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Article

Application of Synephrine to Grape Increases Anthocyanin via Production of Hydrogen Peroxide, Not Phytohormones

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Abstract: The global warming has caused poor grape skin coloration and reduced the production of high-quality berries. To overcome these problems, the effect of synephrine (Syn) on anthocyanin accumulation was investigated. Syn treatment above 1 mM increased anthocyanin content in cultured grape cells in a molecular-structure-specific and concentration-dependent manner, and increased only the anthocyanin without affecting the sugar/acid ratio in grape bunches. It increased the expression of genes linked to anthocyanin synthesis after 24 h of treatment, without affecting phytohormone levels. Syn treatment induced the expression of *SOD3*, elevating hydrogen peroxide (H_2O_2) production from 3 to 24 h after treatment in cultured cells. Subsequent increases in *CAT* and *APX6* expression occurred, which encode H_2O_2-eliminating enzymes. In cultured grape cells, Syn and H_2O_2 treatments upregulated the H_2O_2-responsive gene *Chit4* and the anthocyanin-biosynthesis-related genes *mybA1* and *UFGT* 4 days after the treatments and promoted anthocyanin accumulation 7 days after the treatments. In berries, both treatments enhanced anthocyanin accumulation after 9 days. These results suggest that Syn promotes anthocyanin accumulation through the production of H_2O_2 without the upregulation of phytohormone biosynthesis. Syn treatment is expected to improve poor grape coloration and contribute to higher-quality fruit production.

Keywords: Anthocyanin; Grape; bioactive natural products; phytohormone regulator; Hydrogen peroxide; Synephrine

1. Introduction

It is a fact that global warming will have an extremely negative impact on plant growth. Grapevine (*Vitis* spp.) is an economically important berry-bearing plant that is widely grown throughout the world for wine production and as table grapes. The ripening of grape berries is controlled by a variety of environmental factors including sunlight [1], water [2], and temperature [3]. Fruit coloration is mainly regulated by phytohormones such as abscisic acid (ABA) and ethylene (ET), which promote berry ripening [4–7], and jasmonic acid (JA), which promotes anthocyanin biosynthesis and enhances disease resistance in plants [8]. In grape berries, anthocyanin accumulation during ripening is mainly promoted by ABA [9]. Increases in average temperature due to global warming reduce the ABA content of grapes and inhibit anthocyanin accumulation [10,11], resulting in poor grape coloration, which, in turn, degrades the quality of grapes and red wine. Further increases in average temperature are predicted to cause significant economic damage to not only grape growers but also the winemaking industry [12].

Although methods such as girdling [13], leaf removal [14,15], and cluster thinning [16] have been found to solve the problem of poor grape coloration, these methods require skill and labor for application. The direct spraying of ABA [5,17–19] is unpopular because of high cost and side effects such as grapevine defoliation [20]. On the other hand, simple methods to promote grape coloration have been reported, including stimulation with sound [7] and fragrance [21]. In recent years,

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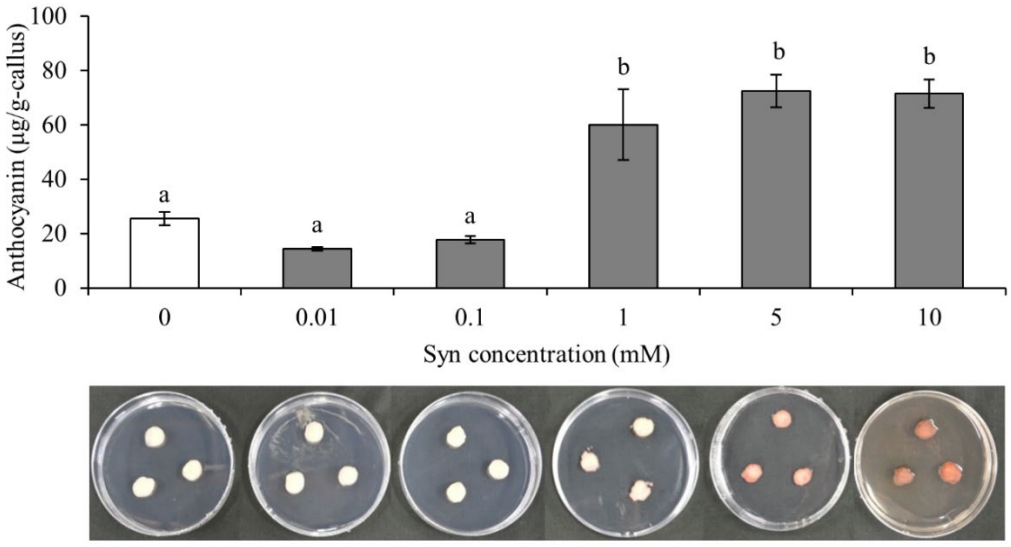
bioactive natural products with high specificity of effect have been focused on as an alternative to synthetic chemicals such as ABA. The direct spraying of bioactive natural products such as allantoin [6], amino acids [22], and vanillyl acetone [4] has been shown to improve grape coloration. The application of bioactive natural products is expected to have advantages over the other techniques, including ease of application, less stress on vines and the environment, and lower cost.

We conducted a screening for bioactive natural products and found that synephrine (Syn) has a color-improving effect on grapes (Enoki, personal communication). Syn is an alkaloid with a phenethylamine skeleton, and its IUPAC nomenclature is 4-[1-hydroxy-2-(methylamino)ethyl]phenol. Syn is found in some orange species [23,24]. It has a lipolytic effect in the human body and is used as a dietary supplement [25,26]. However, there are no reports on the effects of Syn on agricultural plants. Therefore, in this study, we aimed to examine the effect of Syn on anthocyanin biosynthesis in grape berries and to elucidate the mechanism underlying its effect in grape cells, with the goal of improving grape quality by enhancing grape coloration with Syn. Our findings show that Syn induces anthocyanin accumulation via the production of hydrogen peroxide (H_2O_2), not phytohormones.

2. Results

2.1. Syn induces Anthocyanin Accumulation in VR Cells

To investigate the effect of Syn on anthocyanin accumulation, VR (*Vitis* Red) cells were used (Figure 1). Anthocyanin content was significantly higher in VR cells treated with Syn at concentrations of 1 mM or higher than in Control, and no significant difference was found among VR cells treated with Syn at concentrations of 1 mM or higher (Tukey, $P < 0.01$ or 0.05; Figure 1a). Syn is biosynthesized in the phenylalanine and tyrosine pathways via L-(-)-tyrosine (L-Tyro), tyramine (Tyra), and octopamine hydrochloride (Oct) using phenylalanine (Phe) as the substrate [27]. The effect of Syn and the aforementioned compounds on anthocyanin accumulation in the Syn biosynthetic pathway tended to decrease in the order of Phe, Syn, Oct, Tyra, and L-Tyro. Anthocyanin contents in Syn- and Phe-treated VR cells were significantly higher than that in Control (Dunnett, $P < 0.05$ or 0.01; Figure 1b). These results suggest that Syn induces anthocyanin accumulation in VR cells in a concentration-independent and molecular-structure-specific manner.



