Full Length Article

Effect of Abscisic Acid on Expression of its Key Enzyme Synthesis Genes, *RiNCED1* and *RiCYP707A1*, and Quality of Raspberry (*Rubus idaeus*) Fruits

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Abstract

In the present study, two ABA synthesis key enzyme genes, *RiNCED1* and *RiCYP707A1*, were cloned from 'Heritage' raspberry. The ABA accumulation in raspberry fruits reaches its maximum at the ripe stage, is 26.92 μg/g, meanwhile, a complete correspondence of the expression of *RiNCED1* in time to the accumulation of ABA, which indicate that ABA plays an important effect in regulating ripening of raspberry fruits. Applied exogenous ABA for unripe green raspberry fruits detached to the plant could significantly promote ABA, soluble sugar and anthocyanins contents, softening of fruit and decreasing of chlorophyll in fruits. Fluridone, as an ABA biosynthesis inhibitor, could inhibit increase of ABA, soluble sugar and anthocyanins contents, delay softening of fruit and decrease of chlorophyll in raspberry fruits. Exogenous ABA also prompted an increase of *RiNCED1* expression and made it reach the maximum level in advance for 12 h, and the expression of *RiCYP707A1* also was promoted. The expression of *RiNCED1* after fluridone application were only little lower than that of control and the process of reaching the maximum level were delayed. The expression of *RiCYP707A1* in each period was lower than that of control after fluridone treatment, but it was not significant. The results above indicated that exogenous ABA could promote the synthesis of endogenous ABA and it has an affirmative effect to the softening and ripening of raspberry fruits. © 2020 Friends Science Publishers

Keywords: ABA; Raspberry; *RiNCED1*; *RiCYP707A1*; Softening

Introduction

Red raspberry (*Rubus idaeus* L.) is a popular berry due to its organoleptic characteristics and rich nutritional value with kinds of antioxidants, such as vitamins, anthocyanins, ellagic acid and other phenolic compounds which are beneficial to human health (Rao and Snyder 2010; Škrovánková *et al*. 2015; Lamanauskas *et al*. 2016). Red raspberry is widely consumed as fresh and frozen fruit, or processed to jelly, jam and juice and other products, or as ingredients in various foods (Rao and Snyder 2010). When raspberry is harvested for the fresh market, they can only be stored for a few days, which limits shelf-life and commercial value badly (Rao and Snyder 2010). Therefore, understanding the softening process of raspberry fruit could provide a theoretical basis for increasing its postharvest life. During ripening, soluble solid contents increase and titratable acidity contents of raspberry fruit decrease (Perkins-Veazie and Nonnecke 1992). However, underlying molecular mechanisms of governing the natural ripening and postharvest behavior of raspberry fruit need to be investigated.

Fruit, based on the distinct respiratory patterns during ripening, is generally divided into climacteric fruit and non-climacteric fruit (Osorio *et al*. 2013). During non-climacteric fruit ripening, there is no respiratory peak. Currently, it was demonstrated that there is no apparent burst in respiration rate as the fruit matures and ethylene production rate in raspberry fruit increases with fruit ripening, though no mass of ethylene releasing (Fuentes *et al*. 2015). Ethylene accelerates abscission zone formation and induces softening and colour formation, but many of the changes in raspberry fruit associated with ripening begin at or before the yellow stage and are independent of ethylene production changes (Fuentes *et al*. 2015). Therefore, ripening of raspberry fruits may be governed by phytohormones other than ethylene. ABA was thought to play an effect in regulating non-climacteric fruit ripening (Balbontin 2018), such as citrus (Zhang *et al*. 2014), grape (Jia *et al*. 2017), strawberry (Jia *et al*. 2011). The direct molecular level evidence for the effect of ABA in strawberry fruit was demonstrated by the restrain of *FaNCED1* expression, which is the key ABA biosynthetic gene, could block ABA accumulation and

result for a fruit uncoloured partly which still can be coloured by exogenous ABA (Jia *et al.* 2011). ABA is an effective promoter of grape berry maturation in grapes. ABA not only induces the ripening-related gene expression levels, but also promotes decrease of fruit firmness, accumulation of sugar, anthocyanin and phenolic compounds, including flavonols and proanthocyanidins, in berries (Oh *et al.* 2018).

