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RESEARCH ARTICLE



## Study on russet-related enzymatic activity and gene expression in ‘Shine Muscat’ grape treated with GA<sub>3</sub> and CPPU

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### ABSTRACT

Physiological (metabolite analysis) and molecular (gene expression) approaches were used to understand the mechanism underlying russet formation in response to the application of GA<sub>3</sub> and CPPU (Forchlorfenuron) in a Japanese table grape cultivar ‘Shine Muscat’. Several different concentrations of GA<sub>3</sub> and GA<sub>3</sub> + CPPU [25 mg L<sup>-1</sup> GA<sub>3</sub> (A), 25 mg L<sup>-1</sup> GA<sub>3</sub> + 5 mg L<sup>-1</sup> CPPU (B), 25 mg L<sup>-1</sup> GA<sub>3</sub> + 10 mg L<sup>-1</sup> CPPU (C), and 25 mg L<sup>-1</sup> GA<sub>3</sub> + 15 mg L<sup>-1</sup> CPPU (D)] were applied to grape berry clusters at two weeks after flowering (WAF). No russet was observed on the berries treated with the ‘C’ combination. Lower levels of phenylalanine ammonia-lyase (PAL) activity was observed in the treated samples, relative to the untreated material. Reduced peroxide (POD) activity was also observed in response to different treatments, while the expression of *Peroxidase 17* and *Phenylalanine ammonia-lyase G1* genes mirrored lignin content. Increased activity of 4-coenzyme A ligase (4CL) may contribute to decreasing the level of russet and help to improve grape berry quality.

### ARTICLE HISTORY

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### KEYWORDS

GA<sub>3</sub> and CPPU; ‘Shine Muscat’ grape; russet; lignin; enzyme activity

### Introduction

The Japanese diploid grape ‘Shine muscat’ is a hybrid between *Vitis labruscana* Baily and *Vitis vinifera* L. and is mainly consumed as a table grape (Yamada et al. 2008). This cultivar exhibits strong muscat flavor (Suehiro et al. 2014), a charismatic appearance, crisp and juicy flesh texture, and a high concentration of soluble solids. In recent years, the cultivation area of this variety in Japan, as well as China, has noticeably increased (Yu and Yu 2013; Suehiro et al. 2014). It has become very popular among grape growers due to several aspects of its phenology, including high levels of flower bud differentiation, as well as its late-maturity and long fruiting period. The grape berries of this variety, however, tend to have small size and are susceptible to russet; both of which have an impact on the value of the commodity (Li et al. 2016). Therefore, commercial cultivation usually involves the application of gibberellic acid (GA<sub>3</sub>) and N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) one or more times at different stages of fruit development (Wang et al. 2016). GA<sub>3</sub> can break dormancy and promote cell elongation, and also plays an important role in fruit expansion and preventing fruit drop. CPPU increases cell division and fruit enlargement, thus increasing the yield and commodity value (Gu et al. 2015).

Fruit russet, also known as water russet, is a physiological disorder that is manifested as a layer of reddish or yellowish brown corky tissue on the fruit surface (Pauwels 2015). Yellowish-brown spots are present on the peel when the disorder is present, which negatively impacts the appearance of the fruit and decreases fruit value (Cáceres Vargas et al. 2014; Liang et al. 2015). russet may be induced by a range of environmental conditions, including high humidity, low temperature, poor ventilation, and insufficient light (Eccher

et al. 2006; Knoche and Grimm 2008). Mechanical damage can also induce fruit russet (Andersen 2010). russet can be substantially reduced by applying gibberellins, silica, bactericide, or by fruit bagging (Falginella et al. 2015). Among these measures, the application of gibberellins and bagging has the greatest effect (McCartney et al. 2007; Knoche et al. 2011; Curry 2012).

Chen et al. (2011) reported that high temperature and humidity promote fruit russet, and also increases peel lignin and cellulose content. This study also reported that the content of peel lignin and cellulose was higher in areas of russet than in normal, intact pericarp tissue.

Lignin synthesis is a complex process that involves three pathways or stages; specifically, the shikimic acid, phenylpropanoid and lignin synthesis pathways (Tobimatsu et al. 2013). Zhu et al. (2016) reported that the phenylpropanoid pathway and lignin synthesis pathway are mainly responsible for lignin biosynthesis. Phenylalanine ammonia lyase (PAL) is the first key enzyme in the synthesis of lignin monomer and functions as a rate limiting enzyme in the phenylpropanoid pathway (Lepelletier et al. 2012). Kawaoka et al. (2006) reported that inhibition of *Ntliml* gene expression in tobacco resulted in lower expression levels of PAL, cinnamyl alcohol dehydrogenase (CAD), and 4-coenzyme A ligase (4CL), which reduced lignin content by approximately 27%. Cinnamic acid-4-hydroxylation enzyme (C4H) is the second key enzyme in the phenylpropanoid pathway. Lignin content is reduced in plants when C4H activity is inhibited (Heo et al. 2012). 4CL is the principle enzyme in the phenylpropanoid pathway. CAD plays a key role in lignin monomer synthesis (Saathoff et al. 2011). CAD expression in *Panicum virgatum* is down regulated (Legay et al. 2015) when decreased lignin content is observed. Peroxidase (POD) plays a key role in

