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High Content of Resveratrol in Lettuce Transformed with a Stilbene Synthase Gene of *Parthenocissus henryana*

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Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a plant phytoalexin which has positive effects on human health. Stilbene synthase (STS) is a key enzyme involved in resveratrol biosynthesis. To construct a vector for STS expression in lettuce plant, a cDNA-encoding STS of *Parthenocissus henryana* was fused to the Cauliflower mosaic virus (CaMV) 35S promoter, and the bar gene was used as a selective marker gene. To increase the expression of STS, the expression cassette was flanked by MARs. In transgenic lettuce plants, an additional compound was identified as resveratrol by HPLC and ESI-MS. Quantitative analysis showed that the average content of resveratrol reached 56.40 ± 5.52 μg/g leaf fresh weight, which was comparable to the amount in grape skin. Anticancer assay in HeLa cells revealed that apoptosis was induced by 200 μM of resveratrol extracted from transgenic lettuce.

KEYWORDS: Lettuce; resveratrol; stilbene synthase (STS); MARs; *Parthenocissus henryana*

INTRODUCTION

Stilbenes are phenylpropanoid derivatives and their biosynthesis is induced by fungal infection and abiotic stress such as wounding and UV irradiation (*1*). Simple stilbenes exist in several distant plant species including peanut (Leguminosae), grapevine (Vitaceae) and pine (Cyperaceous) (*2*).

The physiological functions of most stilbenes, especially resveratrol, are well recognized. Resveratrol shows cancer chemopreventive activity and plays a role in prevention of coronary heart disease and arteriosclerosis (*3*).

Stilbene synthase (STS) in phenylpropanoid branch pathway is the key enzyme in resveratrol biosynthesis. It is known that STS and chalcone synthase (CHS) each catalyze the formation of a tetraketide intermediate from a CoA-tethered phenylpropanoid starter and three molecules of malonyl-CoA. STS and CHS are related polyketide synthases which use the same substrates but produce different products (*4*). Therefore, a single STS gene is sufficient to synthesize resveratrol in heterologous plant species.

In recent years, several STS genes have been isolated and characterized (*5*−*7*, *17*). The STS gene has been expressed in transgenic plants such as tobacco (*7*, *8*), apple (*9*), kiwifruit (*10*), papaya (*11*), alfalfa (*12*), white poplar (*13*), rice (*14*), barley (*15*), and wheat (*16*). In these transgenic plants, resveratrol or resveratrol glucoside was detected. Most transgenic plants show enhanced disease resistance to phytopathogenic fungi, but they do not contain a high level of free resveratrol.

Lettuce contains the substrates for STS, but no resveratrol was detected. In our previous work, an STS gene has been isolated from *Parthenocissus henryana* (*17*). In this research, a construct was generated containing the bar gene and the STS gene driven by the CaMV35S promoter; this fragment was flanked by two MARs to increase STS expression (*17*−*21*). The construct was transformed into lettuce plants, and a high level of free *trans*-resveratrol was obtained in transgenic lettuce.

MATERIALS AND METHODS

Plant Material and Transformation Procedure. Lettuce (*Lactuca sativa* L.) seeds were purchased from The Chinese Academy of Agricultural Sciences. The structure of plasmid was shown in **Figure 1**. pMT1ΩBM contains the *P. henryana STS* cDNA (*17*) and the bar gene, both driven by the CaMV35S promoter; this sequence was flanked by MAR sequences. The plasmid was transferred into *A. tumefaciens* strain GV3101 using the freeze−thaw method (*22*). The cotyledons of 7-day-old lettuce seedlings were used for the genetic transformation via the *Agrobacterium*-mediated procedure. MS medium used for shoot regeneration contained 0.5 mg/L benzyladenine, 0.1 mg/L naphthaleneacetic acid, 1 mg/L phosphinothricin, and 500 mg/L carbenicillin (*23*, *24*). And MS medium containing 1 mg/L phosphinothricin and 500 mg/L carbenicillin was used for rooting.

Identification of Transgenic Plants by PCR. To confirm transformation of the STS gene in lettuce, genomic DNA was extracted from leaves by the SDS method. The primers used for PCR were: (STS)FP, 5′-GGGATCCGCCATGGCTTCAGTTGAGAAATTTAG-3′, and (STS)RP, 5′-GTGAGCTCGAAGGGTAAACCATTCTCTTTTAT-3′. The binary vector construct pMT1ΩBM served as positive control. PCR was performed under the following conditions: denature at 94 °C for 5 min, 30 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension for 10 min at 72 °C.

