Induction of Resveratrol Biosynthesis in Skins of Three Grape Cultivars by Ultraviolet Irradiation

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Summary

Resveratrol production and expression of the genes related to resveratrol biosynthesis were investigated in the skins of three *Vitis vinifera* cultivars Chardonnay, Koshu and an American hybrid grape, Muscat Bailey A (Bailey × Muscat Hamburg). Resveratrol concentration in the skins of all the grapes increased significantly when exposed to ultraviolet (UV-C, 254 nm) irradiation. The UV-induced resveratrol concentration in the grape skins was lower after veraison (onset of ripening) than before it. The maximum concentration of the UV-induced resveratrol in ‘Muscat Bailey A’ was higher than those in the other two cultivars. The relative mRNA expression levels of stilbene synthase (STS), phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes in grape skins 8 hr after UV irradiation were determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results revealed that STS- and PAL-mRNA expressions were significantly increased by UV irradiation. STS-mRNA expressions in ‘Muscat Bailey A’ were higher than those in ‘Chardonnay’ throughout berry development. The UV-induced CHS-mRNA expression in the grape skins decreased before veraison and subsequently increased.

Key Words: grape, resveratrol, stilbene synthase, UV, *Vitis*.

Introduction

Grape plants synthesize resveratrol, a stilbene phytoalexin, as part of their defense system against fungal disease (Dai et al., 1995; Langcake, 1981; Langcake and McCarthy, 1979; Langcake and Pryce, 1976; Pool et al., 1981; Sarig et al., 1997; Stein and Blach, 1985). The amount of resveratrol induced by pathogen invasion or elicitor treatment is positively correlated with disease resistance to a gray fungus (*Botrytis cinerea*) and may therefore be used as an indicator to screen for disease-resistant cultivars (Barlass et al., 1987; Creasy and Coffey, 1988; Sbaghi et al., 1995; Stein and Blach, 1985). The key element of resveratrol biosynthesis is thought to be stilbene synthase (STS), which synthesizes the stilbene backbone from phenylpropanoid precursors (Fig. 1, Schroder and Schroder, 1990). STS is rapidly and transiently induced in grape cell cultures after elicitation with *B. cinerea* cell wall (Liswidowati et al., 1991). Melchior and Kindl (1991) found that the expression of phenylalanine ammonia-lyase (PAL) and STS mRNAs (*SV21* and *SV25*) was simultaneously induced by fungal cell walls of cultured grape cells. When STS genes *Vst1* and *Vst2* were transferred into tobacco, the plant produced resveratrol upon elicitor stimulation and

its disease resistance to *B. cinerea* was significantly increased (Hain et al., 1993). These findings suggest that disease-resistant plants may be genetically engineered by introducing the STS gene.

In our previous study (Takayanagi et al., 2002), we investigated resveratrol induction and STS gene expression in the leaves of two grape cultivars (*Vitis vinifera* cv. Chardonnay and *V. rupestris* cv. Saint George) and suggested that the difference in the amount of resveratrol induced between the two grape cultivars is due to the transcription of the STS gene. In this study, we focused on the production of resveratrol in berries of ‘Koshu’ (*V. vinifera*) and ‘Muscat Bailey A’ (Bailey × Muscat Hamburg), and the ‘Chardonnay’ (*V. vinifera*). ‘Koshu’ and ‘Muscat Bailey A’ are the leading grape cultivars in Japan for white and red wines, respectively, whereas ‘Chardonnay’ is a popular white grape cultivated all over the world. Although ‘Koshu’ and ‘Muscat Bailey A’ exhibit high resistance against *B. cinerea* (Goto and Aono, 1981), the resveratrol production in these cultivars has not yet been investigated. The berries of the three cultivars were irradiated with ultraviolet (UV) radiation, an abiotic elicitor, and the subsequent production of resveratrol and the expression of the genes related to resveratrol biosynthesis, namely, STS, PAL and chalcone synthase (CHS) genes, were followed.

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Table 2. PCR primers of STS, PAL and CHS genes and 18S rRNA.