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the lignin biosynthesis pathway (Heng et al. 2014) which can catalyzes the polymerization of lignin monomers to form lignin. Zhang et al. (2008) reported that a reduction in polyphenol oxidase (PPO) and POD activity reduces lignin synthesis. Wang, Li, et al. (2014) reported that a decline in PAL, PPO, and POD enzyme activity was associated with a reduction in wax secretion and lignin synthesis in epidermal cells, as well as an inhibition of the activation of cork cambium (Wang, Zhang, Dai and Shi, 2014).

In the current study, changes in lignin content and lignin-associated enzyme activity were examined during different stages of grape berry development and in response to the application of different concentrations of GA<sub>3</sub> and CPPU, as well as the impact of these growth regulators on the level of russet. A transcriptomic analysis of the fruit peel during the critical period of fruit russet formation was also carried out, and the differential expression of genes related to the phenylpropanoid pathway were evaluated based on the RNA-seq results. The objective of current study was to characterize the physiological and molecular mechanism underlying fruit russet in grape berries before and after the application of GA<sub>3</sub> and CPPU. The study provides a foundation to better understand the mechanism of fruit russet which will ultimately be helpful for improving the quality of table grapes.

## Materials and methods

### Plant material

Six-year old 'Shine Muscat' (*Vitis labruscana* Bailey × *V. vinifera* L.) grape vines, spaced at 4 m × 8 m and planted under a rain shelter in sandy soil, were used in the study. The planting was supplemented with drip irrigation. The study site was at the Nanjing Agricultural University vineyard located in the Tangshan Valley, Nanjing, Jiangsu Province, China. Trees were planted in an 'H' shape and grown using standard cultivation conditions. Similar vines were selected for the current study. The berries were treated with different concentrations of GA<sub>3</sub> and CPPU at two weeks after flowering. Five treatments were applied: water (control); 25 mg L<sup>-1</sup> GA<sub>3</sub> + 0 mg L<sup>-1</sup> CPPU (A); 25 mg L<sup>-1</sup> GA<sub>3</sub> + 5 mg L<sup>-1</sup> CPPU (B); 25 mg L<sup>-1</sup> GA<sub>3</sub> + 10 mg L<sup>-1</sup> CPPU (C); and 25 mg L<sup>-1</sup> GA<sub>3</sub> + 15 mg L<sup>-1</sup> CPPU (D).

### Methods

#### Sampling and observation

Berry samples were collected in triplicate nine weeks after flowering. Each sample consisted of approximately twenty randomly-collected grape berries, taking into consideration berry size, and a balance between sunlit vs. shaded berries. Samples were transported immediately to the laboratory on ice. Individual grape berries were manually peeled and the pericarp tissues were frozen in liquid nitrogen and stored at -80°C until further processing. A stereomicroscope (LEICA S8AP0) was used at 25X to observe differences in the peel of berries at the same stage of maturity (17 weeks after flowering), from the treated and control samples.

#### Lignin content

Lignin content was measured using the method described by (Hatfield et al. 1999). Berry skins from the different treatments, collected at the 14th week after flowering, were

wrapped with aluminum foil and put into an oven for 24 h at 80°C for drying. After drying, the samples were ground and accurately weighed in triplicate to 0.02 g. The powder was then transferred to a plastic tube containing 2 mL 25% (w/w) acetyl bromide in glacial acetic acid and 0.08 mL perchloric acid. After sealing, the tubes were placed in a water bath at 70°C for 30 min with shaking every 10 min. A quarter of the reaction solution was subsequently transferred to a volumetric flask containing 1 mL 2 mol L<sup>-1</sup> NaOH and 2.5 mL glacial acetic acid, mixed well and then diluted with glacial acetic acid to 10 mL. Lignin content in the sample solutions was measured as absorbance at 260 nm using a ELIASA (TECAN, Switzerland) spectrophotometer. Glacial acetic acid was used as a blank. Lignin content was determined using the formula:

$$\text{Lignin} = \text{Abs} \times \text{liters} \times 100\% / W \text{ sample}$$

where lignin represents the lignin content, Abs is the absorbance of the sample solution, liters is the solution constant volume (mL), and *W* represents the dry mass of the individual samples.

