Natural Product Sciences
10(5) : 248-251 (2004)

A Molecular Switch for the Induction of Resveratrol Biosynthesis in Grapes

Mi Sook Lee and Jae ho Pyee*

Department of Molecular Biology and Institute of Nanosensor and Biotechnology, Dankook University, Seoul 140-714, Korea

Abstract – Resveratrol has been reported to possess a variety of biological and pharmaceutical activities. Regardless of its beneficial effects on health, the amount of resveratrol in grapes is very low. In order to induce the resveratrol biosynthesis, the promoter region of a genomic fragment encoding the resveratrol synthase was isolated and a molecular switch was identified which provides us with defining biotic or abiotic inducers that transcriptionally up-regulate the gene expression involved in the resveratrol biosynthesis. We could successfully increase the amount of resveratrol in grapes up to 3-fold by using these environmental factors.
Keywords – resveratrol, resveratrol synthase gene, promoter, up-regulate, biotic or abiotic inducers

Introduction

Resveratrol and it's derivatives are naturally occurring phytoalexins belonging to a group called stilbenes, one of the group of phenolics found in grapes and wines. Resveratrol has been reported to possess a variety of bi ological and pharmacological activities such as anticancer chemopreventive activities (Jang *et al.*, 1997), inhibition of free radical generation (Olas *et al.*, 1999) and anti-inflammatory activities (Donelly *et al.*, 2004). Regardless of its beneficial effects on health, the amount of re sveratrol in grapes is unfortunately very low.

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 (*stsy*) genes have been isolated from grapevines and these genes are transcriptionally up-regulated by various environmental factors (Hain *et al.*, 1990; Melchior *et al.*, 1991; Sparvoli *et al.*, 1994; Wiese, 1994; Schubert, 1997). Hence, treatment of grapes with these factors to increase the resveratrol content may generate a great interest to pharmacologists.

In order to identify biotic or abiotic factors which can be easily applied to grapes to up-regulate the resveratrol biosynthesis pathway, we isolated a genomic fragment encoding the resveratrol synthase and the promoter region was fused to β-glucuronidase (GUS) reporter gene. Our studies have defined biotic or abiotic factors by analysis of transgenic *Arabidopsis* plants harboring the fusion gene as a molecular switch. We report here a successful ap plication of these factors to turn on the molecular switch which in turn increased the resveratrol biosynthesis in grapes.

*Author for correspondence
Fax: +82-2-709-2818; E-mail: jpyee1@dankook.ac.kr

Experimental

Plant Materials – Ten-day-old transgenic *Arabidopsis* seedlings were incubated in distilled water as a control, irradiated with UV at 254 nm for 15 min or treated with a solution containing 0.1% or 0.5% yeast extract or 75 mM or 100 mM $AlCl_3$ (Difco, USA) for 24 hr. After treatment, the plants were subjected to fluorometric assay of the GUS activity. A red table grape variety (*Vitis vinifera* cv. Campbell Early) was used to induce the resveratrol biosynthesis in this experiment. Berries, harvested at ripe stages, were irradiated with UV or treated with a yeast extract solution as described above. After treatment the samples were subjected to analysis of resveratrol by HPLC or stored at –70°C for further use.

DNA analysis – Genomic DNA was extracted as described (Doyle and Doyle, 1990). Genomic walking was performed on *V. labruscana* cv. Kyoho DNA to isolate 5'-upstream fragment of *stsy*1 gene according to the Genome Walker kits (Clontech, CA). To obtain the 5'-terminal sequence of grapevine *stsy*1 transcript, 5'-RACE was performed using the SMART RACE cDNA am plification kit (Clontech). *pstsy*1, a 896-bp fragment of *stsy*1 promoter, was obtained by PCR and inserted into the *Xba*I-*Bam*HI sites of pBI101, forming *pstsy1*::GUS.

Agrobacterium*-mediated transformation of *Arabidopsis – The *pstsy1*::GUS construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and subsequently transferred into *Arabidopsis* using a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on media containing 50 μg/ml of kanamycin.

Fluorometric assay of the GUS activity – After treatment with various signals, the transgenic plants were homogenized in the extraction buffer (50 mM phosphate buffer (pH 7.4), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium-lauryl sarcosine), and the supernatant was sub jected to Bradford protein assay (Bradford, 1976) and qu antitative β-glucuronidase (GUS) assay (Jefferson *et al.*, 1987). Fluorescence was measured in a fluorometer (Ve rsaFluor, Bio-Rad) and GUS activities were expressed as nmole of 4-methylumbelliferone produced per mg of total protein per min.

HPLC analysis of resveratrol – Grapes treated with inducing factors 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 resvertrol peak was monitored at 307 nm and the standard was purchased from Sigma (USA).