Some researchers suggested that the ABA is more crucial than ethylene in fruit ripening (Lado *et al.* 2018). The effect of ABA in many fruits natural ripening process has been researched over the years, but it is not clear during raspberry fruit ripening. At present, very little is known about the expression of ABA biosynthetic genes during raspberry ripening. In ABA biosynthesis pathway, 9-cis-epoxycarotenoid dioxygenase (NCED), as the key enzyme, catalyzes the cleavage of either 9-cis-neoxanthin or 9-cis-violaxanthin or both to xanthoxin. Active ABA can be inactivated by 8'-hydroxylation, in which cytochrome P450 (CYP) monooxygenase is a key enzyme (Nambara and Marion-Poll 2005). A dynamic balance of endogenous ABA levels in fruits was regulated by the processes of the biosynthesis and catabolism. At present, *NCED* and *CYP707A* cDNAs have been cloned and characterized in different fruits, such as strawberry (Kadomura-Ishikawa *et al.* 2015), citrus (Rodrigo *et al.* 2006; Rehman *et al.* 2018), grape (Koyama *et al.* 2010) and so on. Currently, some genes in the *NCED* and *CYP707A* gene families, are expressed related to the trend of ABA accumulation and look like to play a leading effect in the regulation of ABA accumulation in many fruits. For example, *FaNCED2* and *FaCYP707A1* in strawberry (Ji *et al.* 2012), *PacNCED1* and *PacCYP707A2* in sweet cherry (Ren *et al.* 2010). However, the relationship between the expression of *NCEDs* and *CYP707As* and the ABA accumulation have not been studied in raspberry fruit.

The aim of the present study was to explore the effect of ABA on the fruit ripening and the expression of ABA synthesis key enzyme gene *RiNCED1* and *RiCYP707A1* and quality of raspberry fruits. One ABA key synthetase gene *RiNCED1* and one ABA key degradation enzyme gene *RiCYP707A1* were cloned firstly. ABA accumulation, physiological parameters changes of fruit and the transcriptional regulations of both genes during raspberry fruit natural ripening process were demonstrated. Furthermore, the effect of exogenous ABA and fluridone, which could inhibit the NCED enzyme in fruit, on raspberry fruit softening and ripening was also investigated.

Materials and Methods

Plant materials and growth conditions

The study was conducted from 2017 to 2018 in the Northeast Agricultural University of China. Perennial 'Heritage' raspberry with planting row spacing 1 m × 2 m, conventional soil and fertilizer management, its fruits were harvested from Horticultural Institute in the Northeast Agricultural University between the 4th and 27th September. The fruit development was divided into five periods subjectively based on berry size and colour (green: small green berry; white: expanded white berry; mottled: middle size mottled berry; ripe: big red berry; overripe: big purple berry), as described by Perkins-Veazie and Nonnecke (1992). Fruits were harvested with receptacle still attached by cutting the pedicel which is about 1 cm. Berries at different developmental stages were randomly collected from each plant and the berries in same stages were similar in size and weight roughly.

Postharvest treatments

The fruits in the white stage were harvested and allocated into three groups at random. The fruits were treated with 1 mM ABA [(±)-abscisic acid; Sigma, United States] and 0.6 mM fluridone (analytical standard, United States), respectively. The fruits were treated with distilled water as a control. The optimum treatment concentration was selected by pre-experiment before the test. Approximately 80 berries were utilized per treatment. Fruits were subsequently immersed in three liquids about 10 min, then, be well ventilated for about 30 min to dry. The fruits were maintained at 25 ± 5°C in 55 ± 5% RH and fruit samples were collected after 0, 12, 24, 36, 48 and 60 h from the first treatment, some fruits were used to measure the hardness, and others were promptly frozen in liquid nitrogen, then stored at −80°C until used for the determined of other parameters.

Determination of ABA content

The ABA content in the fruit were determined as reported earlier by Ma *et al.* (2014) with slight modification. About 2 g of raspberry sample was weighed and ground in liquid nitrogen, homogenized in 50 mL 80% (v/v) methanol/water, fully shaken and extracted at 4°C for 24 h. The extract was filtered, and the filtrate was evaporated at 40°C until no methanol remained. The remaining aqueous phase was transferred to a flask and extracted to decolourization using 30 mL petroleum ether for three times. Then the pH of the aqueous phase solution was adjusted to 8.0. About 1.0 g of crosslinking polyvingypyrrolidone (PVPP) was added to the aqueous phase solution for three times, which was ultrasound for 1 h and was filtered. The pH of the aqueous phase solution was adjusted to 3.0, extracted with 30 mL of ethyl acetate three times, and the ester phases were combined. The ester phase was evaporated at 40°C until no solution remained. About 2 mL of chromatographic pure methanol was dissolved in the evaporated residue and filtered through a 0.22 μm disposable microfiltration membrane. The test solution was stored at -80°C for testing.