RNA Extraction and Transcriptional Analysis of STS in Transgenic Plants. Total RNA was extracted from leaves using Trizol Reagent (Invitrogen, American) following the manufacturer's instruction. 10 μg of total RNA was separated on a 1.2% agarose formaldehyde gel, blotted onto Hybond-N^+ membrane (Amersham) by capillary

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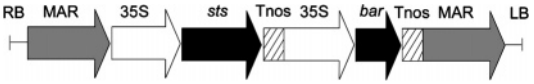

Figure 1. Schematic map of the T-DNA region of plasmid. RB: right border of T-DNA; LB: left border of T-DNA. Phosphinothricin resistance is conferred by the the bar gene and STS gene under the control of the 35S promoter, and the transcriptional unit is between MAR sequences.

transfer, and exposed to UV light for covalent cross-linking (Stratalinker, Stratagene, CA). The hybridization and detection were carried out according to the manufacturer's instructions (Roche, Germany) with digoxigenin-dUTP-labeled STS fragment by PCR as the probe.

Resveratrol Identification by HPLC and ESI-MS Analysis. Fully expanded leaves at the same maturity were collected from transgenic and nontransgenic lettuce. Leaves were dried at 50 °C and ground to fine powder in mortar. Metabolites were extracted from 1.0 g of dried powder with 40 mL of 70% methanol for 24 h. Resveratrol was extracted and purified by thin layer chromatography (TLC) on silica gel (GF254) according to the method of <crn>Subíková (*25*). All these steps were carried out under light protection. HPLC was performed at room temperature on a HPCHEM high performance liquid chromatograph (Shimadzu, Japan) using a Nucleosil C18 column (4.6 × 150 mm, 5 m) and H_2O−acetonitrile as eluent (acetonitrile: H_2O = 35:65, flow rate 0.6 mL/min), and 306 nm of detection wavelength was used. The compound with the same retention time as the free *trans*-resveratrol standard (Sigma) was collected for ESI-MS analysis. Mass spectrometric analysis was carried out on Micromass LCMS2010 system (Shimadzu, Japan).

Effect of Resveratrol Extracted from Transgenic Lettuce on HeLa cell Morphology. HeLa (cervix carcinoma) cells were maintained in DMEM medium at 37 °C in a humidified incubator containing 5% CO_2. A stock solution of resveratrol was dissolved in DMSO at 200 mM and stored at −20 °C with light protection. Resveratrol extracted from transgenic lettuce was dissolved in DMSO at 200 mM. Working solutions were diluted with DMEM medium. Cells were seeded at a density of 1.0 × 10^3/well, and cultured for 24 h. After preincubation, the culture medium was changed to experimental medium supplemented with resveratrol at the final concentration of 200 μM. Photos were taken 24 h after treatment with resveratrol.

RESULTS

Plant Transformation Experiment. Cotyledons of seedlings were inoculated with *A. tumefaciens* (GV3101) carrying the STS gene of *Parthenocissus henryana.* After approximately 3 weeks, adventitious shoot buds differentiated, mainly from the proximal cutting edge. The 2−3 cm shoots were excised and transferred into selective medium, and some of the shoots successfully rooted. Twelve independent phosphinothricin-resistance lettuce lines were obtained from 100 infected explants.

Polymerase Chain Reaction (PCR) Analysis. The presence of the transgene was confirmed by PCR using primers specific for STS (**Figure 2A**). All the phosphinothricin−resistance lines showed the predicted 1.2kb band, while no fragment was amplified in control lines. Putative transgenic lines were transplanted into potted soil and cultured in a growth chamber for further analysis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis. RT-PCR and Northern blot were performed to identify the expression of STS. RT-PCR results indicated that out of 12 independent transgenic lines, 9 lines showed the predicted 1.2kb fragment (**Figure 2B**), while no fragment was amplified in control cDNA samples. Northern blot results in **Figure 2C** show that the highest level of STS transcript was detected in line 4 among all the transgenic lettuce plants, but no correlation between the transcript level and resveratrol content was observed.