#### Enzyme activity

PAL activity was assayed as described by Assis et al. (2001) with some modifications. A total of 0.1 g fresh of berry skin from the different treatments was weighed and ground in 1 mL 0.1 mol L<sup>-1</sup> borate buffer (PH = 8.8), and then centrifuged. The enzyme reaction mixtures contained 1 mL crude extract, 2 mL borate buffer, 1 mL 0.02 mol L<sup>-1</sup> L-phenylalanine in a total volume of 4 mL. The mixture was mixed and placed in a water bath at 30°C for 1 h and OD was then measured at 290 nm on a spectrophotometer.

Extraction and assay of C4H activity using crude enzyme extracts was performed as described by Maurer and Schmid (2005) with slight modifications. The 1 mL 50 mM, pH = 8.9 Tris-HCl extracts which contained 15 mM mercaptoethanol, 4 mM MgCl<sub>2</sub>, 2.5 mM vitamin C, 10 μM leupeptin, 0.15% polyvinylpyrrolidone and 10% glycerin. The 50 mM, pH = 8.9 Tris-HCl reaction mixture which contained 2 μM trans-cinnamic acid, 2 μM sodium oxide coenzyme and 5 μM disodium glucose disodium phosphate. The mixtures were shaken at 25°C for 30 min, then 100 μL 6 M HCl was added. The reaction mixtures were then centrifuged (5000 g; 5 min; 4°C) to remove denatured proteins, after which absorbance was measured at 340 nm.

4CL activity was assayed according to the method previously described by Zhou et al. (2014). The 1 mL 0.2 M, pH = 8.0 Tris-HCl extracts which contained 25% glycerin and 0.1 mM DTT. The 0.2 M, pH = 8.0 Tris-HCl reaction mixture which contained 15 μM MgCl<sub>2</sub>, 5 μM P-Coumaric acid and 50 μM ATP 1 μM CoA. The extracts were added to the reaction mixture and placed in a water bath at 40°C for 10 min. The reaction mixtures were completed by adding 100 μL 6 M HCl. Afterwards, the mixtures were then centrifuged (5000 g; 5 min, 4°C) to remove the denatured protein. 200 μL reaction mixtures were measured at 333 nm absorbance.

POD activity was determined as described by Allison and Schultz (2004) with some modification. The extracts contained 1 mL 0.2 M, pH = 6.0 phosphate buffer, while the reaction mixture was comprised of 0.2 M, pH = 6.0 Phosphate buffer (28 μL guaiacol and 19 μL 30% H<sub>2</sub>O<sub>2</sub>). The final

mixture was placed for 10 min at 23°C, after which the OD at 470 nm was determined.

PPO activity was assayed following the method reported by Mastuti et al. (2015) and Lin et al. (2005). Absorbance was measured at 410 nm.

A total of 0.1 g fresh berry peel was used to determine enzyme activity in each assay. The total measuring volume was 3 mL and contained 1 mL enzyme extract and 2 mL of reaction mixture. The following formula was used to calculate enzyme activity:

$$U = \frac{(\Delta A \cdot Vt)}{(W \cdot Vs \cdot t \cdot 0.01)}$$

$U$  represents enzyme activity,  $W$  represents the mass of the sample (g),  $t$  is the reaction time (min),  $Vt$  is the total volume of enzyme solution (mL),  $Vs$  is the measured volume of enzyme solution (mL). One activity unit was defined as the change in 0.01 unit of absorbance per minute ( $U \text{ g}^{-1} \text{ min}^{-1}$ ).

### RNA-seq analysis and reverse transcription – quantitative PCR (RT-qPCR)

Total RNA was extracted by using Trizol reagent (Foregene, Chengdu, China) according to the manufacturer's instructions. The concentration and quality of each RNA sample was determined by absorbance at 260 and 280 nm and the integrity of all of the RNA samples was assessed on a 1% agarose gel when the A260/A280 ratio was greater than 1.8. Total RNA samples were pooled into three sample stages and 10 µg of total RNA was used to build each Illumina RNA-seq library. Sequencing was conducted using an Illumina HiSeq<sup>TM</sup> 2000 platform following the manufacturer's protocol.

A total of 1 µg of purified RNA per reaction was reverse transcribed to generate cDNA using a cDNA synthesis kit (Takara) according to the manufacturer's instructions. The first strand cDNA was diluted 5 times for further use. Six genes were selected for validation of the RNA-seq data using reverse transcription-quantitative PCR (RT-qPCR). Gene specific primers for the selected genes were designed using Beacon Designer 7 (PREMIER Biosof company) and the ubiquity gene was used as a reference gene.