Chromatographic separation was performed on the Waters Xevo TQ-S using a C18 chromatographic column (2.1x 100 mm, 1.7 μm; WATERS). Liquid phase conditions:

mobile phase A phase is acetonitrile, phase B is 0.05% $NH_3 \cdot H_2O$, gradient elution conditions are shown in Table 1 (30ºC column temperature, 10.0 μL injection volume, 0.3 mL/min flow rate). Mass spectrometric analyses were performed using a Thermo Q-Exactive high resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA) with negative ion scanning (ESI) electrospray ion source, multiple reaction detection mode (Table 2), 3000 V capillary voltage, 1000 L/h desolvent flow rate, 150 L/h cone gas flow rate and 450ºC ion source temperature. The retention time and mass spectrometry information of ABA were determined with standard substance.

RNA isolation, reverse-transcription PCR and sequencing

Total RNA was isolated from 0.2 g of fruit. All plant materials were finely ground in liquid nitrogen. Total RNA extraction was used OmniPlant RNA kit (CW2598S CWBIO) according to instructions. About 100 mg raspberry fruits were ground into powder in liquid nitrogen, and 500 mL Buffer RLS was added to mix them immediately. Centrifugation for 2 min at 4ºC 12,000 rpm. The supernatant was transferred into the filter column (Spin Columns FS) which has been loaded into the collection tube. Centrifugation for 1 min at 4ºC 12,000 rpm. The supernatant was absorbed from the collection tube and was transferred to the new RNase-Free centrifugal tube. Absolute ethanol, about 0.5 times the supernatant volume, was slowly added to the supernatant. The mixture was transferred the adsorption column (Spin Columns RM) which has been loaded into the collection tube. Centrifugation for 1 min at 4ºC 12,000 rpm. The waste liquid was discarded and the adsorption column was put back into the header. The adsorption column RM was centrifuged for 1 min with 350 mL Buffer RW1 and 4ºC 12,000 rpm and the waste liquid was discarded and the adsorption column was re-placed in the header, which were conducted for two times. The adsorption column RM was centrifuged for 1 min with 500 mL Buffer RW2 and the waste liquid was discarded and the adsorption column was re-placed in the collector, which were conducted for two times, too. Then the RM column was loaded into a new RNase-Free Centrifuge Tubes (1.5 mL) and 30-50 mL RNase-free water was suspended in the middle part of the adsorption membrane. It was placed at room temperature for 2 min and centrifuged for 1 min at 4ºC 12,000 rpm. The obtained RNA solution was stored at -80ºC to prevent degradation. RNA integrity and purity were respectively analyzed by electrophoresis on agarose gel 1.0% (w/v) and A260/A280 rate. The cDNA were obtained by using Trans Script® First-Strand cDNA Synthesis Super Mix (TransGen Biotech) following instruction. 0.2 μL total RNA, 10.0 μL 2xTS Reaction Mix, 1.0 μL Anchored Oligo(dT)$_{18}$ and 1.0 μL *TransScript*® RT/RI Enzyme Mix were mixed and ddH$_2$O was added to 20.0 μL in 0.2 mL PCR tube. Mixture was incubated at 42ºC for 0.5 h and reacted at 85ºC for 5 s. Continue the follow-up test on ice or store at -20ºC.

