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Farnesyl diphosphate synthase may determine the accumulation level of (−)-rotundone in 'Syrah' grapes

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Summary

(−)-Rotundone is an oxygenated sesquiterpene responsible for the peppery aroma in grapes, wines, herbs, and spices, and it was first identified in 'Syrah' wine from Australia. In this study, we demonstrated the expression profiles of genes related to (−)-rotundone biosynthesis during the maturation of 'Syrah' grapes from two different vineyards, namely, the Iwaimura and Johnohira vineyards in Japan. The α-guaiene and (−)-rotundone accumulation levels in the grape exocarp from the Johnohira vineyard, which has a cool climatic condition located at a high altitude, were extremely higher than those from the Iwaimura vineyard. Among the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway genes, the transcript levels of 1-deoxy-D-xylulose-5-phosphate synthase gene (*DXS*) in the grape exocarp from the Johnohira vineyard were higher than those from the Iwaimura vineyard after véraison. The expression levels of the mevalonate pathway genes, *Vitis vinifera* terpene synthase gene (*VvTPS24*) and cytochrome P450 gene (*CYP71BE5*) in the final step of (−)-rotundone biosynthesis were not significantly different between samples from the two vineyards during grape maturation. In contrast, the farnesyl diphosphate synthase gene (*FPPS*) expression level was considerably higher in the grape exocarp from the Johnohira vineyard than in that from the Iwaimura vineyard. Consistent with these observations, *FPPS* was constantly expressed at higher level in 'Syrah' grape exocarp compared with 'Merlot' grape which is a low-rotundone cultivar. These findings suggest that *FPPS* may play a key role in determining the accumulation level of (−)-rotundone, which can provide abundant substrates for *VvTPS24* catalysis to produce α-guaiene as a precursor of (−)-rotundone. In addition, among the MEP pathway genes, *DXS* may have a regulatory role for a precursor supply from the plastids to (−)-rotundone biosynthesis.

Key words: *CYP71BE5*; farnesyl diphosphate synthase; guaiene; rotundone; *VvTPS24*; *Vitis vinifera.*

Introduction

(−)-Rotundone, an oxygenated sesquiterpene, was first identified in 'Syrah' wine as a molecule responsible for its peppery aroma (Wood *et al.* 2008). It is an important molecule in wines and grapes because of its low sensory threshold (16 ng·L^{-1} in red wine, 8 ng·L^{-1} in water) and characteristic aroma properties (Siebert *et al.* 2008). (−)-Rotundone is distributed in 'Syrah' (regionally called 'Shiraz') as well as various grape varieties including 'Durif', 'Mourvèdre', 'Duras', 'Grüner Veltliner', 'Vespolina' and 'Schioppettino' (Caputi *et al.* 2011, Geffroy *et al.* 2014, Herderich *et al.* 2012, Mattivi *et al.* 2011). (−)-Rotundone is localized in the grape exocarp and increases dramatically from véraison to harvest, but its extraction rate from the grape exocarp during the vinification process is very low and limited because of its high hydrophobic properties (Caputi *et al.* 2011). Therefore, (−)-rotundone concentration in finished wine is relatively dependent on that in grape berries. The accumulation of (−)-rotundone is affected by various environmental factors, such as cool climate (Caputi *et al.* 2011), soil properties and topography (Scarlett *et al.* 2014), soil moisture from irrigation, and light exposure of the bunch zone after leaf removal (Geffroy *et al.* 2014). Furthermore, it has been more recently proposed that the biotic stress by infection with powdery mildew could enhance the production of (−)-rotundone in grape berries, resulting from grapevine defence response to powdery mildew (Geffroy *et al.* 2015). However, the biological function of (−)-rotundone was not yet determined.

Sesquiterpenoids are biosynthesized from the two isomeric five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are condensed into farnesyl diphosphate (*FPP*) by farnesyl diphosphate synthase (*FPPS*), and then various sesquiterpene synthases transform *FPP* to a wide variety of sesquiterpenoids. In plants, IPP and DMAPP are formed *via* two independent pathways, the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway operating in plastids and the mevalonate (MVA) pathway operating in the cytosol. 1-Deoxy-D-xylulose-5-phosphate synthase (*DXS*), 1-deoxy-D-xylulose5-phosphate reductoisomerase (*DXR*), and

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(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*HDR*) for the MEP pathway, and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*) for the MVA pathway are considered as the rate-limiting enzymes (Ikram *et al.* 2015). Generally, for sesquiterpenoid biosynthesis, IPP and DMAPP are primarily provided via the MVA pathway (Bohlmann and Keeling 2008). On the other hand, intermediates can be exchanged between the MVA and MEP pathways across the plastid membrane in some plants (Hemmerlin *et al.* 2003, Hampel *et al.* 2006). Previous studies have shown that precursors from both the MVA and MEP pathways were incorporated into sesquiterpenoids (May *et al.* 2013), and monoterpenoids are almost exclusively synthesized *via* the MEP pathway in grape berries (Luan and Wüst 2002).