(**a**)

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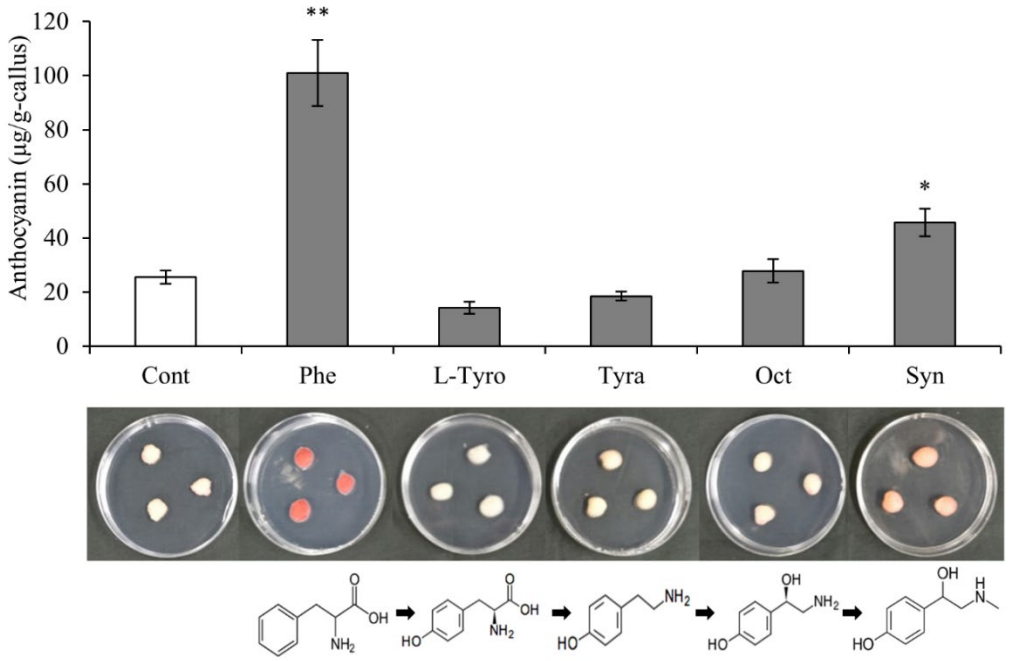
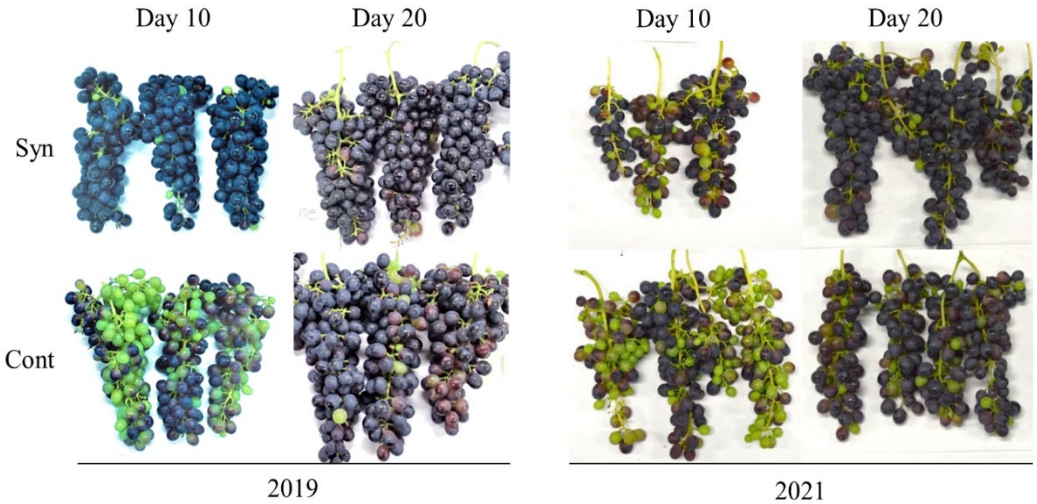


(b)

Figure 1. Effects of Syn on anthocyanin accumulation in VR cells. (a) Effects of Syn at concentrations of 0, 0.01, 0.1, 1, 5, and 10 mM on anthocyanin accumulation. Different letters (a, b) above the bar graphs indicate statistically significant differences (Tukey, $P < 0.01$ or 0.05). (b) Molecular structure specificity of Syn. Effects of Phe, L-Tyro, Tyra, Oct, and Syn (each 5 mM) on anthocyanin accumulation. * and ** indicate significant difference at $P < 0.05$ and 0.01, respectively, relative to Control (Dunnett). VR cells were cultured at 27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day for 7 days. Data are means ± S.E. (n = 3).

2.2. Syn Promotes Anthocyanin Accumulation in Grape Skin in Field Trials

We investigated whether Syn affects the ripening of grape bunches in the field (Figure 2). Field trials were conducted in 2019 and 2021 (Figure 2a). In 2019, anthocyanin content was significantly higher in Syn-treated berries than in Control on days 10 and 20 after treatment ($P < 0.01$ or 0.05; Figure 2b). Similarly, in 2021, anthocyanin content was significantly higher in Syn-treated berries than in Control on day 20 after treatment ($P < 0.01$; Figure 2c). However, in both years, the sugar/acid ratio, a ripeness index, was not significantly different between Syn-treated berries and Control (Figures 2d and 2e). Therefore, these results suggest that Syn promotes only anthocyanin accumulation in grape skin and not fruit ripening.



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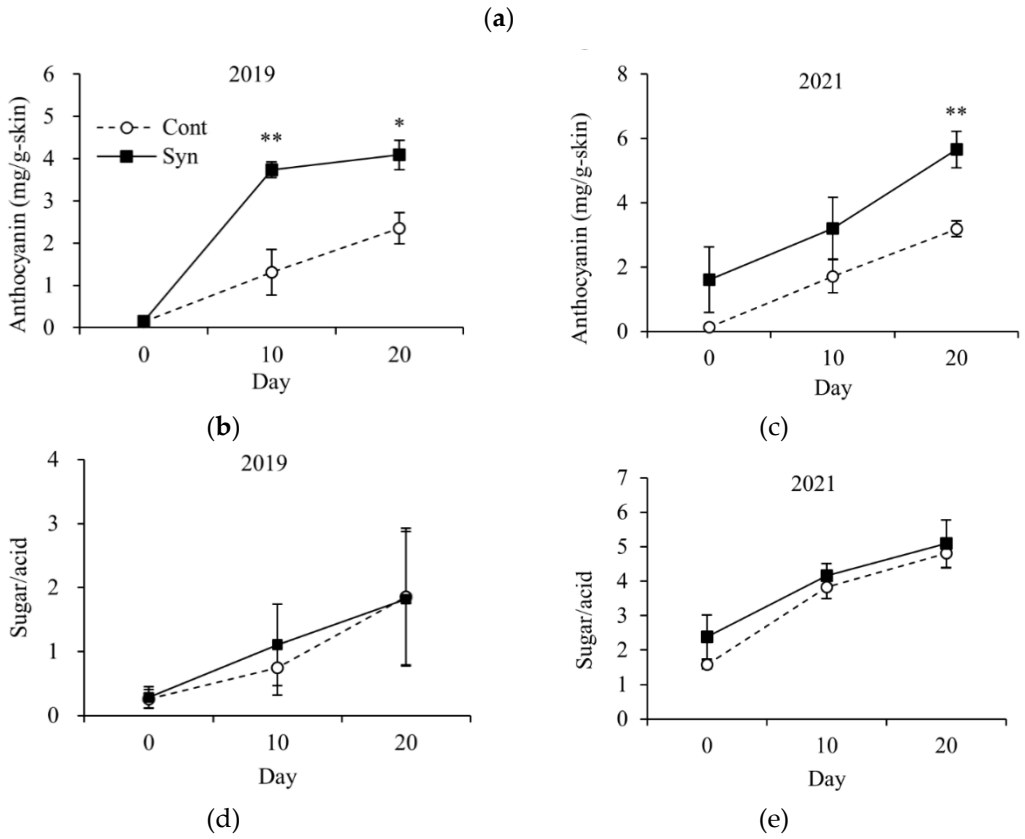

Figure 2. Effect of Syn on fruit quality. Photographs of fruit bunches 10 and 20 days after (1 mM Syn treatment in 2019 and 2021 (a). Anthocyanin content in berry skin (b, c). Sugar/acid ratio of juice (d, e). Data represent means ± SE (n = 3). * indicates significant difference at $P < 0.05$ and **, at 0.01 (*t*-test).

