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RESEARCH ARTICLE

Construction and transformation for the antisense expression vector of the polyphenol oxidase gene in Yali pear

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Abstract **To inhibit the browning process in fruits of Yali pear, in this paper, antisense gene techniques were used to reduce the expression of *PPO* gene. A cDNA fragment of 450 bp, which is located at the 3′ terminal of the polyphenol oxidase (*PPO*) gene, was amplified from Yali pear using the RT-PCR method, then the antisense expression vector was constructed by inserting the fragment of the Yali pear *PPO* gene between the CaMV promoter and NOS terminator of the expression vector pBI121 in a reverse orientation. After that, with the agrobacterium-mediated method, the *PPO* antisense gene was transformed into Yali pear shoots. Northern blot analysis and enzyme activity assay showed that the PPO activities in the transgenic Yali pear shoots were significantly decreased, compared with the non-transformed Yali pear shoots. This lays a good foundation for breeding new varieties of pears with browning resistance in the future.**

Keywords Yali pear, polyphenol oxidase, antisense expression vector, genetic transformation

Introduction

Yali Pear (*Pyrus bretschneideri* Rehd.), a cultivar of pear, originates from China. In China, although the Yali pear has won general popularity and has been widely cultivated in this country for its excellent taste with crisp, sweet flavor and juicy flesh, it is often subject to rapid enzyme browning after harvest, causing serious deterioration in the appearance and processing qualities of the fruit, which has become one of the serious problems in Yali pear production and urgently needs to be solved now.

It has been confirmed by most previous researches that PPO plays a very important role in the fruit browning process, and when preventing the expression of the *PPO* gene, it will induce the fruit's browning greatly (Vámos-Vigyázó, 1981; Vaughn et al., 1988). Accordingly, in our investigation we attempted to inhibit the expression of the *PPO* gene in Yali pear by using antisense gene techniques (Van der Krol et al., 1988). With gene cloning, construction of a plant antisense expression vector and agrobacterim mediated-transformation, we obtained the transgenic Yali plants that contain the antisense *PPO* gene. It provided some excellent materials for studying the molecular mechanism and physiologic function of PPO in plant tissue browning, and moreover, the obtained transgenic plants with low PPO activity would be of great value in Yali pear production to improve its browning resistance.

Materials and methods

Materials

Yali pears grown at the orchard of the Hebei Academy of Agriculture and Forestry Sciences (Shijiazhuang, China) were harvested at the mature stage (October 25, 2008). The fruit tissues were then frozen in liquid nitrogen and stored at −80°C until use.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from fruit tissues using the modified CTAB method (Dong, 2007), then the RNA samples (5 μg) were reversely transcribed to cDNA using the M-MLV RTase cDNA Synthesis Kit (Takara Biotechnology) following the manufacturer's instructions. The synthesized cDNAs were

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used as a template for RT-PCR using degenerate oligonucleotide primers on the basis of the *PPO* gene (DDBJ Accession No. EU048225) (Li et al., 2008) from Yali pear. The primers that gave positive results were as follows: 5′-AAG CT G GGA GTT GCT CAC GCA ACT GT-3′ (sense) and 5′-TTA AGA AGC AAA CTC AAT CTT GAT ACC ACC-3′ (antisense). Reactions were carried out under the following conditions: initial denaturation at 94°C for 5 min, then 30 cycles of 30 s at 94°C, 45 s at 62°C and 1 min at 72°C, and final extension at 72°C for 10 min. The amplified cDNA fragments with the proper size were cloned into the pGEM-T vector (Promega) by TA cloning to obtain the plasmid pGEM-PPO. The nucleotide sequence of the cDNA was determined with an ABI 3730 sequencer (Applied Biosystems) using either the T7 or SP6 primer.

Vector construction for antisense PPO cDNA

The cDNA of the *PPO* gene was excised from pGEM-PPO using *Xba*I and *Sac*I restriction endonucleases. This fragment was cohesive-end-ligated into the pBI121 plasmid and replaced the *GUS* gene to generate the antisense construct pBI121-PPO(-). This construct contained the *nptII* gene that allowed us to select kanamycin-resistant transgenic plants. The constructed vectors were introduced into the *Agrobacterium tumefaciens* strain EHA105.