Several sesquiterpene synthases such as (−)-valencene synthase and (−)-germacrene D synthase have been isolated from grapevines (Lucker *et al.* 2004, Martin *et al.* 2009). Recently, it has been demonstrated that at least two alleles of *Vitis vinifera* terpene synthase gene (*VvTPS24*) exist in grapevines, both of which encode functional sesquiterpene synthases (*VvGuaS* and *VvPNSeInt*), and *VvGuaS* can transform *FPP* to mainly α-guaiene (Drew *et al.* 2016). Furthermore, α-guaiene 2-oxidase cytochrome P450 (*CYP71BE5*), which transforms α-guaiene to (−)-rotundone, was identified in grapevines (Takase *et al.* 2016). However, it has not been clarified, which genes actually regulate the accumulation level of (−)-rotundone in grapevines. The information of genes regulating the (−)-rotundone accumulation is needed for the further investigations to determine why the level of (−)-rotundone accumulation in the grape berries is variable depending on the wine production regions, and what environmental factor critically impact on (−)-rotundone accumulation in the grape berries.

Our objective of this study is to obtain a better understanding of the regulation of (−)-rotundone biosynthesis in grapevines at genetic level. Therefore, we demonstrated the expression profiles of genes related to (−)-rotundone biosynthesis during the maturation of Japanese 'Syrah' grapes from two different vineyards that showed different accumulation patterns of α-guaiene and (−)-rotundone. These profiles revealed the correspondence of *FPPS* expression with the levels of α-guaiene and (−)-rotundone accumulation in the grape exocarp, suggesting that *FPPS* may play a key role in determining the accumulation level of (−)-rotundone.

Material and Methods

Chemicals: (−)-Rotundone, deuterated $^{2}H_{5}$-rotundone, and α-guaiene were prepared as previously described (Takase *et al.* 2015). All chemicals were purchased from Sigma-Aldrich Japan (Osaka, Japan). Milli-Q water was prepared using a Milli-Q purification system (Merck Millipore, Tokyo, Japan).

Experimental sites and plant materials: Samples were obtained from grapevines (*Vitis vinifera* 'Syrah') grown in two vineyards, namely, the Iwaimura vineyard (lat. 35°39’31”N; long. 138°43’25”E; 392 m a.s.l.) and the Johnohira vineyard (lat. 35°38’59”N; long. 138°44’43”E; 589 m a.s.l.) in Koshu City, Yamanashi Prefecture, Japan. These vineyards are located close to each other, separated by a distance of about 2 km, and differ in altitude by approximately 200 m. The grapevines in both vineyards were planted using the single Guyot pruning system and were approximately 10 years old. Their rows in the Iwaimura and Johnohira vineyards are oriented from north to south and from northwest to southeast, respectively. For quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *FPPS* and *VvTPS24* with different grape varieties, samples were obtained from grapevines (*Vitis vinifera* 'Syrah' and 'Merlot') grown in the Mariko vineyard (lat. 36°20’35”N; long. 138°18’5”E; 640 m a.s.l.) in Ueda City, Nagano Prefecture in Japan.

For sampling, one row was selected from each vineyard. Grape berry samples of approximately 1 kg were collected randomly from the same row in each vineyard during grape maturation in the 2013 growing season. After destemming, the berries were immediately stored at -80 °C.

Meteorological data measurement: Regional meteorological data of the nearest weather station (Katsunuma, lat. 35°39’8”N; long. 138°43’5”E; 394 m a.s.l.), such as rainfall and air temperature (maximum, minimum, and mean values) were obtained from the Japan Meteorological Agency website (http://www.jma.go.jp/jma/index.html). The weather station is located approximately 0.5 and 2.4 km from the Iwaimura and Johnohira vineyards, respectively.