2.3. Syn Increases Expression Levels of Anthocyanin-Biosynthesis-Related Genes

As regards genes involved in the anthocyanin-biosynthesis-related pathways, their expression levels relative to actin were measured by real-time RT-PCR (Figure 3). In the phenylpropanoid biosynthetic pathway, the relative expression levels of *PAL* encoding phenylalanine ammonia-lyase [EC 4.3.1.24] and *4CL* encoding 4-coumarate-CoA ligase [EC 6.2.1.12] were significantly higher ($P <$ 0.01 or 0.05) in Syn-treated VR cells than in Control at 24 h after treatment or later. In contrast, the relative expression level of *C4H* encoding cinnamate-4-hydroxylase [EC 1.14.14.91] was significantly higher in Syn-treated VR cells than in Control at 72 h after treatment or later ($P < 0.01$ or 0.05).

Upstream of the flavonoid biosynthetic pathway, the relative expression level of *CHS* encoding chalcone synthase [EC 2.3.1.74] in Syn-treated VR cells was significantly different from that in Control only at 72 h after treatment, whereas the relative expression level of *CHI* encoding chalcone isomerase [EC 5.5.1.6] showed a significant difference as early as 24 h after treatment ($P < 0.01$). Midstream of the flavonoid biosynthetic pathway, the relative expression levels of *F3′H* encoding flavonoid 3'-monooxygenase [EC 1.14.14.82] and *F3′5′H* encoding flavonoid 3',5'-hydroxylase [EC 1.14.14.81], which are related to red and blue anthocyanin pigment biosynthesis, in Syn-treated VR cells were significantly different at 48 h and 96 h after treatment or later, respectively, compared with Control ($P <$ 0.01 or 0.05). The relative expression level of *F3H* encoding flavanone 3-hydroxylase [EC 1.14.11.9] in Syn-treated VR cells was significantly different from that in Control at 48 h after treatment or later ($P < 0.01$ or 0.05). Downstream, the relative expression levels of *DFR* encoding dihydroflavonol 4-reductase [EC 1.1.1.219] and *LDOX* encoding leucoanthocyanidin dioxygenase [EC 1.14.20.4] in Syn-treated cells were significantly different from those in Control at 72 h after treatment or later ($P < 0.01$). In the flavonoid biosynthetic pathway, significant differences in the relative expression levels of these genes were observed in the early stages of the pathway.

The relative expression levels of *UFGT* encoding UDP-glucose:anthocyanidin/flavonol 3-O-glucosyltransferase [EC 2.4.1.115], a key enzyme in the anthocyanin biosynthetic pathway [28], and its transcription factor *mybA1* encoding Myb-related transcription factor A1 [29], were analyzed. The

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relative expression level of *mybA1* in Syn-treated VR cells was significantly higher than that in Control from 24 h after treatment, and that of *UFGT*, from 48 h after treatment ($P < 0.01$ or 0.05). These results suggest that Syn increases the expression levels of genes involved in the anthocyanin-biosynthesis-related pathways as early as 24 h after treatment.