The RT-qPCR assay utilized a Power SYBR Green PCR Master Mix (Applied Biosystems) which was used according to the manufacturer's instructions. The final volume of 20 µL contained 10 µL of Takara SYBR Premix, 0.5 µL of each primer, and 9 µL of water. The PCR cycling conditions were: an initial denaturation step at 95°C for 4 min, then 95°C for 20 s,

60°C for 20 s, 72°C for 30 s, for 40 cycles. All the data were calculated with 2- $\Delta\Delta C_t$  method (Livak and Schmittgen 2001).

### Statistical analysis

The lignin content and enzyme activity data were determined in triplicate and presented as the average of three replicates. SPSS Statistics 17.0 statistical software package was used for all statistical analyses. Analysis of variance was conducted and significant difference were detected using LSD and Duncan's test ( $P < 0.05$ ).

## Results

### Level of russet in response to the different spray treatments

Figure 1 shows the russet on the grape berry fruit surface in response to the different treatments at 17 weeks after flowering. There were significant differences in the level of russet between the different treatments. The distribution and degree of russet in the control samples was the most widespread and intense, occurring on the highest proportion of the peel area. Treatment A, resulted in a much lighter amount of russet, relative to the control, although the russet was still apparent on a number of areas of the grape peel. Treatment B and D resulted in less fruit russet, that was mainly evident around the peduncle. Treatment C had the greatest impact on reducing russet with no apparent russet visible on the grape berry surface.

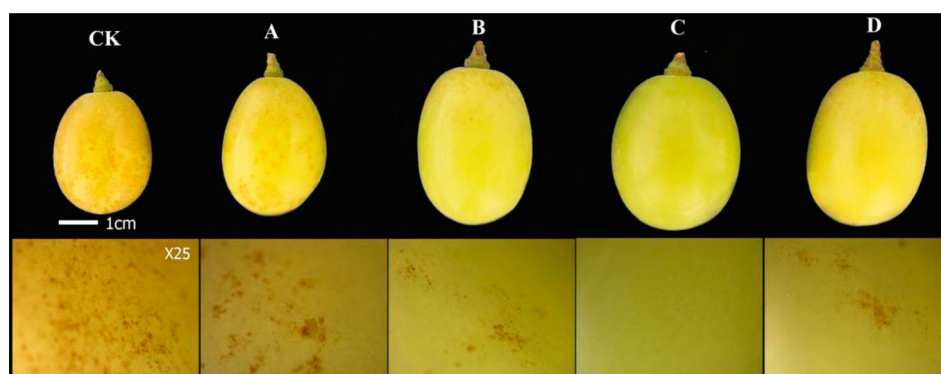
### Differences in lignin content

Figure 2 shows the differences in lignin content in the peels of grape berries in response to the different treatments at 14 weeks after flowering. Lignin content in the control samples was the highest, follow by treatment A. On the other hand, lignin content in treatment C was the lowest, and no significant difference was observed between treatment B and D.

### Differences in enzyme activity

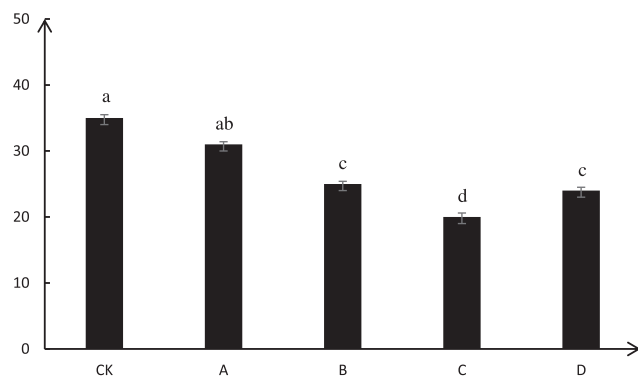
#### PAL activity

PAL (phenylalanine ammonia-lyase) activity in response to the different treatments first decreased and then increased (Figure 3). In the control samples, PAL activity was high relative to the other treatments; especially treatments B and C. In samples collected 9 weeks after flowering, PAL activity was



**Figure 1.** Differences in the level of russet on grape berries in response to the different treatments. Observations were made at 17 weeks after flowering and two weeks after the treatments were applied. The following treatments were applied to the clusters of grape berries: (CK) tap water; (A) 25 mg L<sup>-1</sup> GA<sub>3</sub> + 0 mg L<sup>-1</sup> CPPU; (B) 25 mg L<sup>-1</sup> GA<sub>3</sub> + 5 mg L<sup>-1</sup> CPPU; (C) 25 mg L<sup>-1</sup> GA<sub>3</sub> + 10 mg L<sup>-1</sup> CPPU; and (D) 25 mg L<sup>-1</sup> GA<sub>3</sub> + 15 mg L<sup>-1</sup> CPPU.





**Figure 2.** Differences in lignin content in the peel of grape berries in response to the different treatments at 14 weeks after flowering ( $p < 0.05$ ).

similar among the different treatments, except the control and treatment A. The values of all treatments exhibited a decreasing trend at 11 weeks after flowering. At two weeks after the application of the treatments, PAL activity was highest in the control, followed by treatments D, A, B, and C.

