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A New Process for Mass Production of Resveratrol (I): Analysis of Resveratrol Content and the Expression Profile of a Gene Encoding Resveratrol Synthase in Various Tissues of *Polygonum cuspidatum* Sieb. *Et* Zucc.

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Abstract

The roots of *Polygonum cuspidatum* have been used as herbal medicines in Asia and resveratrol is one of the main active chemicals of root extract. Although a great number of studies have reported its biological and pharmacological activities and purification, resveratrol biosynthesis has never been investigated at the biochemical or genetic level in *P. cuspidatum*. Hence, we tested resveratrol content in various tissues. Roots accumulated approximately 20 to 50-fold higher levels of resveratrol than other parts of *P. cuspidatum* plant. Resveratrol content in roots was increased by about 1.84-fold by irradiation with ultraviolet as compared to untreated roots. RT-PCR analysis also showed that *PcSTSY*, a gene encoding resveratrol synthase (STSY) was expressed at the highest level in roots compared to other tissues in *P. cuspidatum* plant. These results suggest that resveratrol synthesis is spatially regulated in this plant and that the biosynthesis is induced by irradiation with ultraviolet. In conclusion, this biochemical and genetic results will be applicable to development of foods and beverages containing a high level of resveratrol with beneficial effects on health.

Key words: resveratrol, biosynthesis, *Polygonum cuspidatum*

Introduction

The roots of *Polygonum cuspidatum* have been used as herbal medicines in Asia including China, Japan and Korea. Among the main active chemicals of *Polygonum cuspidatum* root extract, resveratrol and its derivatives have been the most extensively investigated (Jayatilake *et al*., 1993). Resveratrol belongs to a group called stilbenes that are a class of biologically active components found in various plants including grapevine and peanut. A great number of studies have reported that resveratrol possess a variety of biological and pharmacological activities (Matsuda *et al*., 2001; Choi *et al*., 2002). Resveratrol has been isolated from the roots of *P. cuspidatum* by various methods (Kubo et al., 1981; Kimura et al., 1983) and its content was much higher in *P. cuspidatum* than in grapes or wines.

Resveratrol is synthesized by a stilbene synthase (STSY), resveratrol synthase, from one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA (Langkake and Pryce, 1976). Stilbene synthase genes have been isolated from several plants including grapevine (Hain *et al*., 1990; Melchior *et al*., 1991; Sparvoli *et al*., 1994; Wiese, 1994; Schubert, 1997; Lee and Pyee, 2004), Scots pine (Fliegmann *et al*., 1992; Preisig-Muller et al., 1999; Chiron *et al*., 2000), Eastern white pine (Raiber *et al*., 1995), Gerbera hybrida (Helariutta *et al*., 1995), Japanese red pine (Koan *et al*., 2002). However, enzymes or genes involved in resveratrol biosynthesis in *P. cuspidatum* have not been isolated and characterized..

In the present study, we tested resveratrol content in roots, stems and leaves from *P. cuspidatum* and also investigated the expression profile of a stilbene synthase-encoding gene from this plant. This research will be

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useful to understand the underlying mechanism of the spatial regulation of resveratrol biosynthesis and also applicable to development of plant resources for food processing using various parts of this plant.

Materials and Methods

HPLC analysis of resveratrol Various tissues of *P. cuspidatum* irradiated with ultraviolet (UV) at 254 nm for 15 min or un-irradiated were homogenized in liquid nitrogen and then extracted with ethanol/water (50:50, v/v) at room temperature for 10 min with ultra-sonication. Cell debris was removed by centrifuge and filtration. Resveratrol was determined by HPLC (Agilent 1100 Series) equipped with a diode-array UV detector using a reverse-phase C-18 column (5 μm, 4.6×150 mm, Hewlett Packard). A linear gradient starting with 95% water, 5% acetonitrile and ending with 30% water, 70% acetonitrile was applied at a flow rate of 1.0 ml/min for 40 min. The resveratrol peak was monitored at 307 nm and the standard was purchased from Sigma-Aldrich (USA).

Reverse transcription and PCR analysis For tissue expression analysis, total RNA was isolated from roots, stems and leaves of *P. cuspidatum* plants and mRNA was purified using a mRNA Mini kit (Quiagen, USA). For first strand cDNA synthesis, 20 ng of mRNA was reverse transcribed using oligo(dT) primer and M-MuLV (Promega, USA). The gene-specific primers for a resveratrol synthase-encoding gene which were designed from alignment of the previously published homologous genes were 5'-GGGTGCTATG CAGGTG GAAC TGTC-3' and 5'-GGCTTGGCCA ACTAAAGAGT CCAA-3. The PCR profile was 94°C for 30 s, 53°C for 30 s, and 72°C for 30s and 30 cycles of PCR amplification were used. *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase) was used for the positive control and mRNA not subjected to reverse transcription was used as the negative control for each sample. Twenty microliters of each PCR product was loaded on a 1.5% (w/v) agarose gel.