Table 1: Gradient elution of the mobile phase

Gradient	Time/min	Flow (mL/min)	Volume fraction of mobile phase/%	
			A	B
1	0.00	0.300	2.0	98.0
2	1.00	0.300	2.0	98.0
3	1.50	0.300	90.0	10.0
4	2.00	0.300	90.0	10.0
5	2.10	0.300	2.0	98.0
6	4.00	0.300	2.0	98.0

Table 2: Condition of MRM

Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone(V)	Collision(V)
Abscisic acid	263.1600	219.00	0.163	32	10
		153.02	0.163	32	8

Table 3: Degenerate primers used for amplification of genes from the raspberry fruits

Name	Oligonucleotides	Accession
NCED1-F	TTCACCGARACSS ASCGYHT	XM_010942986.1, JX013945.1, XM_008380174.2, KJ719311.1,
NCED1-R	TTGAAWATGGART CSGSNGGN	JN602255.1, XM_009356579.2, XM_008228017.1, XM_007214595.1, GQ913652.1, XM_008789586.2
CYP707A1-F	ATGAAAGCVMGV AAGGARYTDG	KP723486.1, XM_008348235.2, XM_007210515.2,
CYP707A1-R	AGTATGGHCCYTT TGCTCTTC	NM_001281052.1, XM_008246871.2, XM_004245970.3, XM_009614183.2, XM_016716782.1, XM_018116623.1

The cDNA was used as a template for amplifying genes with degenerate primers, which were designed from the conserved sequences of other species (Table 3).

PCR was performed using the following conditions: 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s and the final reaction terminated at 72ºC for an additional 7 min. PCR products were ligated into a pEasy-T1 vector and subsequently transformed into Escherichia coli DH5a. Positive colonies were selected, amplified, and sequenced by the Beijing Genomics Institute (Beijing, China).

Multiple sequence alignments were performed using DNAMAN 6.0 (Lynnon Biosoft, Quebec, Canada) to identify the conserved regions. MEGA 6.0 (Tamura *et al.* 2013) was used to construct maximum parsimony (MP) trees with the following parameters: Poisson model, pairwise deletion and bootstrap (1000 replicates; random seed) for phylogenetic analysis.

Quantitative real-time PCR analysis

The isolation of total RNA is same as above. The cDNA was synthesized from 1.0 μg total RNA using the PrimeScript^{TM} RT reagent kit (TaKaRa). Primers used for real-time PCR were given in Table 4. Specific primers were designed by using Primer Premier 5.0 software (Biosoft International, Palo Alto, C.A.). The amplification reactions were performed on qTOWER (analytikjena, Germany),

Table 4: Specific primers used for real-time PCR

Name	Oligonucleotides	Accession
NCED1-F	5'- GCCAACCATGATGCACGACTTC-3'	-
NCED1-R	5'-TCCGGAGCTTCGACCCATTTGATT-3'	-
CYP707A1-F	5'- GCGCGGAAGGAATTTGCTCAGAT-3'	-
CYP707A1-R	5'-GCAAAGACGACGCCGATGAC-3'	-
RiActin-F	5'-GCCAACCATGATGCACGACTTC-3'	HQ439557
RiActin-R	5'-TCCGGAGCTTCGACCCATTTGATT-3'	

in which cDNA was mixed with SYBR Premix ExTaq (TaKaRa, Dalian, China) and distilled water was added to a volume of 10 μL, then in a final volume of 20 mL using each primer pair with the concentration of 0.8 m*M* following the manufacture's procedure. Thermal cycle was: 95ºC for 30 s; 40 cycles of 95ºC for 5 s and 60.9ºC for 30 s; and a melting curve from 60.9ºC to 95ºC. The purity of the amplified products was judged by melting curve analysis and agarose gel electrophoresis. The expression levels of the gene were calculated with $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) by comparison with the reference gene, that is constantly expressed *β-Actin* (GenBank accession NO. HQ439557.1). All reactions were performed three times.

Physical and chemical parameters

Fruit hardness was measured using a digital fruit hardness tester (GY-4, TOP instrument., China) with a 3.5 mm diameter stainless steel probe at a speed of 1.5 mm/s and a depth of one-third of fruit diameter, which was visible through the perspex and expressed as Newton (N). Titratable acidity content was measured referred to Perkins-Veazie and Nonnecke (1992). Total soluble solids content was estimated with a digital refractometer (MASTER-100H ATAGO, Master, Japan) and expressed as % at 20ºC. The extraction of anthocyanins was performed as reported by Karppinen *et al*. (2018). The total anthocyanins were measured spectrophotometrically by reading absorbance at 530 nm and 600nm. The results were expressed as OD/g FW. Chlorophyll determination was referred to Miret *et al*. (2014). About 5.0 g fruits were ground and extracted with 80% acetone. The optical density was measured at 652 nm by 751 spectrophotometer. The content of chlorophyll was calculated according to Amon formula in mg/gFW.