#### C4H activity

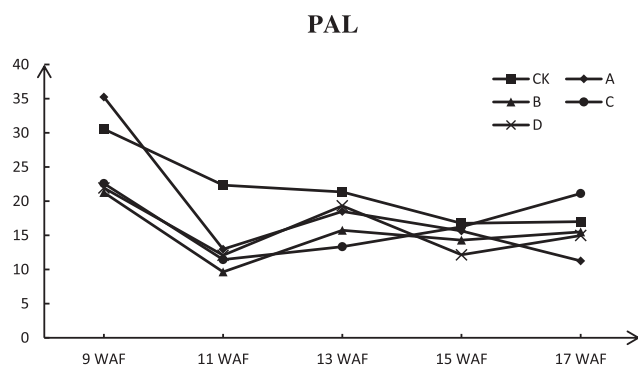
Only minor differences were observed in C4H activity among the control and different growth regulator treatments at 9 weeks after flowering (Figure 4). At 11 weeks after flowering, C4H activity in treatments A, B, and D was lower than in the control. After another two weeks, however, C4H activity in all of the treatments was lower than in the control samples.

#### 4CL Activity

The effect of different treatments on 4CL activity exhibited a 'W' pattern (increasing, decreasing, and then increasing) (Figure 5). From 9 weeks to 17 weeks after flowering, 4CL activity in the control samples was the highest among all of the other treatments, while treatments C and D were lower than A and B. At 11 weeks after flowering, 4CL activity in the B treatment samples was the lowest, relative to the other treatments; while little difference was observed between treatments C and D. No significant differences were observed in 4CL activity among the different treatments, except the control at 15 weeks after flowering.

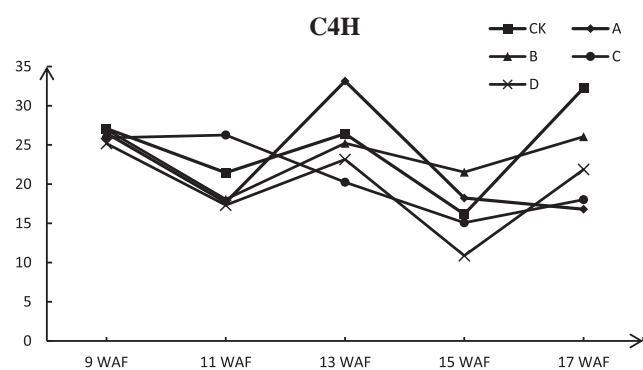
#### POD activity

POD activity first decreased and then exhibited an increasing trend among the different treatments. At nine weeks after flowering, the highest level of POD activity was observed in

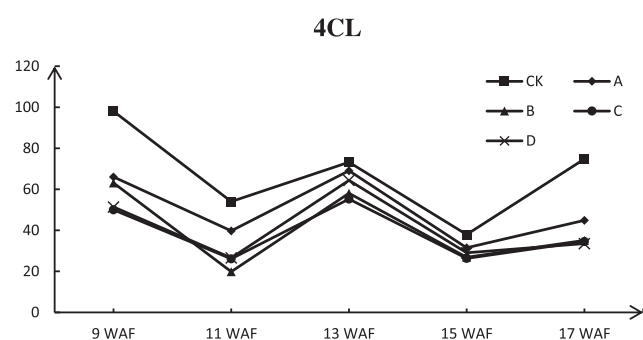


**Figure 3.** Effect of the different concentrations of GA<sub>3</sub> and CPPU applied to grape berries on PAL activity.

Note: 'WAF' indicates weeks after flowering.



**Figure 4.** Effect of the different concentrations of GA<sub>3</sub> and CPPU applied to grape berries on C4H activity.



**Figure 5.** Effect of the different concentrations of GA<sub>3</sub> and CPPU applied to grape berries on 4CL activity.

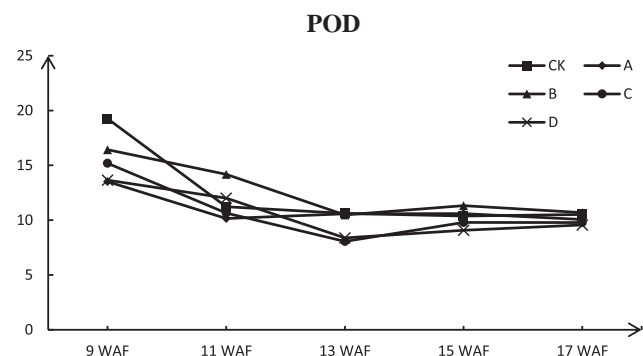
the control samples, followed by treatments B and C. POD activity was low in all of the treatments, except treatment B, at 11 weeks after flowering (Figure 6).