The thermo recorder (TR-51A, T&D Co., Nagano, Japan) was attached adjacent to grape bunches on a representative grapevine in the middle of the row in each vineyard from véraison to harvest (August 10 to October 11, 2013). The recorded meteorological data were used to determinate the mean air temperature and the total growing degree hours at the fruit zone. Total growing degree hours was calculated using a base temperature of 10 °C as previously described (Spayd *et al.* 2002).

Fruit component analysis: Grape berries (200 g) were used for preparing juice samples by homogenizing them in a mill followed by pressing to 60 % of their total weight. Total soluble solids in juice were measured with a refractometer (Pocket PAL-1, ATAGO Co., Tokyo, Japan) and expressed as °Brix. The pH of juice samples was measured with a pH meter (MH-60S, DDK-TOA Co., Tokyo, Japan). Titratable acidity was determined by adding 10 mL of distilled water to 10 mL of juice, which was then subjected to neutralization titration using 0.1 N NaOH. TA was expressed as g tartaric acid per L.

Quantification of α-guaiene and (−)-rotundone in grape exocarp: α-guaiene and (−)-rotundone were quantified by gas chromatography-tandem mass spectrometry (GC-MS/MS) with stir bar sorptive extraction (SBSE). GC-MS/MS analysis was carried out with an Agilent 7890A Series GC system coupled to an Agilent 7000 GC/MS Triple Quad system with a Triple-Axis detector, a thermal desorption unit (TDU, GERSTEL GmbH, Mülheim an der Ruhr, Germany), and a programmed temperature vaporizing injector (CIS4, GESTER GmbH). Extraction of α-guaiene and (−)-rotundone from

the grape exocarp, SBSE, GC separation, and MS/MS detection were performed as previously described (Takase *et al.* 2016).

Quantitative real-time RT-PCR analysis: Total RNA was extracted from the grape exocarp by the CTAB method, treated with an RNase-Free DNase Set (Qiagen, Hilden, Germany), and purified with an RNeasy Plant Mini kit (Qiagen) in accordance with the provided protocol. Quantitative real-time RT-PCR analysis was performed with a One-Step SYBR PrimeScript PLUS RT-PCR kit (Takara Bio Inc., Japan) using an ABI Prism 7300 real-time PCR system (Life Technologies Inc., Rockville, MD, USA) in accordance with the provided protocol. The following primer pairs were used as previously described: the 3-hydroxy-3-methylglutaryl-CoA synthase gene (*HMGS*; Fw, 5'-CTCCAGGCACCCACTATCTT-3'; Rv, 5'-AACATTTCAGCCAACCTCCT-3') and *HMGR* (Fw, 5'-AGAAACCAATCGTGAACAACAA-3'; Rv, 5'-ACTGGACTCCCCCAACTTTAC-3') (D'Onofrio *et al.* 2009), *DXS* (Fw, 5'-CTCATTTCCTGCCCATTTTAGC-3'; Rv, 5'-CTTACTCCTTTGCTGGGATTGG-3'), *DXR* (Fw, 5'-AGAGGCTTTGGCTGACTGTGA-3'; Rv, 5'-AACCTGCGCAACCTACTATTCC-3'), *HDR* (Fw, 5'-TCTTCCTCGTCTGTGGCTGTT-3'; Rv, 5'-GCGATTCATGAGCTCCAGAGT-3'), *FPPS* (Fw, 5'-ATTGCTTATGGCAGGCGAAA-3'; Rv, 5'-CCGTTCCGATCTTACCAATCAC-3') (Martin *et al.* 2012), *CYP71BE5* (Fw, 5'-CTACTGGTCATCTCTTTTCTCCTC-3'; Rv, 5'-ACTTCGGAGCAGGATGGTTG-3') and 18S rRNA (Fw, CGAAAGCATTTGCCAAGGAT; Rv, CCTGGTCGGCATCGTTTATG-3') (Takase *et al.* 2016). The primers of *VvTPS24* that has *VvGuaS* as one of its alleles (accession number, XM_002282452; Fw, 5'-GGTTTCACCACTGCTCACCTTCAG-3'; Rv, 5'-ATGCCTTGCCTCCACCCTTG-3') were designed using Primer 3 software (http://bioinfo.ut.ee/primer3/), and its primer pair was shared with *VvPNSeInt,* which is another allele of *VvTPS24*. Quantitative real-time RT-PCR analysis was performed in triplicate, and all samples were normalized to the data of 18S rRNA as an internal control.