Statistical analysis

All data were measured on three sampling dates and subjected to analysis of variance using SPSS statistical software, an analysis of variance was performed, and significant differences were determined at $P < 0.05$ (LSD test).

Results

Isolation and sequence analysis of NCED and CYP707A genes from raspberry fruit

Two genes fragment cloned from raspberry fruit showed high homology respectively to *NCEDs* and *CYP707As* in other species and were named *RiNCED1* (789 bp) and *RiCYP707A1* (686 bp), which encoded an open reading frame (ORF) of 237 and 227 amino acids, respectively. Multiple alignments of the predicted RiNCED1 protein sequences revealed a sequence homology to wild strawberry (*F. vesca*) (FvNCED3) of 95.93%, grape (*V. vinifera*) (VvNCED1) and peach (*P. persica*) (PpNCED2) of 82.93% and 86.99%, respectively (Fig. 1A). The phylogenetic tree shows the RiNCED1 with NCEDs from strawberry, grape, and so on (Fig. 1B). The RiCYP707A1 protein sequences revealed a sequence homology to wild strawberry (*F. vesca*) (FvCYP707A1) of 89.87%, peach (*P. persica*) (PpCYP707A5) of 83.12%, grape (*V. vinifera*) (VvCYP707A1) and apple (*M. domestica*) (MdCYP707A1) of 81.01% and 78.48%, respectively (Fig. 2A). The phylogenetic tree shows the RiCYP707A1 with the CYP707 As from strawberry, grape, citrus, pear and so on (Fig. 2B).

Physiological parameters and the expression of *RiNCED1* and *RiCYP707A1* in the development of raspberry fruit

The raspberry fruit ripening is characterized by changes in colour and texture. At first, it is the transition from the initial green to white, then followed by anthocyanins accumulation gradually-colouring and softening began to occur. Hardness of raspberry fruit reaches the maximum at the green stage, and it shows a significant downward trend during the whole growth period, and the hardness could not be measured at the overripe stage (Fig. 3A). ABA content increases significantly from the green stage to ripe stage and it reaches the maximum value 26.92 μg/g at the ripe stage. Then ABA content decreases significantly from ripe stage to overripe stage and it is still higher than ABA content at the green stage (Fig. 3B). Red raspberry fruit ripening accompanies a rise of soluble sugar content (Fig. 3C) and a down of titratable acidity content (Fig. 3D). Chlorophyll content decreases from 57.42 (mg/kg FW) at the green stage to 1.63 (mg/kg FW) at the overripe stage and chlorophyll content decreases slowly after the mottled stage (Fig. 3E). Meanwhile, anthocyanins accumulate from 0.08 (OD/g FW) at the green stage to 0.72 (OD/g FW) at the overripe stage, which are about ten times that of the initial anthocyanins and it increased rapidly after the mottled stage (Fig. 3F).