#### PPO activity

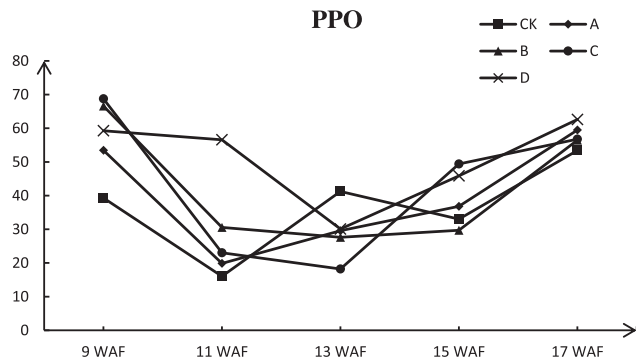
The effect of different concentrations of GA<sub>3</sub> and CPPU on PPO activity exhibited a 'V' pattern in the peel of grape berries. At 9 weeks after flowering, PPO activity in the B and C treatment samples were the most elevated, followed by treatments D and A. In the subsequent time points, a rising trend was observed in all of the treatments, except the control, which first decreased and then increased abruptly. At the final stage of sampling, PPO activity in D treatment samples was the highest, followed by the A treatment (Figure 7).

#### RT-qPCR assay of enzyme-related gene expression

Six genes coding for enzymes whose activity was measured were selected from the RNA-seq data (Table 1). The



**Figure 6.** Effect of the different concentrations of GA<sub>3</sub> and CPPU applied to grape berries on POD activity.



**Figure 7.** Effect of the different concentrations of GA<sub>3</sub> and CPPU applied to grape berries on PPO activity.

expression of three genes related to POD were compared among the different treatments. Results indicated that *peroxidase 17* was up regulated, while *peroxidase 42-like* was down regulated, and the other exhibited no significance difference. Two PAL-related genes exhibited contrasting expression patterns where one was down regulated and the other was up regulated. A gene related to 4CL was down regulated. In general, the results of the RT-qPCR and RNA-seq data were consistent with each other (Figure 8). The expression of *peroxidase 17* exhibited no significant difference between the control and the different treatments. The expression of *phenylalanine ammonia-lyase G1* decreased significantly, relative to the expression in treatments C, A, and the control. Little difference was observed, however, between the control and treatment A. The expression of 4CL related genes was elevated in treatment A samples, relative to control and treatment C samples (Table 2).

## Discussion

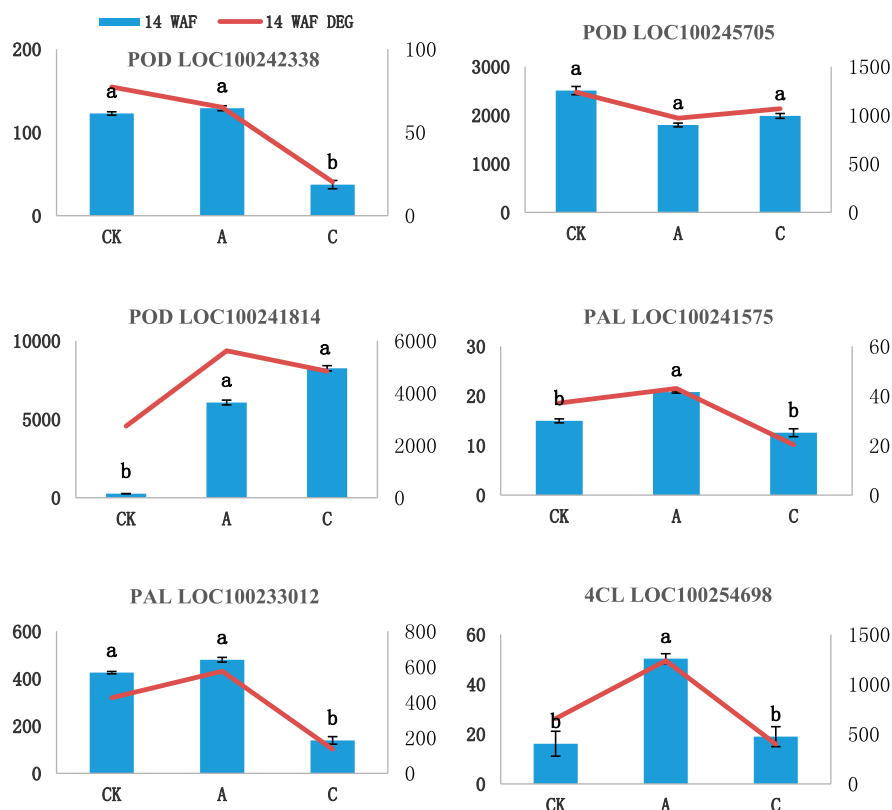
Grape is one of the most common fruit crops grown and is consumed fresh as well as in the form of several products, such as juice, wine, jam, etc. 'Shine Muscat' is one of the most popular table grape cultivars grown in China and Japan, due to its high soluble solid content and muscat aroma. The epidermal layer of the grape berry, however, can suffer from a physiological disorder, called russet or russet, during the growth of the berry clusters. Numerous studies have been conducted to address this problem using different methods, such as bagging and the application of plant growth regulators. Bagging can reduce fruit russet very effectively on 'Cui Guan' pear (Huang et al. 2007). The growth regulators