Results and Discussion

Meteorological characterization and fruit components in two different vineyards were determined. Two different vineyards are closely located with different elevations. Regional meteorological data of the 2013 growing season showed that rainfall from véraison to harvest was 333 mm, and the annual rainfall was 1,056 mm. Furthermore, the mean air temperature, mean maximum temperature, and mean minimum temperature in this region from véraison to harvest were 23.9, 30.9, and 19.2 °C, respectively. The mean air temperatures at the fruit zone of the Iwaimura and Johnohira vineyards were 24.4 and 23.3 °C from véraison to harvest, respectively. This difference is likely due to the temperature drop with increasing elevation at an environmental lapse rate of 0.6 °C per 100 m. In accordance with this finding, the total growing degree hours at the fruit zone of the Iwaimura and Johnohira vineyards were 21,810 and 20,186 degree hours. These results indicate that the Johnohira vineyard was relatively cooler than the Iwaimura vineyard.

Data on berries during grape maturation at each vineyard were determined (Table). The total soluble solids of grape berries from the Iwaimura vineyard were higher than those from the Johnohira vineyard at each sampling period. Furthermore, the titratable acidity of grape berries from the Iwaimura vineyard decreased more rapidly than that from the Johnohira vineyard with increasing pH. These findings were consistent with a previous study demonstrating that grape maturity (sugar and acid levels) is affected by elevation (Failla *et al.* 2004). It is also supported by the finding that a warm climatic condition promotes sugar accumulation and acidity reduction in grape berries (Bonada and Sadras 2015).

Relationship between α-guaiene and (−)-rotundone accumulation during grape maturation was investigated. α-guaiene and (−)-rotundone are notably accumulated in the grape exocarp (Takase *et al.* 2016). The α-guaiene and (−)-rotundone concentrations during grape maturation were quantified in the grape exocarp from the two vineyards by GC-MS/MS with SBSE (Fig. 1A and B). The concentration of (−)-rotundone in the grape exocarp from the Johnohira vineyard extremely increased from 26 August (final period of véraison) to 28 September, and then decreased. This pattern was similar to that of α-guaiene concentration in the grape exocarp from the Johnohira vineyard. On the other hand, the α-guaiene and (−)-rotundone concentrations in the grape exocarp from the Iwaimura vineyard similarly increased moderately and reached maximum on 18 September more rapidly than those from the Johnohira vineyard, and then decreased. However, accumulation of high concentrations of α-guaiene and (−)-ro-

Table

Data on berries from Iwaimura and Johnohira vineyards during grape maturation

Sampling date	Total soluble solids (°Brix)		pH		Titratable acidity ($g \cdot L^{-1}$)	
	Iwaimura	Johnohira	Iwaimura	Johnohira	Iwaimura	Johnohira
13-Aug	14.3	12.7	3.0	2.9	11.4	17.1
26-Aug	17.8	15.9	3.3	3.5	5.1	7.6
18-Sep	19.2	18.9	3.8	3.7	4.3	5.8
28-Sep	20.3	19.8	3.6	3.3	3.7	5.3
10-Oct	21.0	20.3	4.0	3.8	3.0	4.1

