-a and Orin As-i.

Phenotype of Transgenic Apple Shoots. Phenotypes of four transgenic apple shoot lines (Orin 121-a, Orin 121-b, Orin As-a, and Orin As-i) were examined. These four lines were stably maintained on standard medium containing KM for >3 years. Orin 121-a and 121-b were expected to express GUS protein because they are derived explants treated with pBI121. When leaves of 121-a and 121-b were treated by X-Gluc, they turned blue and GUS activity was histochemically detected (Figure 4). GUS activity was clearly expressed in the vascular bundle. Nontransgenic shoots, Orin As-a and Orin As-i, were not stained with GUS. Orin As-a and Orin As-i did not translate active GUS because the GUS gene was downstream connected with the antisense PPO gene.

In Orin As-a and Orin As-i, PPO activities were expected to be reduced because these lines are derived from explants treated with pBI121-AsPPO. Figure 5 shows PPO activity in leaves. The total and specific activities of PPO in Orin As-a were about half those of the other lines. Figure 6 shows the SDS-PAGE and Western blot analysis of PPO in transgenic and nontransgenic apple shoots. The pattern of stained PPO bands with phenolics was very similar in all shoots (Figure 6A). Bands of Orin As-a were weaker than those of nontransgenic control and Orin 121-a. Western blot analysis of transgenic and nontransgenic apple shoots

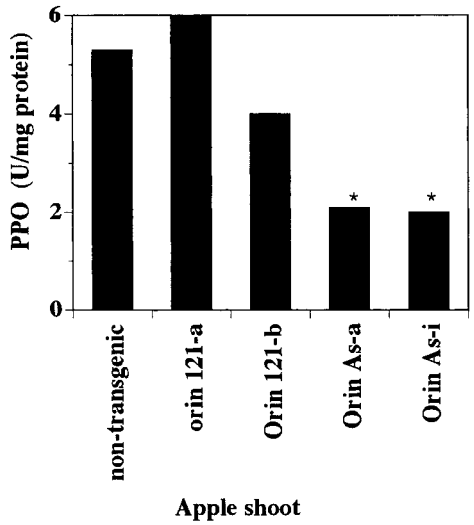

Figure 5. PPO activities in transgenic (Orin 121-a, Orin 121-b, Orin As-a, Orin As-i) and nontransgenic apple shoots. An asterisk (*) indicates a significant difference from nontransgenic shoot ($p < 0.05$).

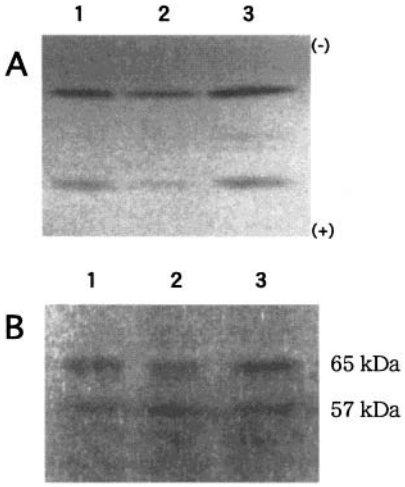

Figure 6. SDS-PAGE (A) and Western blot analysis (B) of transgenic and nontransgenic apple shoots: (A) PPO activity was stained by chlorogenic acid and (−)-epicatechin; (B) PPO protein was immunochemically detected by anti-apple PPO antibody; (lane 1) nontransgenic shoot; (lane 2) Orin As-a; (lane 3) Orin 121-a.

showed that they contained the same PPO, the size of which was about 65 and 57 kDa (Figure 6B). The band of Orin As-a at 65 kDa was weaker than that of control. These results show that there is a difference in the quantity but not the size of PPO proteins between transgenic and nontransgenic apple shoots.

Apple shoots were homogenized in a buffer, and their browning potential was estimated by measuring the absorbance at 400 nm. Figure 7 shows the degree of browning in transgenic and nontransgenic apple shoots. The browning potentials of Orin As-a and Orin As-i were lower than those of other shoots. The amount of residual chlorogenic acid after browning was also examined. The amounts of residual chlorogenic acid in As-a and As-i were higher than those in Orin 121-a, Orin 121-b, and nontransgenic shoot. This result corresponded well to the lower PPO activity in Orin As-a and Orin As-i. There was no difference in the contents of total phenolics (~1 mg/g of fresh weight) and chlorogenic acid (~150 μg/g of fresh weight) between transgenic and nontransgenic apple shoots.

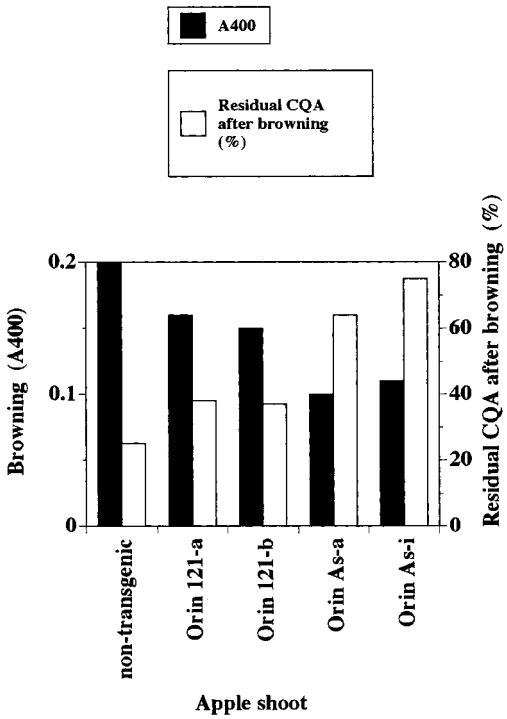

Figure 7. Browning potential and residual chlorogenic acid (CQA) after browning of transgenic and nontransgenic apple shoots.

These results indicate that the browning potential was lowered in a transgenic apple line in which an antisense PPO gene was introduced and the expression of PPO was reduced. These experiments are significant because they demonstrate the central role of PPO in apple browning and confirm the feasibility of controlling this process via antisense technology.

ABBREVIATIONS USED

PPO, polyphenol oxidase; PCR, Polymerase Chain Reaction; cv., cultivar; CaMV, cauliflower mosaic virus; NPT, neomycin phosphotransferase; KM, kanamycin; MS, Murashige−Skoog; NAA, naphthaleneacetic acid; GUS, β-glucuronidase; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide; Nos, nopaline synthase gene.

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