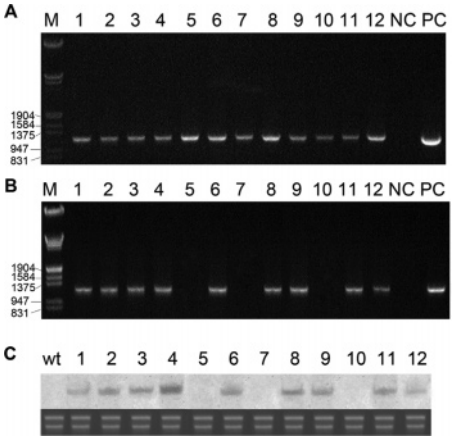

Figure 2. Detection of the STS gene by PCR analysis and STS gene expression in control and transgenic lettuce lines by RT-PCR and Northern blot analysis. A. Detection of the STS gene in transformed lettuce by PCR analysis. B. RT-PCR analysis of STS gene expression in control and transgenic lettuce lines. C. Northern blot analysis of transgenic lettuce with the probe of STS coding sequence. M: Lambda DNA/EcoRI+HindIII, 1–12: transgenic lettuce lines, 13: control lines, 14: pMT1ΩBM plasmid DNA, wt: wild type.

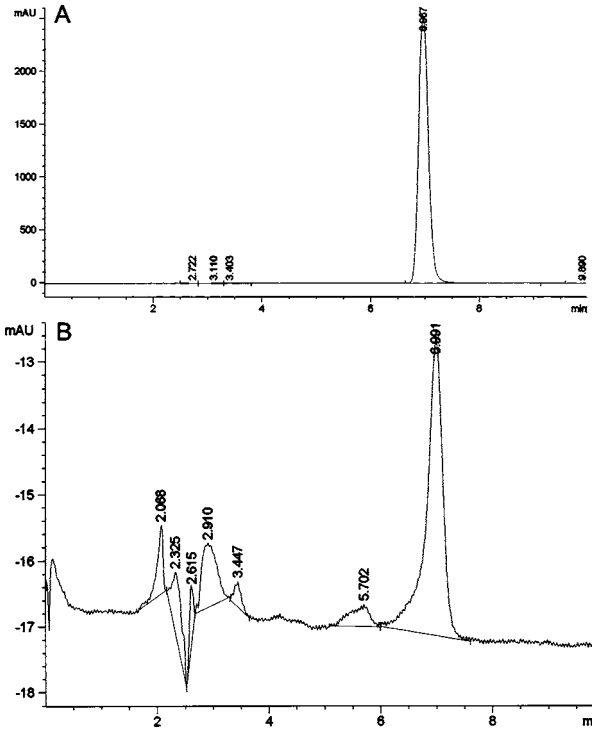

Figure 3. HPLC analysis of resveratrol extracted from transgenic lettuce. A: Free *trans*-resveratrol standard purchased from Sigma. B: Extracts of transgenic lettuce.

Resveratrol Identification and Quantification in Transgenic Lettuce. The partially purified resveratrol extracted from transgenic lettuce, and the extracts from control lines were analyzed by HPLC. Chromatograms of the leaf extracts from transgenic lettuce lines had an obvious peak (**Figure 3**B) which was not detected in the extracts of the control lines. The retention time of the peak was the same as the free *trans*-resveratrol standard (**Figure 3A**), indicating the production of resveratrol in transgenic lettuce lines. Extracts of transgenic lettuce consist of purified resveratrol and other compounds whose R_f value is close to that of resveratrol. Therefore, there were some additional

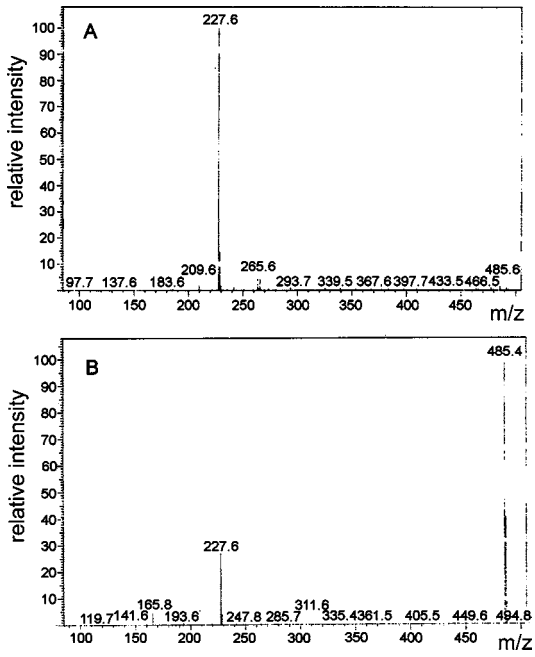

Figure 4. ESI-MS spectra of resveratrol extracted from transgenic lettuce. A: Free *trans*-resveratrol sample purchased from Sigma. B: Sample prepared from extracts of transgenic lettuce.