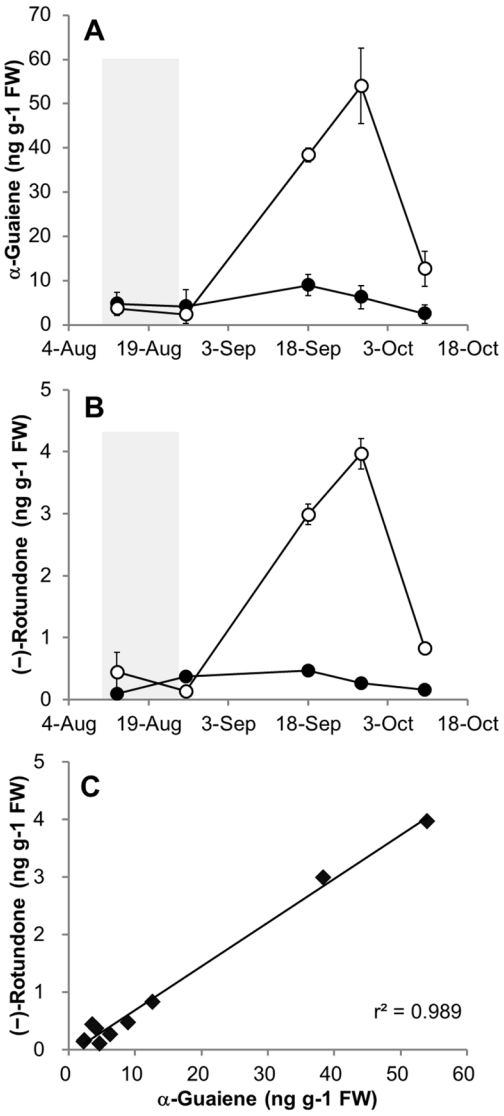

Fig. 1: Relationship between α-guaiene and (−)-rotundone accumulation in 'Syrah' grape exocarp during grape maturation. Concentrations of (**A**) α-guaiene and (**B**) (−)-rotundone, and (**C**) correlation between the concentrations of α-guaiene and (−)-rotundone from both vineyards (n = 10). Filled and open circles indicate samples from the Iwaimura and Johnohira vineyards, respectively. The period of véraison is highlighted by a shade box. The values are means±standard deviation of three replicates. FW indicates fresh weight.

tundone in the grape exocarp from the Iwaimura vineyard was not observed compared with that from the Johnohira vineyard. The microclimatic characteristics of the Johnohira vineyard, such as elevation and air temperature particularly in the fruit zone, might underlie the differences in α-guaiene and (−)-rotundone accumulation levels. A previous report suggests that the biosynthesis of (−)-rotundone occurs following the accumulation of α-guaiene as a precursor (Takase *et al.* 2016). Therefore, (−)-rotundone concentration definitely correlated with α-guaiene concentration in the grape exocarp (Fig. 1C).

Expression analysis of genes related to proposed pathway of (−)-rotundone biosynthesis was performed. (−)-Rotundone accumulated at much higher concentrations in the grape exocarp along with α-guaiene accumulation in the Johnohira vineyard during grape maturation. Therefore, to determine which genes actually regulate (−)-rotundone accumulation at gene expression level, quantitative real-time RT-PCR analysis was performed to compare samples from the Iwaimura and Johnohira vineyards. *DXS*, *DXR* and *HDR* in the MEP pathway and *HMGS* and *HMGR* in the MVA pathway, which are the primary pathway for sesquiterpenoids, and *FPPS*, *VvTPS24* including its *VvGuaS* allele, and *CYP71BE5* in the final step of (−)-rotundone biosynthesis were selected as the target genes (Fig. 2). *DXR*, *HDR* and *FPPS* are singular, and there are several homologues else for *DXS*, *HMGS* and *HMGR* in the 12-fold coverage genome sequence assembly of the grapevine cultivar 'Pinot Noir' PN40024 (Jaillon *et al.* 2007).

In the MEP pathway genes, the transcript levels of *DXS* in the grape exocarp from the Johnohira vineyard were higher than those from the Iwaimura vineyard after the véraison from September 18 (Fig. 3A). On the other hand, the patterns of *DXR* expression in the grape exocarp did not differ between the two vineyards (Fig. 3B), and the patterns of *HDR* expression in the grape exocarp from the Johnohira vineyard were always lower than those from the Iwaimura vineyard during grape maturation (Fig. 3C). These results suggest that among the MEP pathway genes, *DXS* may have a regulatory role for a precursor supply from the plastids to (−)-rotundone biosynthesis. Overexpression of *DXS* from 'Moscato Bianco' grape berries in *Nicotiana tabacum* resulted in a significant increase in monoterpenoid production; however, it was not reported about the sesquiterpenoid production (Battilana *et al.* 2011). Therefore, further studies are needed to determine if *DXS* can really contribute to (−)-rotundone biosynthesis in grapevines.

For the MVA pathway genes, the patterns of *HMGS* and *HMGR* expressions in the grape exocarp were not significantly different between the two vineyards (Fig. 3D and E). Furthermore, for the final step genes of (−)-rotundone biosynthesis, there were no significant differences in the patterns of *VvTPS24* and *CYP71BE5* expressions in the grape exocarp between the two vineyards (Fig. 3G and H). The transcript levels of *VvTPS24* in the grape exocarp from both vineyards increased along with grape maturation. This observation was different from previous report that the sesquiterpene synthase gene corresponding to *VvTPS24* expressed during the period of véraison, but its transcript was not detected at harvest, although the transcript levels of other several sesquiterpene synthase genes increased in the later period of grape maturation (Sweetman *et al.* 2012). It may be due to the differences of environmental conditions, suggesting that some environmental factors in Japan have a positive influence on *VvTPS24* expression. The transcript levels of *CYP71BE5* in the grape exocarp from the Johnohira vineyard increased from August 26 to September 28, and then decreased. This pattern was consistent with the pattern of (−)-rotundone accumulation in the grape exocarp from the Johnohira vineyard. Interestingly, the same tendency as the results from the Johnohira vineyard was also observed in the grape exocarp from the Iwaimura vineyard, although the levels of (−)-rotundone accumulation was lower because of the low availability of