Results and Discussion

Quantitation of resveratrol in various tissues of *P. cuspidatum*

Quantitation for the amount of resveratrol is shown in Fig. 1. It was evident that the level of resveratrol is the highest in roots. Roots accumulated approximately 196.96 μg of resveratrol and stems contained 8.63 μg of resveratrol per gram of dried weight. However, resveratrol was not detected from leaves. The method described was able to determine only *trans*-resveratrol and hence, this was not the total amount of stilbene derivatives including picied, a glucoside, in those tissues.

Stilbene synthase-encoding genes from grapevine, peanut and pine have been reported to be transcriptionally regulated by biotic and abiotic stresses such as fungal infection and UV irradiation (Hain *et al.*, 1990; Fliegmann *et al*., 1992; Wiese *et al*., 1994; Schubert *et al.*, 1997). Hence, UV was selected to be applied to different parts from *P. cuspidatum* to investigate whether this treatment might regulate resveratrol biosynthesis in this plant. UV-irradiation seemed to increase the resveratrol content except for stems in which it did not affect resveratrol synthesis. Cantos *et al.* (2001) also reported that UV-irradiated grapes were enriched about 2-fold in resveratrol. The results suggest that resveratrol synthesis is spatially regulated in *P. cuspidatum* and that the

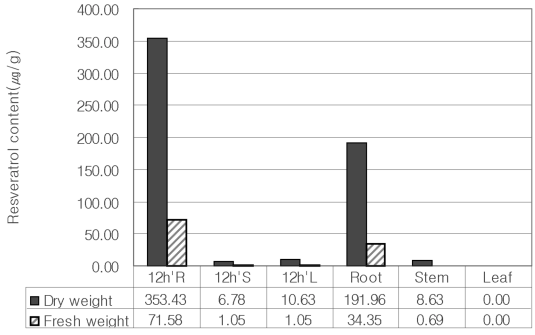

Fig. 1. Resveratrol content of roots, stems and leaves from *Polygonum cuspidatum* plants irradiated with UV or un-irradiated.
Various tissues of *P. cuspidatum* were homogenized and extracted with ethanol/water with ultra-sonication. Resveratrol was determined by HPLC equipped with a diode-array UV detector using reverse-phase C-18 column. A linear gradient starting with 95% water, 5% acetonitrile and ending with 30% water, 70% acetonitrile was applied. Resveratrol content was represented as μg per gram of dry or fresh weight of tissues. 12h'R, 12h'S and 12h'L are leaves, roots and stems irradiated with UV at 254 nm for 15 min and Root, Stem and Leaf are un-irradiated tissues.

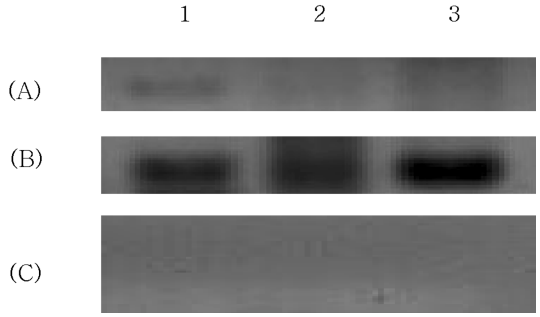

Fig. 2. RT-PCR analysis of *PcSTSY* expression in various tissues from *Polygonum cuspidatum* plants.
mRNA was extracted from different parts of *Polygonum cuspidatum* plants and used for RT-PCR analysis as described in the text. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control. mRNA not subjected to reverse transcription was used as a negative control for each sample. (A) *PcSTSY*, (B) *GAPDH*, (C) mRNA. Lanes 1, root; 2, stem; 3, leaf.

biosynthesis is induced by irradiation with ultraviolet.

RT-PCR analysis of a resveratrol synthase-encoding gene

In order to study the expression profile of a stilbene synthase(STSY)-encoding gene from *P. cuspidatum*, RT-PCR analysis was performed using a pair of primers designed from alignment of the *STSY* homologous genes previously isolated from various plants (data not shown). We could obtain a PCR product from roots of *P. cuspidatum* using the primers and the nucleotide sequence of the product was confirmed to encode a STSY-like protein (data not shown). The gene, designated *PcSTSY*, was expressed in root at the highest level and very little in stem (Fig. 2). However, it was hardly detectable in leaf tissues by RT-PCR. This result suggests that *PcSTSY* a STSY-encoding gene is spatially regulated in *P. cuspidatum*.

In conclusion, these biochemical and genetic results will be applicable to development of foods and beverages containing a high level of resveratrol with beneficial effects on health.

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