Gene name and accession numbers	Nucleotide sequence	Amplified product (bp)
STS (S63225)		
Sense	AAGAGCGAGCACATGACTGA	787
Antisense	ACCTGGGTGAGCAATCCAAA	
PAL (X75967)		
Sense	CCGAAGCAAGATCGATATGCTCTC	498
Antisense	CATGAGCTTCAAGATATCCACCGC	
CHS (AB015872)		
Sense	ATTCTCAAGGAGAACCCCAACGTC	445
Antisense	TGATAATGGCAGCTGCACCATCAC	
18S rRNA		
Sense	GCCTCCGGTGCTGTTACTTTGAAGA	307
Antisense	GGTCGGCATCGTTTATGGTTGAGAC	

18S rRNA ($12.5 \text{ pmol} \cdot \mu\text{l}^{-1}$, Table 2), $2.4 \mu\text{l}$ of EX *Taq* polymerase R-PCR (12 U, Takara), $24 \mu\text{l}$ of $10 \times$ R-PCR buffer, $7.2 \mu\text{l}$ of 10 mM dNTP mixture, $2.8 \mu\text{l}$ of 250 mM Mg, and $192 \mu\text{l}$ of sterile water, was divided into $19 \mu\text{l}$ aliquots in a $200 \mu\text{l}$ PCR tube, to which $1 \mu\text{l}$ of diluted cDNA (2.5 -, 5 -, 10 -, 20 -, 40 -, 80 -, 160 -, 320 -, 640 -, 1280 - or 2560 -fold dilution) was added. The primers for STS, PAL, CHS and 18S rRNA were designed based on the sequences of *SV25* (S63225), PAL cDNA (X75967) that were isolated from *V. vinifera* L.; CHS cDNA (AB015872 and AB066275) were isolated from ‘Cabernet Sauvignon’ and ‘Chardonnay’ 18S rRNA (Takayanagi et al., 2002), respectively.

Thirty (STS, PAL and CHS) or 22 (18S rRNA) PCR cycles were performed in a thermal cycler (Applied Biosystems 2400, USA), using a step-cycle program of 94°C for 1 min, 64°C or 55°C for 1 min and 72°C for 1 min. Following PCR amplification, $8 \mu\text{l}$ of PCR product mixed with $2 \mu\text{l}$ of loading solution (40% sucrose, 0.25% BPB and 1 mM EDTA) was electrophoresed on a 1.8% agarose gel in $1 \times$ TAE buffer. The gel was stained with SYBR GREEN I solution (10000 -fold dilution with $1 \times$ TAE buffer). The fluorescence intensity of each band was measured by a CCD imaging system (ChemiDoc, Bio-Rad, USA) and the intensity data were converted into coefficients reflecting the initial amount of the cDNA templates, according to Horikoshi and Sakakibara (2000). When fluorescence intensities of the PCR products were plotted against the amounts of cDNA template, they were directly proportional. The relative expression levels of STS, PAL and CHS mRNAs were determined by calculating the ratio of the amount of PCR product (fluorescence intensity / cDNA volume) within the linear amplification range of the target genes to that of the endogenous reference gene (18S rRNA) by the following equation: Ratio = (fluorescence intensity / cDNA volume) of target gene fragment / (fluorescence intensity / cDNA volume) of endogenous reference gene fragment.

Results and Discussion

Resveratrol induction by UV irradiation

When berries were irradiated at 30, 60 and 90 DAA with UV and the resulting resveratrol concentrations in the grape skins were measured 0, 24, 48 and 72 hr later, the resveratrol in the control grape skins were below the detectable level in all the sampling days. In berries treated 30 DAA (Fig. 2A), resveratrol concentrations in the skins of all cultivars tested had significantly increased to a maximum 48 hr after UV irradiation. The maximum concentration of resveratrol in ‘Muscat Bailey A’ ($1910 \mu\text{g} \cdot \text{gFW}^{-1}$) was significantly higher than those in ‘Chardonnay’ ($1270 \mu\text{g} \cdot \text{gFW}^{-1}$) and ‘Koshu’ ($1260 \mu\text{g} \cdot \text{gFW}^{-1}$). In ‘Koshu’ and ‘Chardonnay’ berries treated 60 DAA (Fig. 2B), resveratrol concentrations reached a maximum 48 hr after UV irradiation, whereas that in ‘Muscat Bailey A’ grape skins increased continuously to about 72 hr. Likewise, resveratrol concentration in the UV-irradiated berries 90 DAA attained its maximum level in about 72 hr after irradiation in all the cultivars (Fig. 2C); it was significantly lower than those treated 30 and 60 DAA.

The onset of ripening (veraison) of each variety, which was estimated from the sugar content, was around 60 DAA. The Brix values of ‘Muscat Bailey A’ and ‘Chardonnay’ at 90 DAA were 16.0 and 18.9, respectively. Resveratrol concentration in ‘Koshu’ at 105 DAA was lower than that at 90 DAA and the Brix value increased from 12.5 to 16.8 during the same period.