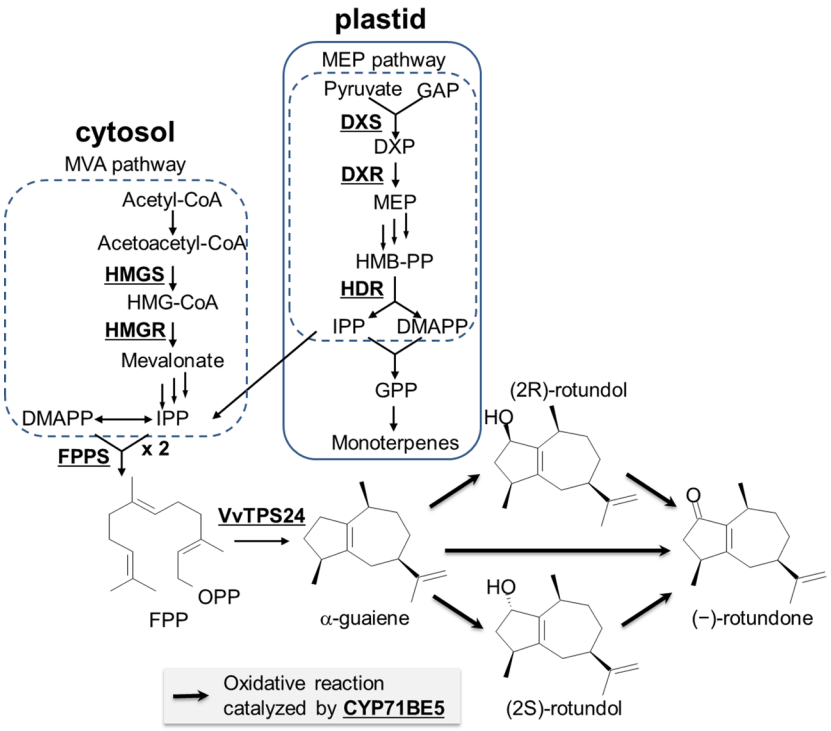

Fig. 2: Proposed pathway for (−)-rotundone biosynthesis. MEP pathway operating in plastids and MVA pathway operating in cytosol. GAP, glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXP, 1-deoxy-D-xylulose-5-phophate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; HMB-PP, HDR, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase, HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; FPPS, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; OPP, diphosphate in the structure of FPP. The underlined enzymes were targeted for quantitative real-time RT-PCR analysis.

α-guaiene, than that in the Johnohira vineyard. These results were in agreement with a previous report that (−)-rotundone accumulation is regulated by *CYP71BE5* expression along with the availability of α-guaiene (Takase *et al.* 2016). On the other hand, among the final step genes of (−)-rotundone biosynthesis, the transcript levels of *FPPS* in the grape exocarp from the Johnohira vineyard were considerably higher than those from the Iwaimura vineyard during grape maturation from August 26 (Fig. 3F). Additionally, it was observed that α-guaiene and (−)-rotundone concentrations rapidly decreased from September 28 to October 10, in particular in the grape exocarp from the Johnohira vineyard (Fig. 1A and B). Concurrently with their decline, most of the genes in the MEP and MVA pathways down-regulated in the grape exocarp from the Johnohira vineyard. Therefore, it may have led to the rapid decline of α-guaiene and (−)-rotundone concentrations. Besides, the degradations and modifications of α-guaiene and (−)-rotundone may have also happened with the catalysis by several enzymes from grapevines at end of grape maturation. The sesquiterpene oxidases capable to transform α-guaiene to unknown sesquiterpenoids, have been identified from 'Syrah' grape exocarp (Takase *et al.* 2016).