Results and Discussion

Stilbene (resveratrol) synthase-encoding genes (*stsy*) from grapevine have been reported to be transcriptionally regulated by biotic or abiotic stresses (Hain *et al.*, 1990; Fliegmann *et al.*, 1992; Wiese *et al.*, 1994; Schubert *et al.*, 1997). Hence, these stress signals have been suggested to be used as inducing regulators for the *stsy* gene expression in grapes. However, a more favorable condition which could be easily applied to grapes was required and a pro bing system was therefore developed to search for biotic or abiotic signals inducing the resveratrol biosynthesis. Four different homologous stilbene synthase genes in cluding 5'-upstream regions were cloned by PCR-based DNA walking from grapevine Kyoho. The *stsy*1 pro moter, the longest one, contained various putative *cis*-ele ments such as GATA motif, GCC-box, CAAT, HES (heat response element), WUN (wound response element), G box, GT 1, AS-1, and other light-responsive elements I box, AE box and GAG motif (data not shown). This leads us to propose that various biotic or abiotic stress signals could be applied to induce the *stsy*1 promoter activity and that this promoter might be used to determine these signals.

The *stsy1* promoter was fused to the GUS reporter gene and incorporated into *Arabidopsis*. The fusion gene was expected to act as a molecular switch in transgenic plants to determine the effects of the selected biotic or abiotic signals (Fig. 1(a)). Ten-day-old transgenic *Arabidopsis* seedlings were incubated in distilled water as a control, irradiated with UV at 254 nm in a clean bench for 15 min or treated with a solution containing 0.1 or 0.5% yeast extract or 75 or 100 mM $AlCl_3$ for 24 hr. After treatment, the plants were subjected to quantitative β-glucuronidase (GUS) assay. Yeast extract, UV and $AlCl_3$ all of the treatments seemed to induce the GUS gene expression from 2.2-fold to 3.7-fold except for treatment with 100 mM $AlCl_3$ which repressed the gene expression on the

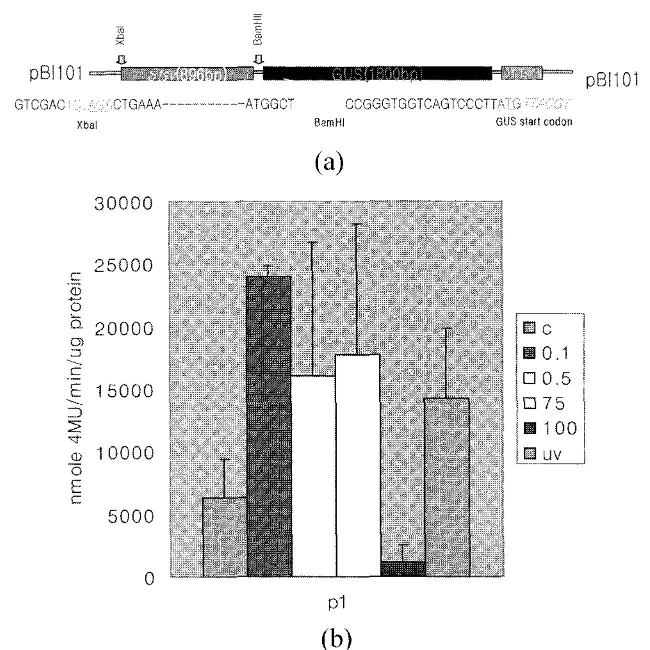

Fig. 1. Florometric assay of the GUS activity of the *stsy 1* promoter in transgenic *Arabidopsis*.
Ten-day-old transgenic *Arabidopsis* seedlings were incubated in distilled water as a control, irradiated with UV at 254 nm for 15 min or treated with a solution containing 0.1 or 0.5% yeast extract or 75 or 100 mM $AlCl_3$ for 24 hr. After treatment, the plants were subjected to Bradford protein assay and quantitative β-glucuronidase (GUS) assay. Specific GUS activities were expressed as nmole of 4-methylumbelliferone per mg of total protein per min. (a) stsy1P::GUS fusion construct in pBI101 vector. (b) Fluorometric assay of the GUS activity. C, control; 0.1, 0.1% yeast extract; 0.5, 0.5% yeast extract; 75, 75 mM AlC13; 100, 100 mM $AlCl_3$; UV, UV at 254 nm. Vertical bars indicate standard errors for the three replicates.

Table 1. Effect of treatments of grapes on the resveratrol content

Treatments	Resveratrol content (ug/g fresh tissue)
Control	1.67 ± 0.8
UV(254 nm)	2.54 ± 0.33
Yeast extract (0.1%)	3.77 ± 0.57

* Values represent the mean ± SE.

contrary (Fig. 1(b)). Yeast extract treatment was the most efficient to induce the *stsy*1 gene promoter and this implies that yeast extract might be applied to grapes to induce the resveratrol biosynthesis.

Yeast extract and UV seemed to induce the GUS reporter gene under the resveratrol synthase gene pro moter in leaves, roots and stems of transgenic plants.

These results suggest that the 5'-upstream region of *stsy*1 region may be applied to construct a probing system for biotic or abiotic signals enhancing the resveratrol content in grapes. In addition, identification and characterization of the *cis*-elements involved in the environmental stress re sponse will result in the development of various post-harvest techniques to increase the resveratrol content.

The selected signals were applied to grapes and confirmed to increase the resveratrol content (Table 1). Cantos *et al.* (2001) also reported that UV-irradiated grapes were enriched about 2-fold in resveratrol.

Consequently, in this investigation we report the successful screening of inducing factors which turned on the molecular switch for up-regulating the resveratrol bio synthesis pathway.

Acknowledgements

This research was supported by Dankook University (2002).

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(Accepted October 15, 2004)