Pear transformation and production of transgenic plants

The leaves were excised from 4-week-old shoots of Yali pear *in vitro* and subcultured in a proliferation medium. Two to three incisions were cut perpendicular to the midrib. The proliferation medium contained MS medium (Murashige and Skoog, 1962) supplemented with 0.5 $mg \cdot L^{-1}$ 6-benzylaminopu- rine (BA), 30 $g \cdot L^{-1}$ sucrose, and 0.8% agar, and was adjusted to pH 5.8. The leaf discs were then inoculated with agrobacterium solution that was prepared as described previously (Gao et al., 2000). The basic medium used in co-cultivation, disinfection, and selection, consisted of MS medium supplemented with 2.2 $g \cdot L^{-1}$ Thidiazuron (TDZ) and 186 $mg \cdot L^{-1}$ 1-naphthaleneacetic acid (NAA). In addition, the co-cultivation medium was solidified with 4 $g \cdot L^{-1}$ Gellan Gum (Sigma Chemical Co., St. Louis, MO) and adjusted to pH 5.2. The disinfection medium was supplemented with 500 $mg \cdot L^{-1}$ carbenicillin (Sangon Biotech Corp., Shanghai, China), and the selection medium was supplemented with 500 $mg \cdot L^{-1}$ carbenicillin and 50 $mg \cdot L^{-1}$ kanamycin, solidified with 0.8% (m/v) agar, and adjusted to pH 5.8. The cultures were kept in the dark in the first 2 d and then cultured in the light for 16 h daily. After several subcultures, the green vigorous calli formed at the cut ends of explants, then the calli were excised and transferred to the fresh selection medium. The adventitious buds that regenerated from the calli were removed and transferred to the proliferation medium supplemented with 50 $mg \cdot L^{-1}$ kanamycin and 500 $mg \cdot L^{-1}$ carbenicillin. Only a single bud was taken from the same callus mass to avoid the confusion of using the same clone in different lines. To maintain the transgenic shoots, the axillary bud was transferred periodically to the fresh 50 $mg \cdot L^{-1}$ kanamycin medium every 6 weeks.

PCR and RNA gel blot analyses

The genomic DNA was extracted from the leaves of non-transformed Yali pear shoots and kanamycin-resistant shoots following the CTAB method (Yamamoto and Hayasi, 2001). For PCR analysis, 50 pg of genomic DNA was used as the template and the *nptII* gene was PCR-amplified with the *nptII* gene-specific primers.

To ensure the same culture conditions applied to both non-transgenic control and transgenic shoots, the axillary buds from the control and transgenic shoots (subcultured in kanamycin medium) were cultured in a non-antibiotic shoot proliferation medium for 30 d prior to the sampling operation. The leaves were harvested from the shoots, frozen immediately in liquid nitrogen, and stored at −80°C before use. The total RNA was extracted from a group of leaf samples weighing 0.5 g (coming from three to five shoots) according to the modified CTAB method (Dong, 2007). The total RNA (5 mg) was separated on a 1% agarose gel containing 2% formaldehyde and then blotted onto a nylon membrane (Hybond-N^+; Amersham Biosciences, Piscataway, NJ). The RNA blots were hybridized with the probes for the *PPO* gene at 68°C. The probes were prepared with a PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH). Stringent washes and chemiluminescence detection for the blots were performed according to the manufacturer’s instructions for the DIG system (Roche Diagnostics GmbH).

Enzyme activity assay

The shoots were subcultured similarly in a kanamycin-free medium for 4 weeks as mentioned above for the RNA blot analysis. The shoot culture (0.5 g) was transferred to 5 volumes of grinding buffer (25 mM Mes, 10 mM ascorbate pH 6.0). Enzyme extracts were prepared by homogenizing the tissue with a Polytron blender (Kinematica AG, Littau Switzerland). Cell debris was removed by filtration through Miracloth (Calbiochem). PPO activity of the extract was measured every minute until 9 min when oxygen was taken up at 25°C in 50 mM sodium phosphate, pH 6.0. The reaction was initiated by the addition of the substrate 4-methyl catechol to a final concentration of 2 mM. One unit of activity was defined as the catalyzed consumption of 1 μmol of oxygen per minute under the assay conditions.