α-Guaiene and (−)-rotundone accumulation during grape maturation was considerably higher in 'Syrah' grape exocarp compared with 'Merlot' grape exocarp, and *CYP71BE5* was expressed in accord with the patterns of (−)-rotundone accumulation (Takase *et al.* 2016). Consistent with our observations with 'Syrah' exocarps from the Iwaimura and Johnohira vineyards, the transcript levels of *FPPS* in 'Syrah' grape exocarp were constantly higher than those in 'Merlot' grape exocarp during grape maturation (Fig. 4A). Furthermore, the transcript levels of *VvTPS24* were not significantly different between both grape varieties, although *VvTPS24* in 'Syrah' grape exocarp was expressed at a relatively high level in the final period of grape maturation (Fig. 4B). *FPPS* catalyzes the formation of *FPP*, which is a common precursor for sesquiterpenoid biosynthesis, including α-guaiene. The overexpression of *FPPS* in a transgenic *Artemisia annua* L plant increases the concentration of artemisinin, which is a sesquiterpene lactone, compared with that in a nontransgenic plant (Han *et al.* 2006). Moreover, in *Arabidopsis thaliana*, overexpression of *FPP2* leads to the synthesis of several new sesquiterpenoids such as *E*-β-farnesene, suggesting that their synthesis could be due to increased availability of *FPP* (Bhatia *et al.* 2015). Although transient expression assays such as overexpression studies of *FPPS* in grapevines remain to be carried out to fully determine the impact of *FPPS* expression on the (−)-rotundone biosynthesis in grapevines, our results suggest that *FPPS* may play a critical role in determining the accumulation level of (−)-rotundone by providing its precursors in abundance, which in turn leads to (−)-rotundone biosynthesis. The Johnohira vineyard was relatively cooler than the Iwaimura vineyard. A cool climate is a well-known environmental factor that