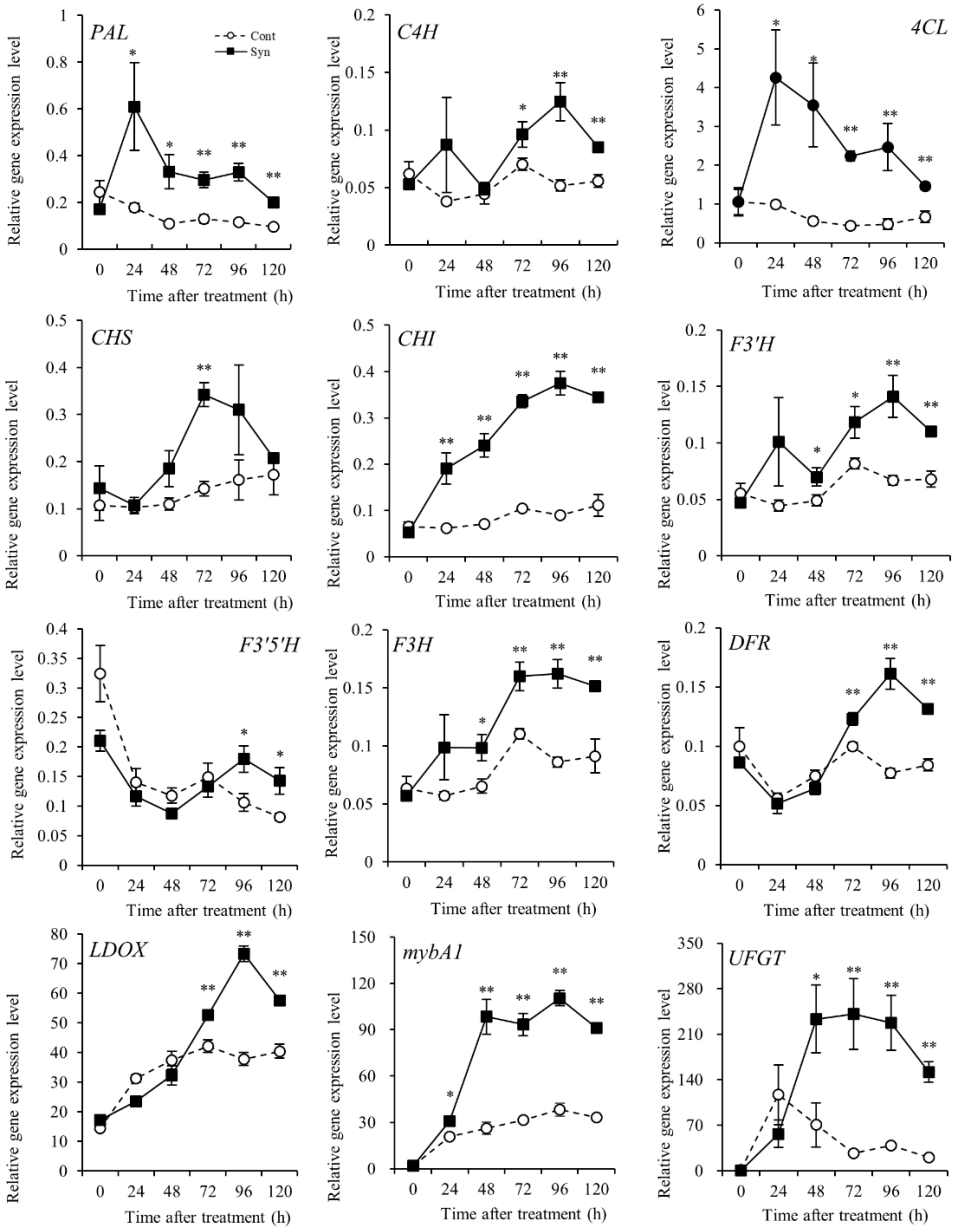

Figure 3. Transcription profiles of genes in anthocyanin-biosynthesis-related pathways in VR cells treated with Syn: *PAL*, *C4H*, and *4CL* in the phenylpropanoid biosynthetic pathway; *CHS*, *CHI*, *F3'H*, *F3'5'H*, *F3H*, *DFR*, and *LDOX* in the flavonoid biosynthetic pathway; and *mybA1* and *UFGT* in the anthocyanin biosynthetic pathway. VR cells were cultured in medium containing 5 mM Syn for 120 h (27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day). The transcription level of each gene was estimated by real-time RT-PCR. Data are relative expression levels to actin. Data are means ± S.E. (n = 4). * and ** indicate significant differences at $P < 0.05$ and 0.01, respectively (*t*-test).

2.4. Syn Does Not Affect the Increase in the Production of Phytohormones, Which Promote Anthocyanin Accumulation

As regards the rate-limiting enzymes of ABA and ethylene, which are phytohormones that have an impact on berry ripening, the relative expression levels of *NCED1* encoding 9-cis-epoxycarotenoid dioxygenase [EC 1.13.11.51] and *ACS3* encoding 1-aminocyclopropane-1 carboxylate synthase [EC 4.4.1.14] in VR cells were measured. Syn neither upregulated *NECD1* expression nor increased ABA

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content at 24 h after treatment (Figures 4a and 4b). The relative expression level of *ACS3* was not significantly different between Syn-treated VR cells and Control at 0 and 12 h after treatment. On the other hand, the relative expression level of *ACS3* showed a significant difference at 24 h after treatment ($P < 0.05$) (Figure 4c). Because it was not technically possible to measure ethylene gas, we measured the relative expression level of *ACO2* encoding aminocyclopropanecarboxylate oxidase [EC 1.14.17.4], which encodes the key enzyme that catalyzes the biosynthesis of ethylene, and found that the expression level was not significantly different between Syn-treated VR cells and Control from 0 to 24 h after treatment (Figure 4d). The effect of Syn on the biosynthesis of JA, a phytohormone involved in fruit coloration and disease resistance, was also investigated. The relative expression level of *LOX* encoding linoleate 13S-lipoxygenase [EC 1.13.11.12], the rate-limiting enzyme in the JA biosynthetic pathway, was significantly different between Syn-treated VR cells and Control at 12 h after treatment (Figure 4e, $P < 0.05$). The endogenous JA content in Syn-treated VR cells was not significantly different from that in Control at 24 h after treatment (Figure 4f). These results suggest that Syn is not involved in the biosynthesis of phytohormones that promote coloration.

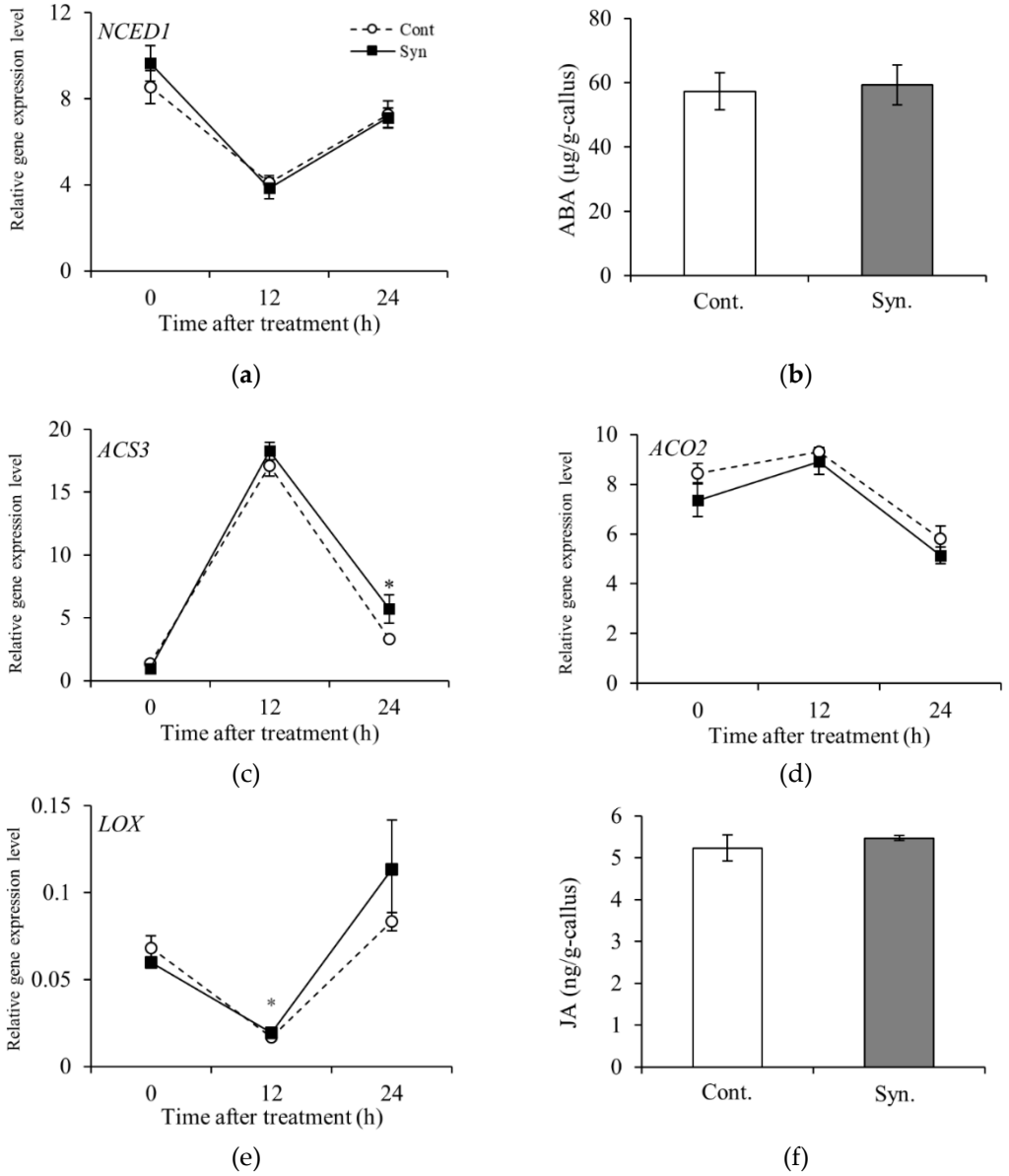

Figure 4. Transcription profiles of various phytohormone biosynthesis genes and their individual phytohormone contents in VR cells treated with Syn. Relative expression level of *NECD1* encoding ABA biosynthesis rate-limiting enzyme (a) and ABA content 24 h after treatment (b). Relative expression levels of *ACS3* (c) and *ACO2* (d), which encode the rate-limiting enzyme of the ET biosynthetic pathway and the enzyme that biosynthesizes ET, respectively. Relative expression level of *LOX* encoding JA biosynthesis rate-limiting enzyme (e) and JA content 24 h after treatment (f). VR

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cells were cultured up to 24 h (27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day) in medium containing 5 mM Syn. The transcription levels of the genes were estimated by real-time RT-PCR. Data are expression levels relative to actin. Each phytohormone content was determined by ELISA. Data are means ± S.E. (n = 4). * and ** indicate significant differences at $P < 0.05$ and 0.01, respectively (*t*-test).