It is usually considered that the cleavage reaction catalyzed by *NCED* is a primary rate-limiting step and point of regulating in ABA accumulation. The *RiNCED1* transcripts are raised during fruit ripening, with the highest
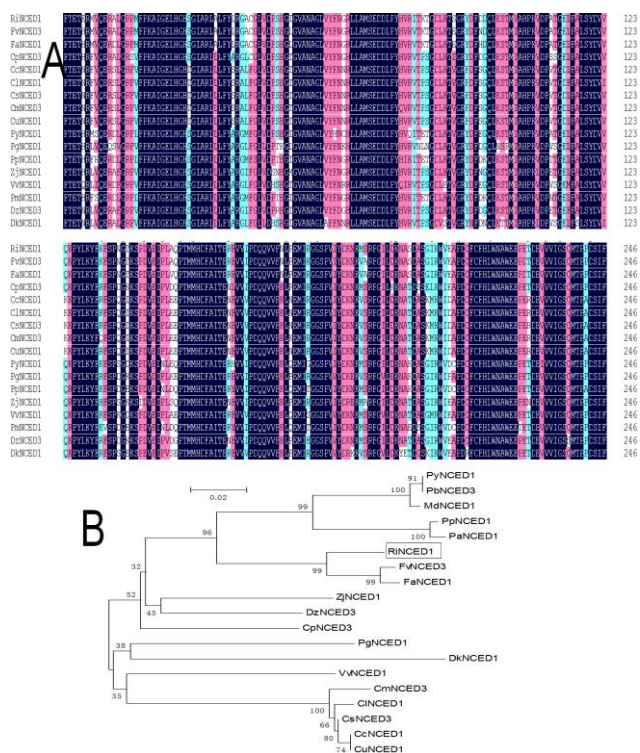
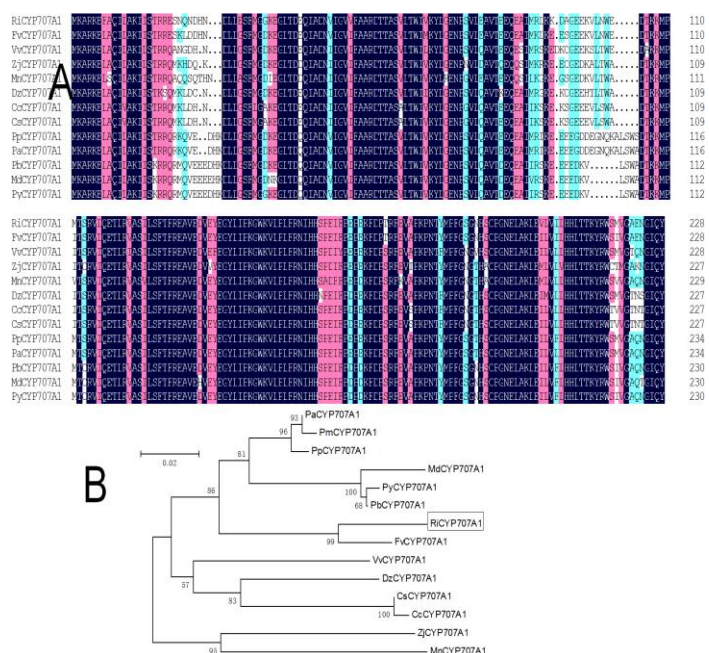**Fig. 1:** Homologous alignment and phylogenetic relationship of RiNCED1 with other reported NCED proteins. Homologous alignment of RiNCED1 with other plant NCED proteins. Positions containing identical residues are shaded in navy blue, while conservative residues are shown in green. This gene fragment is a conserved sequence, which is contained to RPE65 superfamily (**A**). Phylogenetic tree analysis of RiNCED1 and other plant NCED proteins. The tree was constructed by the neighbour-joining method with Mega6. The RiNCED1 is indicated in black box. Numbers on branches indicate bootstrap values (as a percentage) (**B**).

Fig. 2: Homologous alignment and phylogenetic relationship of RiCYP707A1 with other reported CYP707A proteins. Homologous alignment of RiCYP707A1 with other plant CYP707A proteins. Positions containing identical residues are shaded in navy blue, while conservative residues are shown in green. This gene fragment is a conserved sequence, which is contained to p450 superfamily (**A**). Phylogenetic tree analysis of RiCYP707A1 and other plant CYP707A proteins. The tree was constructed by the neighbor-joining method with Mega6. The RiCYP707A1 is indicated in black box. Numbers on branches indicate bootstrap values (as a percentage) (**B**).
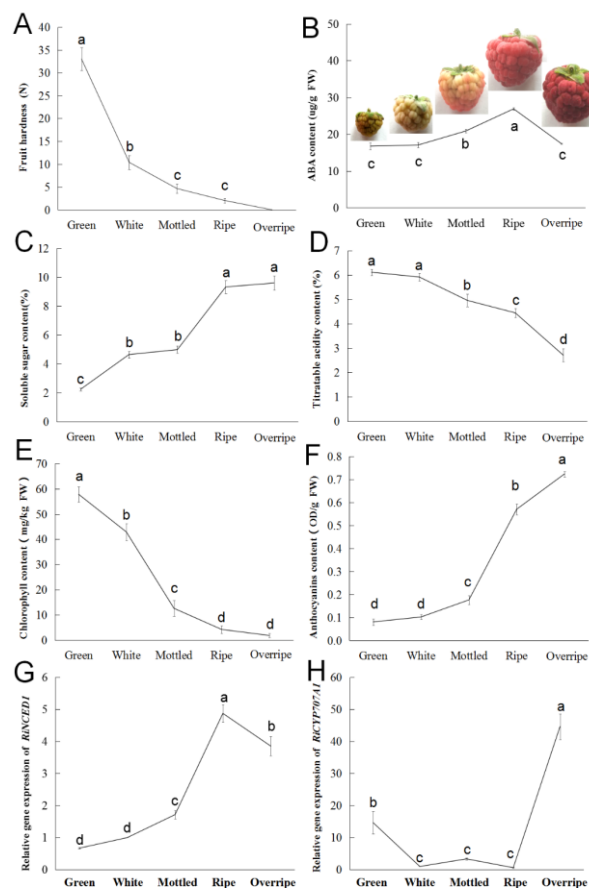
Fig. 3: Physical and chemical parameters, and the expression of *RiNCED1* and *RiCYP707A1* in the ripening of raspberry fruit. (A) Fruit hardness. (B) Fruit appearance and ABA content. (C) Soluble sugar content. (D) Titratable acidity content. (*E*) Anthocyanins content. (F) chlorophyll content. (G) Expression of *RiNCED1*. (H) Expression of *RiCYP707A1*. Each data (mean ± S.D., n = 3) represents the average of three independent plants, error bars indicate the ppercentage deviation. Error bars not visible fall within the symbol (*P* < 0.05).