Results

Cloning of PPO cDNA and vector construction

When the PPO primer pair and the cDNA of pear fruit were

used, a 450 bp amplified band was produced by the PCR reaction. Three clones of the PCR product were sequenced and confirmed to be identical. This fragment showed 100% homology with the *PPO* gene (DDBJ Accession No. EU048225) from the Yali pear, and was at the 1333–1782 bp region in the open reading frame (ORF) for *PPO* gene transcription, named *AsPPO*.

Figure 1 shows the binary vector for transformations of pear with the antisense *AsPPO* gene (namely pBI121-AsPPO). In this construct, only the target (*AsPPO*) gene cassette and selection marker (*nptII*) gene cassette were present, the target gene was under the control of the 35S CaMV promoter, and the selection marker gene was under the control of the NOS promoter.

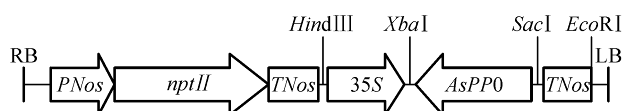

Figure 1 Diagram of antisense express vector of pBI121-AsPPO

Pear transformation

When the inoculated leaf disc explants were cultured in the selection medium at 25°C for about 4 weeks, white calli were generated from the cut surfaces of the leaf explants. The adventitious buds had also regenerated occasionally from the calli (Fig. 2). The earliest adventitious bud regeneration was seen only after 1 month of selection on kanamycin. However, the adventitious buds had been regenerated mostly from the kanamycin-resistant calli that had been in a long-term subculture of over 6 months to 1 year. Eventually, 4 independent kanamycin-resistant shoot lines were obtained, and the transformation frequency based on the number of inoculated explants was about 0.23%.

Figure 2 Generation of adventitious buds from the calli

Genomic confirmation

Using the genomic DNA extracted from the non-transformed Yali pear shoots, the 4 kanamycin-resistant shoots, and the plasmid pBI121-AsPPO as the template, PCR analysis was carried out with the *nptII* gene-specific primers. Results showed that the *nptII* gene was amplified in plasmid pBI121-AsPPO and 1 kanamycin-resistant lines (Fig. 3), indicating that the antisense *AsPPO* gene was successful transformed into only 1 of the 4 obtained kanamycin-resistant shoots in this study.

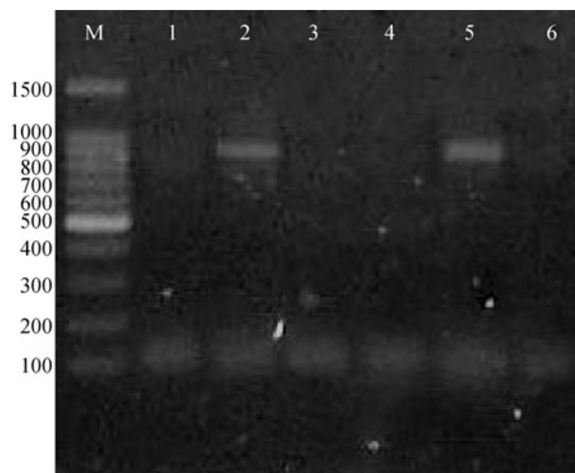

Figure 3 PCR analysis for *nptII* gene *in vitro* leaves
Note: Lane M, Marker of DNA ladder; lane 1, non-transformed Yali pear control; lanes 2, plasmid pBI121-AsPPO; lane 3–6, kanamycin-resistant lines No. 1–4.

Transcription of endogenous genes and transgenes

The RNA gel blot analyses were carried out with a PPO probe. The PPO probe was a fragment that was 1782 bp in length, and which was the full-length ORF for *PPO* gene transcription. The hybridization with the PPO probe resulted in one common signal in the non-transformed line and the transgenic line, but the relative transcription of the non-transformed control was more than ten times when compared with the transgenic line. This result indicated that the transformation treatments seriously affected the expression of the *PPO* gene, and the transcription of endogenous *PPO* gene in the transgenic line was inhibited. (Fig. 4).