2.4. Syn Increases Anthocyanin Content via H_2O_2

The relative expression level of *SOD3* encoding the H_2O_2-generating enzyme superoxide dismutase [EC 1.15.1.1] was significantly higher in Syn-treated VR cells than in Control as early as 3 h to 12 h after treatment. In fact, H_2O_2 content was significantly higher from 3 h to 24 h ($P < 0.01$ or 0.05; Figures 5a and 5b). The relative expression levels of *APX6* and *CAT* encoding the H_2O_2 scavenging enzymes ascorbate peroxidase [EC 1.11.1.11] and catalase [EC 1.11.1.6], respectively, were measured as H_2O_2-responsive genes. The relative expression level of *APX6* was significantly higher ($P < 0.01$ or 0.05) at 24 h and that of *CAT*, at 24 h and 48 h after Syn treatment compared with Control (Figures 5c and 5d).

Chit4 encoding a class 4 chitinase [EC 3.2.1.14] was measured as an H_2O_2-responsive gene. The relative expression level of *Chit4* was significantly higher (Dunnett, $P < 0.01$) in VR cells 4 days after treatment with Syn or H_2O_2 than in Control (Figure 6a). Syn and H_2O_2 treatments also significantly increased the relative expression levels of *mybA1* and *UFGT* 4 days after the treatments and the anthocyanin content 7 days after (Dunnett, $P < 0.01$ or 0.05) compared with Control (Figures 6b–d). In berries, these treatments also promoted anthocyanin accumulation 9 days after treatment (Dunnett, $P < 0.01$ or 0.05) (Figure 6e). These results suggest that Syn induces anthocyanin accumulation via H_2O_2.

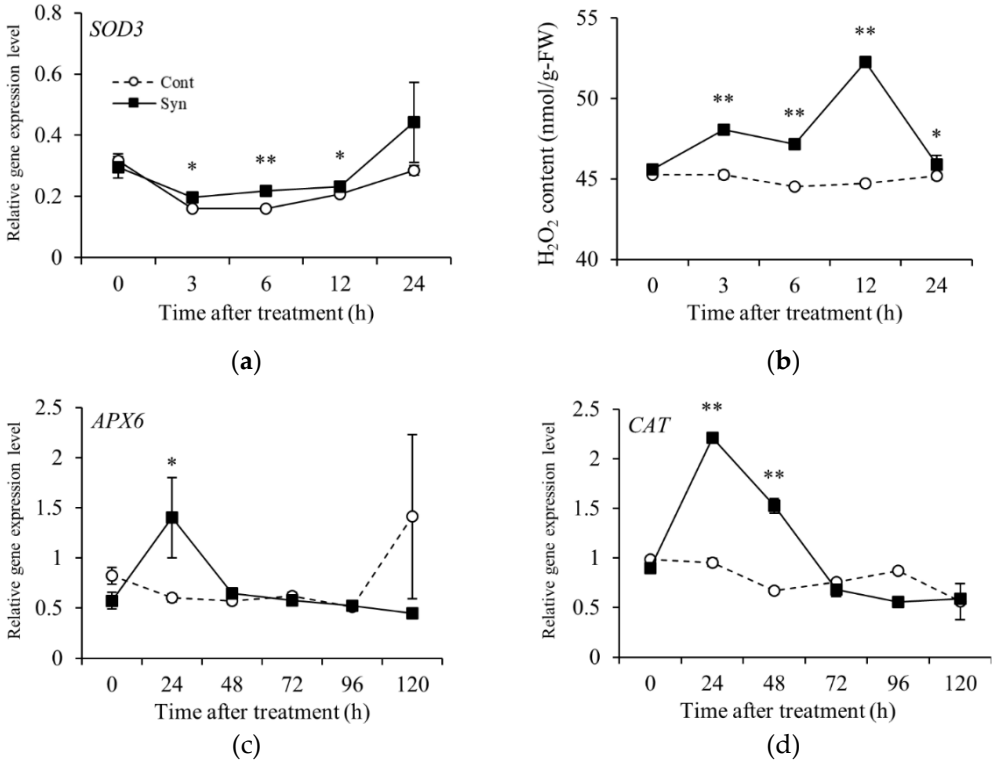

Figure 5. Relative expression levels of H_2O_2-related genes and H_2O_2 contents in Syn-treated cells. *SOD3* encoding H_2O_2-generating enzymes (a) and H_2O_2 content (b). Transcriptional profiles of *APX6* (c) and *CAT* (d) encoding H_2O_2-removing enzymes. VR cells were grown in medium containing 5 mM Syn up to 24 or 120 h (27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day). H_2O_2 content was measured with a fluorescence analysis kit. Transcription levels of the genes were estimated by real-time RT-PCR. Data are expression levels relative to actin. Data are means ± S.E. (n = 4). * and ** indicate significant differences at $P < 0.05$ and 0.01, respectively (*t*-test).