GA<sub>3</sub> and CPPU have a significant impact on berry growth, increasing fruit size at maturity in grapes (Navarro et al. 2001), kiwifruit (Kim et al. 2006), and triploid loquat (Yahata et al. 2005). It also increases parthenocarpic fruit set in apple (Watanabe et al. 2008) and extends the shelf-life of grape and blueberry (Nesmith 2002). Limited studies have been conducted to address the russet problem in 'Shine Muscat' grapes by applying different concentrations of GA<sub>3</sub> and CPPU to gain insight into the molecular mechanism underlying fruit russet (Wang, Li, et al. 2014; Heng et al. 2016; Liang et al. 2016; Wang et al. 2017). The present study is the first one to simultaneously explore the physiological and molecular mechanisms associated with the russet problem in grape berries. A previous study indicated that the russet on the grape berry peel was directly linked to lignin content (Chen et al. 2011). In our study, the proportion of russet on the grape samples of grape peels was highest, and lignin content in the untreated control was also significantly higher than in the other treatments that resulted in less russet. In fact, no russet was observed in C-treatment (25 mg L<sup>-1</sup> GA<sub>3</sub> + 10 mg L<sup>-1</sup> CPPU) berries, which also had significantly lower levels of lignin.

The lignin was formed via the involvement of several enzymes, and the genes coding for these enzymes that regulate the synthesis of those enzymes. Many different enzyme isoforms are present in plants (Anterola and Lewis 2002). Previous studies demonstrated that PAL is the first key enzyme in the biosynthesis of lignin monomers (Lepelley et al. 2012). Its activity has been associated with tan spots on fruits and is positively correlated with lignin content (Mahmoudi et al. 2011; Nazari et al. 2015). Several studies, however, have reported that increased PAL activity does not promote the synthesis of lignin (Assis et al. 2001). In our study, PAL activity was slightly elevated at 13 weeks after flowering which can be inferred that 13 weeks after flowering was the key period of lignin occurs on the peel of berries. PAL activity was highest in the control and treatment C samples. This finding was in agreement with the results of Wang et al. (2013). As reported by Kawaoka et al. (2006), it was interesting to note that the pattern of 4CL activity was similar to PAL and changes in 4CL activity mirrored lignin content. Several studies have reported that the synthesis of lignin in Loquat pulp and soybean roots was related to POD activity (Lin et al. 2005; Cai et al. 2006). In our study, however, results indicated that the different concentrations of plant growth regulators (GA<sub>3</sub> and CPPU) had little effect on POD activity, suggesting that POD is not

**Table 1.** RNA-seq identification of genes related to measured enzymes.

Gene	CK (RPKM)	A (RPKM)	C (RPKM)	q-value of CK vs A (Difference)	q-value of CK vs C (Difference)	q-value of A vs C (Difference)	Function annotation
<i>POD</i>							
LOC100242338	77.3	65.1	20.30	0.44 (noDEG)	4.24E-07 (Down)	0.0001 (Down)	peroxidase 17-like
LOC100245705	1236.8	969.6	1067.40	4.43E-0 (noDEG)	0.6430995 (noDEG)	7.37E-06 (noDEG)	probable glutathione peroxidase 2-like
LOC100241814	2739.7	5621.2	4838.50	6.18E-216 (Up)	1.05E-187 (noDEG)	0.44 (noDEG)	peroxidase 42-like
<i>PAL</i>							
LOC100241575	37.1	43.0	20.3	0.68 (noDEG)	0.143 (noDEG)	0.066 (noDEG)	Phenylalanine ammonia-lyase-like
LOC100233012	425.7	575.00	136.1	2.24E-0 (noDEG)	9.86E-28 (Down)	1.74E-52 (Down)	phenylalanine ammonia-lyase
<i>4CL</i>							
LOC100254698	655.6	1237.10	399.1	6.04E-39 (noDEG)	1.45E-08 (noDEG)	5.63E-77 (Down)	4-coumarate--CoA ligase 1-like



**Figure 8.** RNA-seq identification of genes related to the measured enzymes whose expression was subsequently evaluated by RT-qPCR.