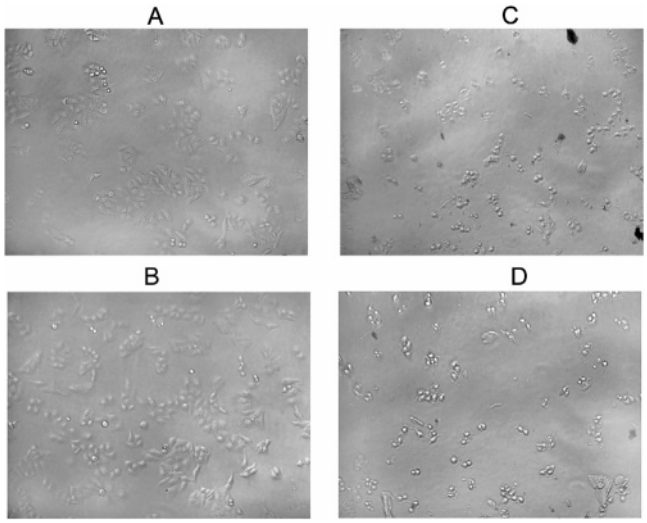

Figure 5. Effect of resveratrol on HeLa cells morphology. A: DMEM culture medium. After preincubation, culture medium was changed to experimental medium supplemented with 0.1% DMSO (B), 200 μM resveratrol standard (C), or 200 μM resveratrol extracted from transgenic lettuce (D).

peaks in HPLC chromatogram. In addition, ESI-MS was performed to further confirm the compound. ESI-MS spectra of the compound showed the same base peak at *m*/*z* 227.6 (**Figure 4**B) as the resveratrol standard (**Figure 4A**).

Effect of Resveratrol Extracted from Transgenic Lettuces on HeLa Cell Morphology. HeLa cells were treated with 200 μM of resveratrol standard or resveratrol extracted from transgenic lettuce. **Figure 5** indicated that in control cultures (A) and medium supplemented with 0.1% DMSO (B), cells adhered to the surface of the dish and showed shuttle shape. In contrast, HeLa cells shrunk in medium supplemented with resveratrol sample (C) or extracted resveratrol (D), indicating that resveratrol extracted from transgenic lettuces has anticancer activity by inducing apoptosis.

DISCUSSION

The plant phytoalexin, resveratrol, is an antimicrobial secondary metabolite and known to reduce heart disease, arteriosclerosis, and cancer mortality. Several plant species, such as grape, peanut, and Japanese fleeceflower, synthesize resveratrol that is absent in garden vegetables like lettuce. Although most of the plant species, including alfalfa, papaya, wheat, barley, and white poplar, were transformed with exogenous STS gene and showed increased resistance to fungal attack, no or marginal levels of free *trans*-resveratol was found in these transgenic plants. At present, free *trans*-resveratrol was merely detected in transgenic tobacco, wheat, and tomato. In transgenic tobacco, the highest content of resveratrol was 9.3 μg/g leaf fresh weight (*17*); in wheat, the resveratrol content is 2.0 μg/g leaf fresh weight. Resveratrol content ranged from 4 to 26 μg/g fruit fresh weight in different tomato lines.

In this study, an STS gene from *Parthenocissus henryana* had been successfully introduced into lettuce, and high expression was confirmed by RT-PCR and Northern blot analysis. As determined by HPLC, high content of resveratrol was obtained in transgenic lettuces, with an average level of 56.40 $\pm$ 5.52 μg/g leaf fresh weight, which was comparable to the amount in grape skin where the highest content of resveratrol was detected (*26*, *27*). Moreover, HeLa cell assay showed that resveratrol extracted from transgenic lettuce has the same anticancer activity with commercial resveratrol.

Resveratrol also exists in the chemical form of *cis*-resveratrol and *trans*- and *cis*-piceid (β-glucosides of resveratrol). Although the STS gene was introduced into white poplar (*13*), and accumulated *cis*-piceid and *trans*-piceid reached a substantial amount (up to 615.2 μg/g leaf fresh weight) with high pharmacological value, resistance against fungal pathogen was not obtained. Resveratrol glucoside synthesis in seeds of transgenic oilseed rape (*Brassica napus* L.) was also at a high level (up to 361g/g in seeds) (*28*). Kiwifruits transformed with a grape stilbene synthase gene produce resveratrol-glucoside at a maximum level of 182μg/ g fresh weight. However, those transformants did not show resistance to pathogen infection (*10*). The maximum amount of *trans*-resveratrol and its piceid in STS gene-transformed tomato fruit was evaluated up to 53μg/g fresh weight (*29*). To our knowledge, the effects of STS gene expression on resveratrol accumulation in differential plant tissues and various plant species has not been assessed. However, metabolite profiling is still an extremely powerful approach to change and enhances the metabolite products in transgenic plants.

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