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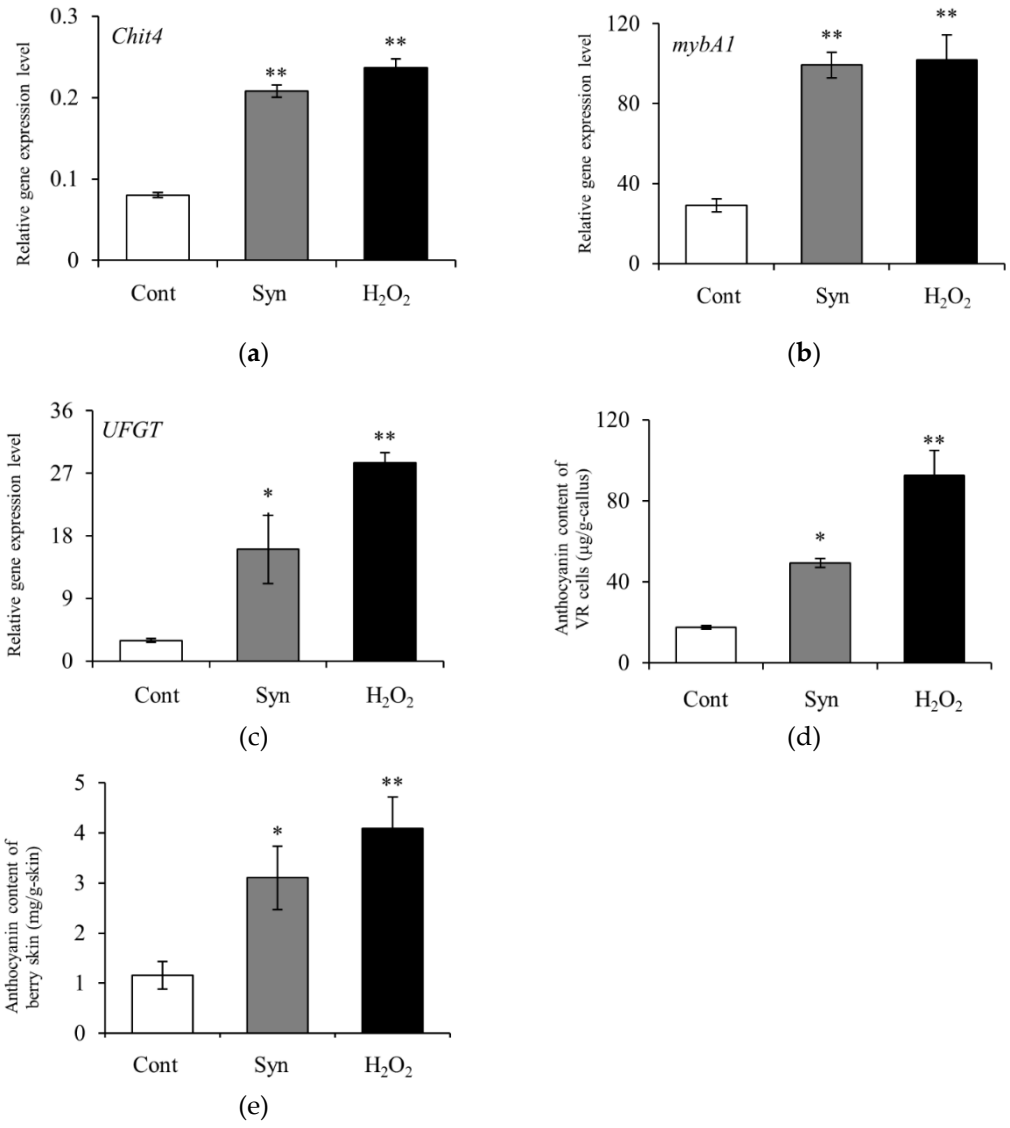

Figure 6. Effects of Syn and H_2O_2 treatments on VR cells and berries. Transcriptional profiles of H_2O_2-responsive gene *Chit4* (a), anthocyanin-biosynthesis-related genes *mybA1* (b) and *UFGT* (c), and anthocyanin content (d) in VR cells. Anthocyanin content (e) in Syn- and H_2O_2-treated grape berries. VR cells were cultured in medium containing 5 mM Syn and 10 mM H_2O_2 for 4 days (for transcriptional profiles) or 7 days (for anthocyanin content) at 27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day. Berries were harvested 9 days after 1 mM Syn and 300 mM H_2O_2 treatments. Transcript levels of the genes were estimated by real-time RT-PCR. Data are expression levels relative to actin. Data are means ± S.E. (n = 4). * and ** indicate significant differences at $P < 0.05$ and 0.01, respectively (Dunnett test).

3. Discussion

The proposed mechanism by which Syn promotes anthocyanin accumulation found in this study is shown in Figure 7. Syn has a phenethylamine skeleton and is biosynthesized from Phe, which is also the substrate for anthocyanin biosynthesis [30] and promotes coloration in grapes [22]. The lack of color in VR cells treated with Syn at intermediate concentrations suggests that the effects of Syn are different from those of Phe (Figure 1). Then, what is the mechanism by which Syn confers coloration? Syn activates *SOD3* and induces H_2O_2 accumulation, as shown in Figure 5. H_2O_2 induces anthocyanin accumulation in many plant species including *Arabidopsis* [31,32] and *Begonia* [33]. On the basis of these findings, we consider that Syn induces anthocyanin biosynthesis in grape cells and berries by activating anthocyanin-biosynthesis-related genes in VR cells, similarly to H_2O_2 treatment (Figure 6). The effects of Syn have been well investigated in the human body, including the lipolytic

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effect [25,26]. However, the effects of Syn on plant coloration have not been reported. This is the first study to demonstrate that Syn promotes anthocyanin biosynthesis by increasing H_2O_2 in grape cells.

Syn upregulated *SOD3* at a very early stage, from 3 h to 12 h after treatment, and promoted H_2O_2 generation from 3 h to 24 h after treatment (Figure 5). Syn is classified as a phenethylamine derivative. In yeast and tobacco, the Syn analog β-phenylethylethylamine promotes H_2O_2 generation [34,35]. These findings suggest that Syn, similar to substances with a phenethylamine skeleton, is an early inducer of H_2O_2 in cells. On the other hand, H_2O_2 also crosstalks with phytohormones and is a signaling molecule that regulates physiological processes such as plant growth and stress response [36–38]. Syn did not markedly alter the expression of genes encoding the biosynthesis rate-limiting enzymes of each phytohormone at 12 h after treatment, and it did not significantly increase ABA and JA contents or the expression level of *ACO2*, which catalyzes ET biosynthesis, at 24 h after treatment (Figure 4). Syn also did not increase the sugar/acid ratio, a ripeness index related to ABA and ET, in grape berries (Figure 2). Therefore, these findings suggest that the H_2O_2 content increased by Syn does not affect the biosynthesis of each phytohormone evaluated in this study at 24 h after treatment, although more detailed evaluation of the crosstalk between H_2O_2 and each phytohormone is needed in the future.

This study revealed that Syn induces anthocyanin accumulation in VR cells by a mechanism similar to that of H_2O_2 production. H_2O_2, a type of reactive oxygen species (ROS), alters the redox state of its surroundings and functions as a signaling molecule *in vivo*, but the balance between ROS production and removal is strictly regulated in cells because excess amounts of ROS cause oxidative injury to cell components [37,39]. In *Arabidopsis*, the accumulation of anthocyanins with antioxidant properties plays an important role in conferring tolerance to ROS-induced oxidative stress [40]. H_2O_2 produced by UV irradiation increased anthocyanin accumulation in radish sprouts [41]. Indeed, H_2O_2 application also increases anthocyanin accumulation in "Kyoho" grapes [42]. On the other hand, oxidative stress due to ROS production induces the expression of *chitinase* [43,44]. H_2O_2 induced by methionine increases the expression level of *class IV chitinase* in grapes [45]. Thus, the expression level of *class IV chitinase* can be used as a marker of H_2O_2-induced oxidative stress response. Although gene expression level analysis and H_2O_2 quantification were not performed on berries, both Syn and H_2O_2 were found to induce the expression of *Chit4* at high levels in VR cells, which in turn induced the accumulation of anthocyanins (Figure 6), indicating that both substances induce oxidative stress in grape cells. Together, these findings suggest that Syn induces the accumulation of the antioxidant anthocyanin by inducing oxidative stress in plant cells similarly to H_2O_2.