Resveratrol production occurred in grape leaves and berries exposed to either biotic or abiotic elicitors (Barlass et al., 1987; Creasy and Coffey, 1988; Sbaghi et al., 1995; Stein and Blaich, 1985). UV irradiation induces the production of a larger amount of resveratrol than other elicitors (Bais et al., 2000; Versari et al., 2001). Although the validity of the response, depending on elicitor type, has not been sufficiently clarified, the use of UV irradiation as a model system for resveratrol

Fig. 2. Resveratrol production in grape skins at 30 (A), 60 (B), 90 (C) DAA of 'Muscat Bailey A' (○), 'Koshu' (△) and 'Chardonnay' (□) induced by UV irradiation. The concentration of resveratrol in 'Koshu' grape skin at 105 DAA was also measured (▲ in C). Bars represent SE ($n = 3$). The vertical bar of SE is not shown when SE is smaller than the size of the symbol.

induction provides clear and detailed results (Bais et al., 2000). A UV irradiation experiment of grape leaves revealed a good correlation between resveratrol production and resistance to gray mold (Sbaghi et al., 1995). 'Muscat Bailey A' showed a higher resveratrol accumulation by UV irradiation than did 'Chardonnay' and 'Koshu', whereas no significant difference in the maximum concentration of accumulated resveratrol was

observed between 'Chardonnay' and 'Koshu' (Fig. 2). Bais et al. (2000) reported no major differences in UV-induced resveratrol accumulation between white and red *V. vinifera* grapes. 'Muscat Bailey A' is a red grape variety that exhibits high disease resistance (Goto and Aono, 1981). The accumulation of a large amount of resveratrol in Muscat Bailey A may be unrelated to the presence of anthocyanins, but related to the inherent high disease resistance.

STS, PAL and CHS gene expression by UV irradiation

The relative mRNA expression levels of STS, PAL and CHS genes were determined in grape skins 8 hr after UV irradiation. The RT-PCR product of each gene showed a single band, the size of which agreed with that expected from the nucleotide sequence of each gene (Table 2). The relative mRNA expression level calculated by the ratio of the PCR product generated by linear amplification of the cDNA fragments to that of the 18S rRNA fragment as reference, showed that STS mRNA expression was markedly increased by UV irradiation (Fig. 3), whereas its expression in the control was undetectable in all sampling days. STS mRNA expression in 'Chardonnay' grapes was decreased during grape development, whereas that in 'Muscat Bailey A' was increased. STS mRNA expression in 'Muscat Bailey A' was always higher than that in 'Chardonnay' at 30, 60 and 90 DAA. PAL mRNA expression was also increased by UV irradiation (Fig. 4). Similar to STS, PAL mRNA expression in 'Muscat Bailey A' grapes were higher than those in 'Chardonnay' grapes at 30, 60 and 90 DAA. In contrast, CHS mRNA expression after UV irradiation decreased in both 'Chardonnay' and 'Muscat Bailey A' 30 DAA, but increased 90 DAA (Fig. 5).

Resveratrol is biosynthesized in plant cells by a stepwise decarboxylative condensation of three malonyl-CoA molecules with one 4-coumaroyl-CoA molecule (Fig. 1) (Abe et al., 2001; Morita et al., 2001). STS, which catalyzes this reaction, is thought to be a key enzyme for resveratrol biosynthesis. STS mRNA expressions in both 'Muscat Bailey A' and 'Chardonnay' increased markedly 8 hr after UV irradiation (Fig. 3), indicating that the mRNA expression is induced rapidly. Northern blot analysis of elicited grape berries, leaves and cells reveals a rapid increase in STS mRNA expression within 8 hr after treatment (Bais et al., 2000; Hain et al., 1993; Melchior and Kindl, 1991). The finding that STS mRNA expression in 'Muscat Bailey A' was higher than that in 'Chardonnay' (Fig. 3) explains the difference in resveratrol accumulation between the two cultivars. However, the amount of resveratrol that accumulated in 'Muscat Bailey A' grapes after UV irradiation decreased with the ripening of the grapes, whereas STS mRNA expression increased. This finding suggests that not all differences in resveratrol induction during grape development can be explained by the transcription of the STS gene detected

Fig. 3. Relative expression levels of stilbene synthase (STS) mRNA in 'Muscat Bailey A' (A) and 'Chardonnay' (B) grapes. Quantitative RT-PCR analysis was performed using total RNA isolated from the grapes 8 hr after UV irradiation. The relative mRNA expression level was determined by calculating the ratio of cDNA fragments of STS to that of 18S rRNA. STS mRNA expression in the control grapes was below the detectable level in all the sampling days. Bars represent SE (n = 3).