Note: The blue bar graph and the left ordinate represent the results of RT-qPCR assay. The red line and the right ordinate represent the results of the RNA-seq analysis. Gene ID LOC100242338, Gene Name *peroxidase 17*; Gene ID LOC100245705, Gene Name *probable glutathione peroxidase 2*; Gene ID LOC100241814, Gene Name *peroxidase 42-like*; Gene ID LOC100241575, Gene Name *phenylalanine ammonia-lyase-like*; Gene ID LOC100233012, Gene Name *phenylalanine ammonia-lyase G1*; Gene ID LOC100254698, Gene Name *4-coumarate--CoA ligase 2*.

a key enzyme in lignin biosynthesis in grape berries. Additionally, C4H may play a key role in lignin synthesis since the different treatments had little impact on C4H activity and changes in C4H activity did not correspond with changes in lignin content at 13 weeks after flowering. Our results indicated that PPO activity exhibited the lowest level of enzyme activity and that lignin reached its highest level during 11 to 13 weeks after flowering. These results were in contrast to the results reported by Zhang et al. (2008), who indicated that a decrease in PPO activity led to reduced levels of lignin content.

Six genes coding for family members of the measured enzymes were selected for RT-qPCR analysis to confirm the accuracy and reproducibility of the results obtained in the transcriptome analysis. RT-qPCR results indicated that the level of expression of the analyzed genes closely corresponded

to the transcript levels obtained by RNA-seq of each of the treatments. More specifically, *peroxidase 17* and *peroxidase 42-like* were regarded to regulate the biosynthesis of peroxidase among the POD-related genes. Down regulation of *peroxidase 17* was observed in treatment C which corresponded with lignin content. Our data also indicate that POD activity was regulated by *probable glutathione peroxidase 2* gene, whose expression level exhibited little change among the various treatments at the different time points. Treatment C significantly inhibited PAL activity and reduced lignin content. In treatment C samples, the expression of the *phenylalanine ammonia-lyase G1* gene, coding for phenylalanine ammonia-lyase was significantly lower, relative to the other treatment groups. The *phenylalanine ammonia-lyase G1* gene plays an important role in the formation of fruit russet, which infers that this enzyme plays a key role in the biosynthesis of lignin. However, *4-coumarate--CoA ligase 2* coding for a 4-coumarate CoA ligase related to 4CL, may not play a key role in regulating 4CL activity. This scenario is plausible since its expression was significantly higher in treatment A, relative to the other treatments, and its expression was not correlated with changes in 4CL activity.

## Conclusions

The present study illustrated that treatment A, B, and D, representing different concentrations of GA<sub>3</sub> and CPPU, could reduce but not completely inhibit russet in 'Shine Muscat' grape berries. Treatment C (10 mg L<sup>-1</sup> CPPU and 25 mg L<sup>-1</sup> GA<sub>3</sub>), however, completely eliminated russet on the surface of grape berries. It is likely that this occurred by

**Table 2.** Gene-specific primers used in the RT-qPCR assay.

	Gene ID	Gene name	Primer sequence (5'→3')
POD	LOC100242338	<i>peroxidase 17</i>	F:GTGGCATTAGAGAACTTAGATG R:GCAACCTCCTCACTGATTA
POD	LOC100245705	<i>probable glutathione peroxidase 2</i>	F:GATAAGGTTGAGGTGAATGG R:AGAGGTGATGTGGTAGGA
POD	LOC100241814	<i>peroxidase 42-like</i>	F:CTTGAGAGGATGAAGATG R:ACCATAACGCCATTGTAAC
PAL	LOC100241575	<i>phenylalanine ammonia-lyase-like</i>	F:AACACCAGATTAGCAATAGC R:GATTCTTCCACCAGAGA
PAL	LOC100233012	<i>phenylalanine ammonia-lyase G1</i>	F:CTGAAGCCGTGGACATAT R:GCCAATGGTTAGGACTCT
4CL	LOC100254698	<i>4-coumarate--CoA ligase 2</i>	F:TCTTCGTAGAGACAATTCCT R:ATGGTTCTCAGCTCATT

inhibiting the expression of *phenylalanine ammonia lyase G1*, decreasing PAL activity, and ultimately controlling the biosynthesis of lignin to reduce pericarp suberification. 4CL may play a supporting role in reducing russet. For the commercial production of 'Shine Muscat' grapes, fruit russet can be controlled by the application of 10 mg L<sup>-1</sup> CPPU and 25 mg L<sup>-1</sup> GA<sub>3</sub> (treatment C), which prevents fruit drop as well.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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## Author contributions

Development and design of experiments: JMT and XDH; Performed the experiments: XDH and JF; Writing of the manuscript: LLW, XDH and JJZ. Data analysis: XDH and YSX. All authors read and approved the final version of the manuscript.

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