How can Syn be used in viticulture? Syn promotes anthocyanin biosynthesis by increasing H_2O_2 production without phytohormones such as ABA, suggesting that Syn can improve grape coloration without undesirable side effects such as defoliation when ABA is used. On the other hand, the safety of Syn for use in the agricultural and food industry needs to be considered. Syn can be safely used as a dietary supplement [46,47], although there have been concerns about the health hazards of Syn because of its structural similarity to the doping agent ephedrine. On the other hand, H_2O_2 is a highly reactive deleterious substance; thus, many countries have set criteria for its content in foods. H_2O_2 in small amounts functions as a signaling molecule in cells, but H_2O_2 in excess amounts is toxic; thus, organisms have mechanisms to remove excess H_2O_2 through catalase and other enzymes. [37]. The findings that Syn rapidly increased the transcription levels of *APX6* and *CAT* and reduced H_2O_2 content in Syn-treated VR cells to the same levels as those in Control at 24 h (Figure 5) suggest that Syn can be used as a transient and safe H_2O_2 inducer. Further evaluation of its possible undesirable effects such as defoliation and analysis of H_2O_2 residues in Syn-treated grapevines in field trials are required to confirm the usefulness of Syn as a safe grape color quality enhancing agent, thereby contributing to viticulture and the wine industry.

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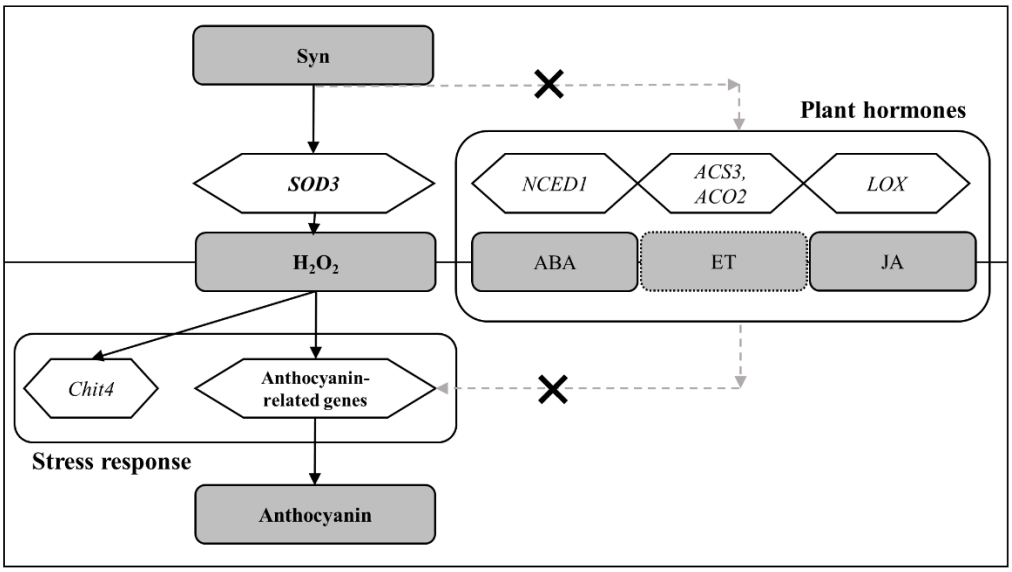

Figure 7. Predicted pathway of anthocyanin accumulation promoted by Syn.

4. Materials and Methods

4.1. In Vitro Trials

Cultured grapevine cells (VR cells) were used in this study. VR cells (PRC00003) were provided by the RIKEN BioResource Center Research (RIKEN BRC) through the National BioResource Project of MEXT/AMED, Japan. The cell line was derived from *Vitis* hybrid cv. Bailey Alicante A, which has high anthocyanin-biosynthesizing ability [48]. Modified Linsmaier and Skoog (LS) medium (pH 6.1) containing 3% (w/v) sucrose, 0.05 mg/L 2,4,-dichlorophenoxyacetic acid (2,4-D), and 0.2 g/L kinetin was used. The medium was autoclaved (1.06 kg cm^{-2}) at 121°C for 15 min, gelled with 1.2% (w/v) agar, and poured into disposable sterile plastic petri dishes. Only white VR cells without red coloration were subcultured every week under sterile conditions and grown in a dark incubator at 27°C.

In the coloration experiments, because phytohormones 2,4-D and kinetin inhibit anthocyanin accumulation during maturation, 10 mL of modified LS medium without phytohormones was autoclaved and dispensed into 70×16.5-mm-diameter petri dishes. One dish was inoculated with 3–4 VR cells (each approximately 5 mm in diameter) under sterile conditions and incubated for up to 7 days in an incubator at 27°C, 54.2 μmol m^{-2} s^{-1}/16 h/day. The final concentrations of the test solutions in the medium were as follows: Syn concentrations of 0.01, 0.1, 1, 5, and 10 mM; molecular structure specificity, 5 mM each of phenylalanine (Phe), L-(-)-tyrosine (L-Tyro), tyramine (Tyra), octopamine hydrochloride (Oct), Syn, and control (Cont) (Tokyo Chemical Industry, Japan); H_2O_2 test, 10 mM H_2O_2 (30% H_2O_2, Fujifilm Wako, Japan). Stock solutions of reagents were prepared and sterilized by filtration using a sterile syringe (2.5 mL SS-02SZ, Terumo, Japan) and a sterile filter (Minisart® 0.45 μm syringe filter, Sartorius, Germany). Each sterile solution was added into the autoclaved medium and adjusted to the above concentrations.

4.2. Field Trials

Vitis vinifera cv. Syrah grapevines in the experimental vineyard (2019, 2021) of The Institute of Enology and Viticulture and an affiliated farm (2022) of the University of Yamanashi (Yamanashi, Japan) were used. The grapevines were approximately 30 years old and grown using the double-cordon-style training method.

A solution of 1 mM Syn with 0.01% (v/v) Approach BI (Kao, Japan) was prepared. The grapevines were defoliated in the fruit zone before veraison and were sprayed with 500 mL of water (as control) or Syn solution per grapevine at veraison (July 30, 2019; July 18, 2021). Thereafter, grape

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bunches were sampled every 10 days. The berries were photographed and stored at -80°C until analyses.

Solutions of 1 mM Syn and 300 mM H_2O_2 with 0.01% (v/v) Approach BI (Kao, Japan) were prepared (August 10, 2022). Nine grape bunches were randomly selected from one grapevine. Three bunches each were sprayed with water (Control), Syn, and H_2O_2. On days 0 and 9 after spraying, bunches were harvested and 10 berries per bunch were randomly collected to determine anthocyanin content.

4.3. Total RNA Isolation

VR cells, 300 μL of Fruit-mate™ for RNA Purification (TaKaRa, Japan), and 300 μL of Buffer RLT for use with an RNA extraction kit (RNeasy Plant Mini kit, QIAGEN, Germany) were added to a 2.0 mL tube. The mixture was homogenized (30.0 Hz, 3 min) using TissueLyser II (QIAGEN) and centrifuged at 120 rpm for 3 min at 4℃. Total RNA was then extracted from 450 μL of supernatant using QIAcube (QIAGEN) with the RNeasy Plant Mini Kit and accessories in accordance with the product manual.