PPO activity *in vitro* shoots

To further analyze the effect of transformation treatment in

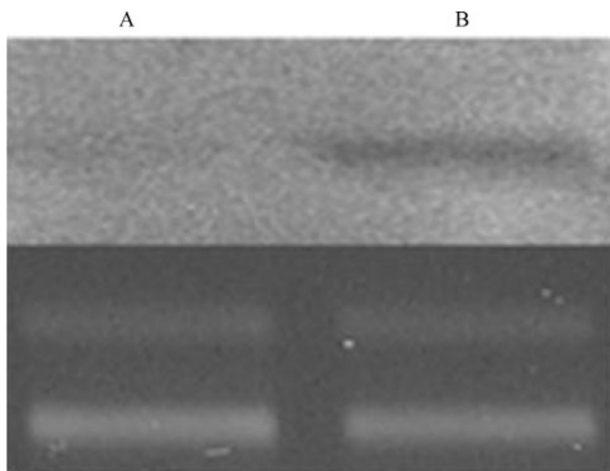

Figure 4 RNA blot analysis of *PPO* gene in transgenic shoots and non-transformed shoots

Note: The blot was hybridized with a DIG-labeled PPO probe. Lane A, transgenic shoots; lane B, non-transformed control. The equivalence of RNA was demonstrated by ethidium bromide staining of RNA on the gel.

this study, the PPO activity in transgenic and non-transgenic shoots was examined with PPO extracted from 0.5 g *in vitro* shoots. The result showed that the PPO activity in transgenic shoots was significantly lower than the non-transgenic control under the same reaction time (Fig. 5), indicating that the translation of the endogenous *PPO* gene in transgenic shoots was significantly inhibited by the antisense *PPO* gene.

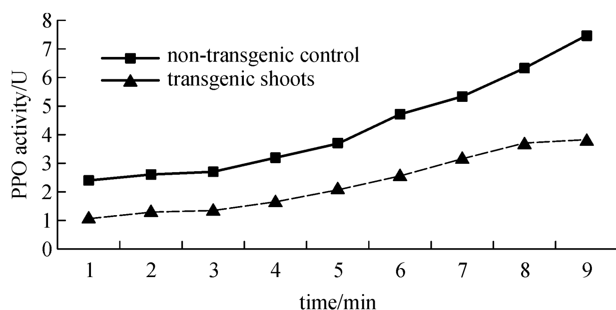

Figure 5 Analysis of PPO activity in the control and transgenic plants

Discussion

Many pear species are difficult to be transformed, especially those that are commercially important cultivars (Matsuda et al., 2005). In our study, we successfully transformed the Yali pear with leaf discs for the first time, and this work will provide great application references for the Yali pear's genetic modification in the future research. However, on the other hand, though we obtained one transgenic line, the transformation frequency was just around 0.06% based on the number of inoculated explants, at a very low level when compared with analogous researches in other pear species (Gao et al., 2007), still showing many defects in our transformation techniques. Therefore, it essentially needs more efforts for improvement.

It has been well known that the PPO are encoded by a multigenic family in plants (Cary et al., 1992; Newman et al., 1993). In this study, a *PPO* cDNA from the ripe Yali pear fruits was obtained as a browning-related gene in the production of fruit's PPO, because its transcript became increasingly abundant during fruit ripening (Li et al., 2008). It is unclear whether this *PPO* gene transcription existed in the vegetative tissues, such as the leaves and roots. But with the PPO probe in the RNA gel blot analysis, we detected the expression of the endogenous *PPO* gene in leaves of non-transgenic shoots, and it was also found that the level of endogenous *PPO* gene transcripts could be altered by the expression of antisense *PPO* gene in transgenic shoots. Many researches have proved that the PPO proteins encoded by different members of the multigenic family are highly homologous, and these genes share identical two Cu^{2+} binding sequence motifs: CuA and CuB (Newman et al., 1993; Joy et al., 1995; Thygesen et al., 1995; Chevalier et al., 1999). These results led us to consider the possibility that an integration of *PPO* antisense cDNA into the pear genome would suppress all PPO familial transcriptions.

The ultimate goal of our research is to improve the browning resistance of pear fruit by the means of PPO activity reduction. However, it is impossible to study the fruits from our transgenic pear tree now because of the nature of woody plants. Instead, we have developed a potential *in vitro* selection method that would allow us to evaluate the transgene effects on PPO activity to select the most promising transgenic lines in the early stages. Previously, Bachem et al. (1994) and Wang et al. (2007) reported that the transgenic potato with antisense *PPO* gene appeared to have browning resistance in the fruits. Their results make us believe that the transgenic plants with a greater degree of suppression for PPO transcripts obtained in our study would also have improved fruit browning resistance.

Acknowledgements

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