Fig. 4. Relative expression levels of phenylalanine ammonia-lyase (PAL) mRNA in 'Muscat Bailey A' (A) and 'Chardonnay' (B) grapes. Quantitative RT-PCR analysis was performed using total RNA isolated from the grapes 8 hr after UV irradiation (solid column) or control grapes (open column). The relative mRNA expression level was determined by calculating the ratio of cDNA fragments of PAL to that of 18S rRNA. Bars represent SE (n = 3).

Fig. 5. Relative expression levels of chalcone synthase (CHS) mRNA in grapes of 'Muscat Bailey A' (A) and 'Chardonnay' (B) grapes. Quantitative RT-PCR analysis was performed using total RNA isolated from the grapes 8 hr after UV irradiation (solid column) or control grapes (open column). The relative mRNA expression level was determined by calculating the ratio of cDNA fragments of CHS to that of 18S rRNA. Bars represent SE (n = 3).

by the PCR primers used in the present study (Bais et al., 2000). Southern blot analysis of genomic DNA extracted from *V. vinifera* cv. Lambrusco seedling shows that STS and PAL genes are part of multigene families, each of which consists of 15–20 members (Sparvoli et al., 1994). Sequence analysis of the STS DNA fragment amplified by RT-PCR revealed that it contains two kinds of fragments that possess sequences similar to STS cDNAs, *SV25* and *SV21* (data not shown).

The relative mRNA expression level of PAL, which catalyzes the first reaction in the plant phenylpropanoid pathway, was increased by UV irradiation (Fig. 4), suggesting that UV irradiation activated the phenylpropanoid pathway and increased the amount of 4-coumaroyl-CoA as a substrate for STS (Fig. 1). The coordinated induction of STS and PAL mRNA expressions suggests that these genes have similar UV-responsive promoter regions (Bais et al., 2000). CHS, on the other hand, catalyzes the first step in the biosynthesis of flavonoid compounds, that is, the synthesis of chalcone by the condensation of 4-coumaroyl-CoA with three malonyl-CoA molecules (Fig. 1). CHS uses the same substrates as STS; the amino acid sequence of CHS has a high similarity to that of STS (Goto-Yamamoto et al., 2002). In this study, to detect CHS mRNAs as comprehensively as possible, we used the primers based on the common sequences of *Chs1* (AB015872) and *Chs2* (AB066275), because *Chs1* and *Chs2* mRNAs accumulate in many grape tissues, namely leaves and berry skins of white and red grape cultivars (Goto-Yamamoto et al., 2002). The expression pattern of the CHS gene after UV irradiation differed from that of the STS gene. CHS mRNA expression after UV irradiation decreased before veraison and subsequently increased as the berries matured (Fig. 4), suggesting that the regulation of CHS gene expression in grape berries is unrelated to that of STS gene expression.

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紫外線を照射したブドウ果皮におけるリスベラトロール合成の誘導

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摘 要

3ブドウ栽培品種(マスカット・ベリー A, シャルドネ, 甲州)の果実に紫外線(254 nm, UV-C)を照射し, 果皮に誘導されるリスベラトロールとその合成に関連した酵素遺伝子の発現レベルを測定した。UV照射によりブドウ果皮のリスベラトロール濃度は著しく増加した。この増加は, 果実の成熟がはじまるベレゾーン前の果実で大きく, ベレゾーン後の果実で小さかった。マスカット・ベリー A のリスベラトロール誘導量が3品種のなかで最も大きかった。紫外線照射8時間後のスチルベンシンターゼ(STS), フェニルアラニ

ンアンモニアリアーゼ(PAL), カルコンシンターゼ(CHS)遺伝子の mRNA 発現レベルを定量 RT-PCR 法により測定した。STS と PAL 遺伝子の mRNA 発現レベルは紫外線照射により増加した。リスベラトロール合成のキー酵素である STS の mRNA 発現レベルは, 全ての採取時期において, マスカット・ベリー A のほうがシャルドネに比べて大きかった。一方, 紫外線照射後の CHS 遺伝子の mRNA レベルはベレゾーン前の果実では減少したが, ベレゾーン後の果実では増加し, STS 遺伝子の発現とは異なった挙動を示した。