4.4. Real-Time RT-PCR Analysis

Single-stranded cDNA was synthesized from total RNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa) and TaKaRa Cycler Dice™ mini (TaKaRa) in accordance with the manufacturer's manual. Real-time RT-PCR was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) with Thermal Cycler Dice Real Time System III (TaKaRa) in accordance with the manufacturer's manual. Data were analyzed using Thermal Cycler Dice® Real Time System Single Software ver. 5.11. Actin was used for normalization because it is recommended as a reference gene for grapes, and expression levels are shown as relative values [49]. The real-time RT-PCR conditions were as follows: 37 ºC for 15 min for RT reaction and 85 ºC for 5 s for cDNA synthesis, and then 40 cycles at 95 ºC for 5 s and at 60 ºC for 30 s for PCR amplification. The nucleotide sequences of the primers were as follows: *Vvactin* (5′-CAAGAGCTGGAAACTGCAAAGA-3′ and 5′-AATGAGAGATGGCTGGAAGAGG-3′, GenBank accession no. AF369524), *PAL* (5′-AAACAAGGTGGTGCCCTTCA-3′ and 5′- GGTGTTGATCCTCACGAGCA-3′, NM_001397918), *C4H* (5′-AAAGGGTGGGCAGTTCAGTT-3′ and 5′-GGGGGGTGAAAGGAAGATAT-3′, XM_002266202), *4CL* (5′-AGATGGGGATCAAGCAAGGC-3′ and 5′-ATCTCGGCCGGCATGTAAAA-3′, XM_002272746), *CHS* (5′-TCTGAGCGAGTATGGGAACATG-3′ and 5′-CTGTGCTGGCTTTCCCTTCT-3′, NM_001280950), *CHI* (5′-GACGGGTCGCCAGTATTCAG-3′ and 5′-GCTTTGGCTTCTGCGTCAGT-3′, NM_001281104), *F3′H* (5′-TATGGGCTGACCCTACAACGA-3′ and 5′-CCTGGGCAAACAACCTCATT-3′, NM_001280987), *F3′5′H* (5′-AGGGTCGGAGTCAAATGAGTTC-3′ and 5′-CGCTGGATCCCTTGGATGT-3′, NM_001281235), *F3H* (5′-CCAATCATAGCAGACTGTCC-3′ and 5′-TCAGAGGATACACGGTTGCC-3′, NM_001281105), *DFR* (5′-AACTGCTCTTTCCCCGA-3′ and 5′-AACGTCCCTCTGCCTTAGGATTC-3′, NM_001281215), *LDOX* (5′-GCGATATGACCATCTGGCCTAA-3′ and 5′-ATCCCAACCCAAGCGATAGC-3′, NM_001281218.1), *mybA1* (5′-GCAAGCCTCAGGACAGAAGAA-3′ and 5′-ATCCCAGAAGCCCACATCAA-3′, AB111101), *UFGT* (5′-CTTCTTCAGCACCAGCCAATC-3′ and 5′-AGGCACACCGTCGGAGATAT-3′, NM_001397857.1), *NCED1* (5′-GAGACCCCAACTCTGGCAGG-3′ and 5′-AAGGTGCCGTGGAATCCATAG-3′, NM_001281270.1), *ACS3* (5′-CCACCCCATACTACCCAGGA-3′ and 5′-TTGAGGCTGCGTTTTTGAGC-3′, XM_003635528.3), *ACO2* (5′-CAAATGGACGCTGTGGAAAA-3′ and 5′-ATGGCGGAGGAAGAAGGTACT-3′, NM_001280942.1), *LOX* (5′-TGGGCTGAAGCTTTTGATAG-3′ and 5′-CTTGGGCTTGGGTAGTAGT-3′, FJ858257)[50], *SOD3* (5′-GGCGATTCATCTACGGTTGTC-3′ and 5′-CCTCCGCCGTTGAACTTG-3′, NM_001281206)[51], *APX6* (5′-GCCCACTCTCCCCATTCTC-3′ and 5′-TGGAGTTTTGGCGGGAAAT-3′, XM_002282641)[51], *CAT* (5′-GGAGGATGAAGCCATAAGAG-3′ and 5′-

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GGCTGCAAGGGCAAGATA-3′, XM_003631877)[52], and *chit4* (5′- CAATCGGGTCCTTGTGATTC-3′ and 5′- CAAGGCACTGAGAAACGCT-3′, U97522, respectively).

4.5. Total Anthocyanin Content

Anthocyanins in berry skins or VR cells were extracted using the procedure of Yokotsuka et al. (1999) [53] with modifications. Briefly, 10 randomly selected berries per bunch were peeled and crushed with liquid nitrogen using a mortar and pestle. One gram of crushed skin or weighed VR cells was immersed in an appropriate amount of 1% HCl–methanol overnight in the dark. The mixture was centrifuged at 10,000 rpm for 5 min, and the supernatant was diluted with 1% HCl–methanol. After mixing, absorbance was measured at 520 nm using a spectrophotometer (ASV11D-S, AS ONE, Japan). Total anthocyanin content (malvidin-3-*O*-glucoside equivalent) in skin and VR cells was calculated according to a published formula [51].

4.6. Sugar/Acid Ratio

Ten berries per bunch were pressed to obtain grape juice. The juice was centrifuged at 10,000 rpm for 5 min. The sugar (Brix)/acid ratio of the supernatant was measured using a pocket refractometer (PAL-BX|ACID2, ATAGO, Japan) in accordance with the manufacturer's instructions.

4.7. Phytohormone Contents

Each phytohormone was quantified by ELISA. JA content in VR cells was measured in accordance with the manual for plant JA using an ELISA kit (MyBioSource, USA), as reported by Tsai et al. (2019) [55]. Briefly, VR cells cultured for 24 h and PBS (100 μL of PBS/10 mg of tissue) were added to a 2 mL Eppendorf tube and homogenized (30.0 Hz, 3 min) using TissueLyser II (QIAGEN). Then, 50 μL of the supernatant was centrifuged in a tabletop centrifuge and dispensed into a 96-well plate. Within 15 min after the addition of Stop Solution in the kit, absorbance t was measured at 450 nm using an absorbance microplate reader, and JA content was calculated by the calibration curve method. Similarly, ABA content in VR cells cultured for 24 h was measured using a plant hormone ABA ELISA kit (CUSABIO, USA) as reported by Enoki et al. (2017) [4].

4.8. H_2O_2 Content

H_2O_2 content was determined using a Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit *Green Fluorescence* (AAT Bioquest, USA) on the basis of the method of Nie et al. (2020) [56] with modifications. Briefly, VR cells and Component C assay buffer (200 mg/mL) were added to a 2 mL Eppendorf tube and homogenized (30.0 Hz, 3 min) using a TissueLyser II (QIAGEN). The homogenate was separated using a tabletop centrifuge, and 50 μL of the supernatant was used as a test sample. After the reaction solution was added in accordance with the manufacturer's instructions, the mixtures were incubated at room temperature for 20 min, and fluorescence intensity was measured at Ex/Em=485/538 nm using a fluorescence microplate reader. H_2O_2 content was calculated using the calibration curve method.

4.9. Statistical Analysis

Data are presented as means ± standard error (SE). Statistical analysis was performed using BellCurve for Excel software (Social Survey Research Information, Japan) with the Student's *t*-test, Tukey test, or Dunnett test.

5. Conclusions

This study demonstrated the molecular mechanism of the effect of synephrine (Syn) on anthocyanin accumulation. The anthocyanin accumulation was increased by induction of hydrogen peroxide, but not phytohormones, and the upregulation of a group of anthocyanin biosynthesis-related genes in Syn-treated cells. The results of upregulated *chitinase* gene, a stress response marker, by Syn treatment suggest that Syn increases the accumulation of the antioxidant anthocyanin by

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inducing oxidative stress due to hydrogen peroxide. In the future, the new approach of promoting coloration by synephrine (Syn) application to grape berries may serve as an alternative to the conventional use of phytohormone-related agents for improving grape quality.

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