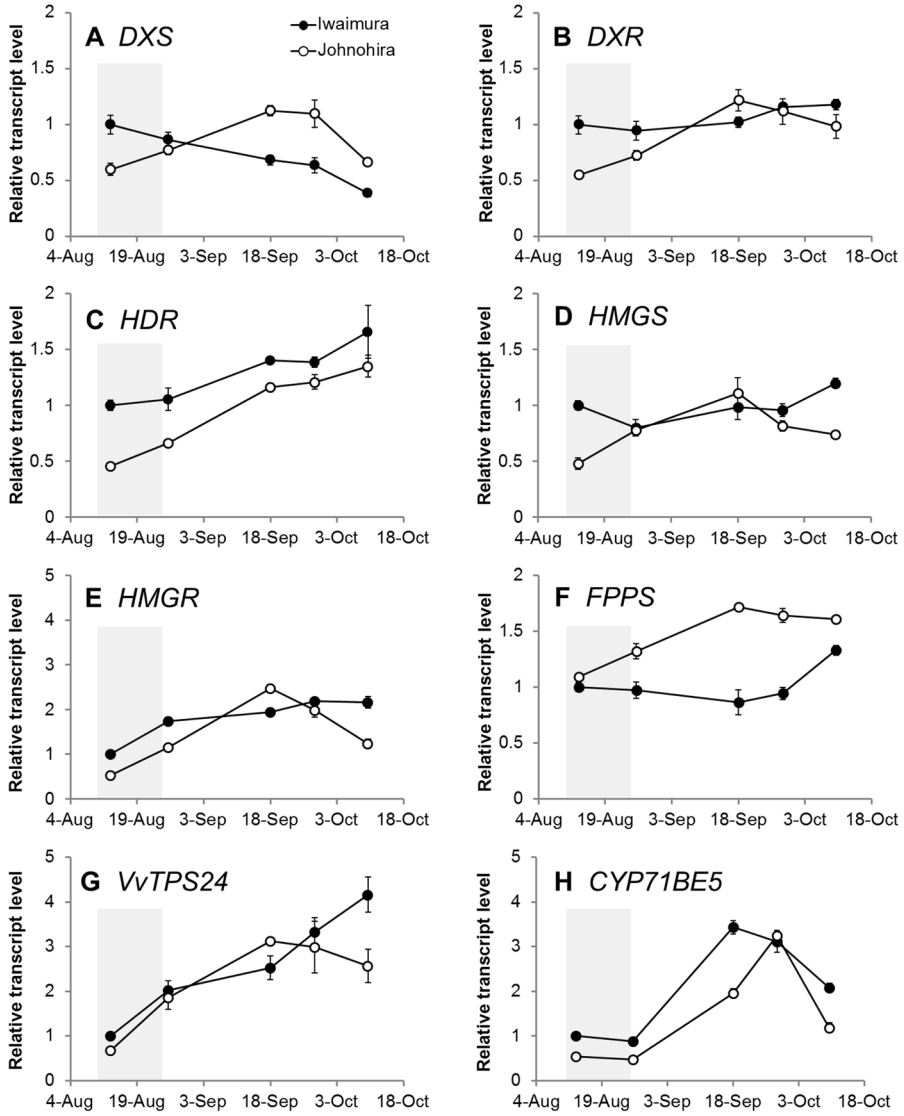

Fig. 3: Expression profiles of genes related to (−)-rotundone biosynthesis in 'Syrah' grape exocarp during grape maturation. (**A**) *DXS*, (**B**) *DXR* and (**C**) *HDR* in MEP pathway; (**D**) *HMGS* and (**E**) *HMGR* inMVA pathway; (**F**) *FPPS*, (**G**) *VvTPS24*, and (**H**) *CYP71BE5* in final step of (−)-rotundone biosynthesis. Filled and open circles indicate samples from the Iwaimura and Johnohira vineyards, respectively. The period of véraison is highlighted by a shade box. The transcript levels in 'Syrah' grape exocarp from the Iwaimura vineyard on 13 August were set to 1 to calculate the relative transcript levels of each gene, respectively. The values are means±standard deviation of three replicates.

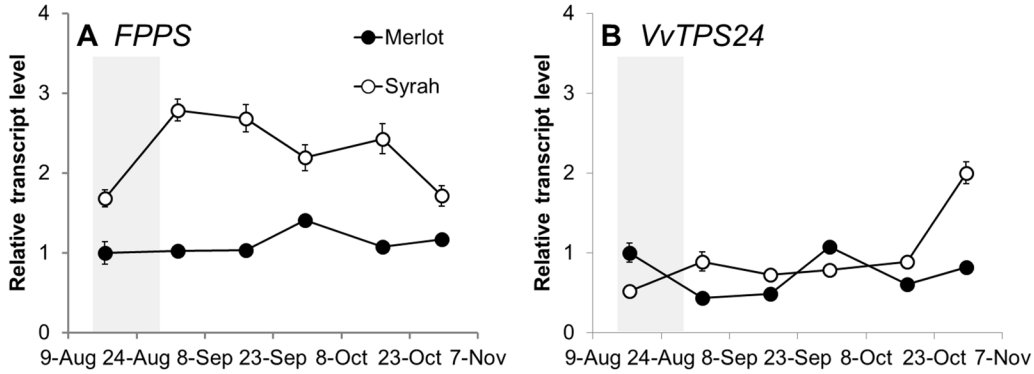

Fig. 4: Expression profiles of *FPPS* and *VvTPS24* in Merlot and 'Syrah' grape exocarps during grape maturation. (**A**) *FPPS* and (**B**) *VvTPS24* in pathway of (−)-rotundone biosynthesis. Filled and open circles indicate samples from Merlot and 'Syrah' grapes, respectively. The period of véraison is highlighted by a shade box. All samples were normalized using 18S rRNA as an internal control. The transcript levels in Merlot grape exocarp on 17 August were set to 1 to calculate the relative transcript levels of each gene, respectively. The values are means±standard deviation of three replicates.

promotes (−)-rotundone accumulation (Caputi *et al.* 2011). Besides, in several plants, *FPPS* expression is induced in response to mechanical wounding, insect attacks (Richter *et al.* 2015), and treatment with methyl jasmonate (Zhao *et al.* 2015), which is a lipid-derived compound with signal functions in plant responses to abiotic and biotic stresses, as well as in development (Wasternack and Hause 2013). These environmental factors such as cooler climatic condition and biological stresses arising from the microclimate in the Johnohira vineyard might affect *FPPS* expression during grape maturation, which in turn leads to high accumulation levels of (−)-rotundone in the grape exocarp. To fully understand the interaction between grapevines and microclimate affecting (−)-rotundone accumulation, further investigations using genetic markers such as *FPPS*, *VvTPS24,* and *CYP71BE5* over several years in multiple vineyards having different characteristic microclimates for comparison are required.

Conclusion

Transcriptional analysis of genes related to (−)-rotundone biosynthesis during the maturation of Japanese 'Syrah' grapes demonstrated that *FPPS* may play an important role in determining the accumulation level of (−)-rotundone, which can provide abundant substrates for *VvTPS24* catalysis to produce α-guaiene as a precursor of (−)-rotundone. In addition, among the MEP pathway genes, *DXS* may have a regulatory role for a precursor supply from the plastids to (−)-rotundone biosynthesis. Furthermore, it also showed that *CYP71BE5* regulated the (−)-rotundone biosynthesis along with the availability of α-guaiene. *FPPS* may serve as a crucial target gene together with *VvTPS24* and *CYP71BE5* for enhancing (−)-rotundone biosynthesis in grapevines, depending on various environmental factors.

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