Fig. 4: The colouration of raspberry fruits which were collected at 0, 12, 24, 36, 48 and 60 h after 1 m*M* ABA and 0.6 m*M* fluridone applied.

level in the ripe stage (Fig. 3G). Also, the maximum expression coincides with the ABA accumulation. The *RiCYP707A1* transcription level varies with a higher expression in the green and overripe stages and the minimum expression at the ripe stage during raspberry fruit ripening (Fig. 3H). It seems that the lower ABA accumulation in the green and overripe stages compared to other stages is related to a higher expression of *RiCYP707A1* in the green and overripe stages.

Effect of abscisic acid and fluridone on *RiNCED1* and *RiCYP707A1* gene expression and physiological parameters

To explore the effect of ABA on the ripening of raspberry fruit and the accumulation of anthocyanin, the unripe white raspberry fruits detached to plants was immersed in
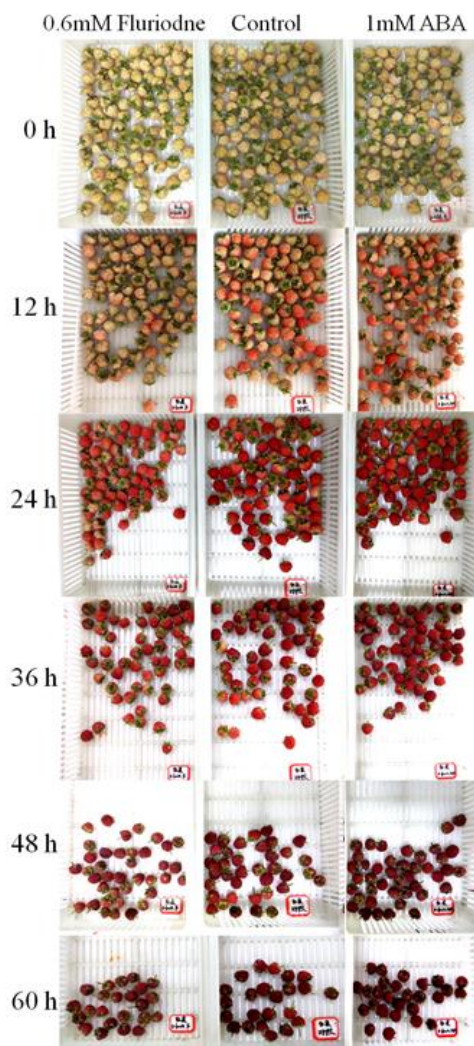
exogenous ABA and ABA inhibitor fluridone. The fruit appearance after treatment is shown in Fig. 4. With the extension of processing time, the number of fruit colouring increases, the colour of the fruit changes from pink to deep red and even the fruit loses water and becomes small. The depth of fruit colouration and the number of colouring fruit of ABA treatment were higher than that of control at the same prcocessing time. According to the colouration of the treated fruit, the fruit of the main colouring state at the current time is collected. Determination of the physiological parameters related to the fruit can be more understood the effect of exogenous ABA on raspberry fruit. As is shown in Fig. 5A, the fruit hardness has been in a downward trend with the extension of processing time. Fruit hardness of ABA-treated has always been significantly lower than control. While fluridone-treated fruit hardness has always been higher than control. The change trend of chlorophyll
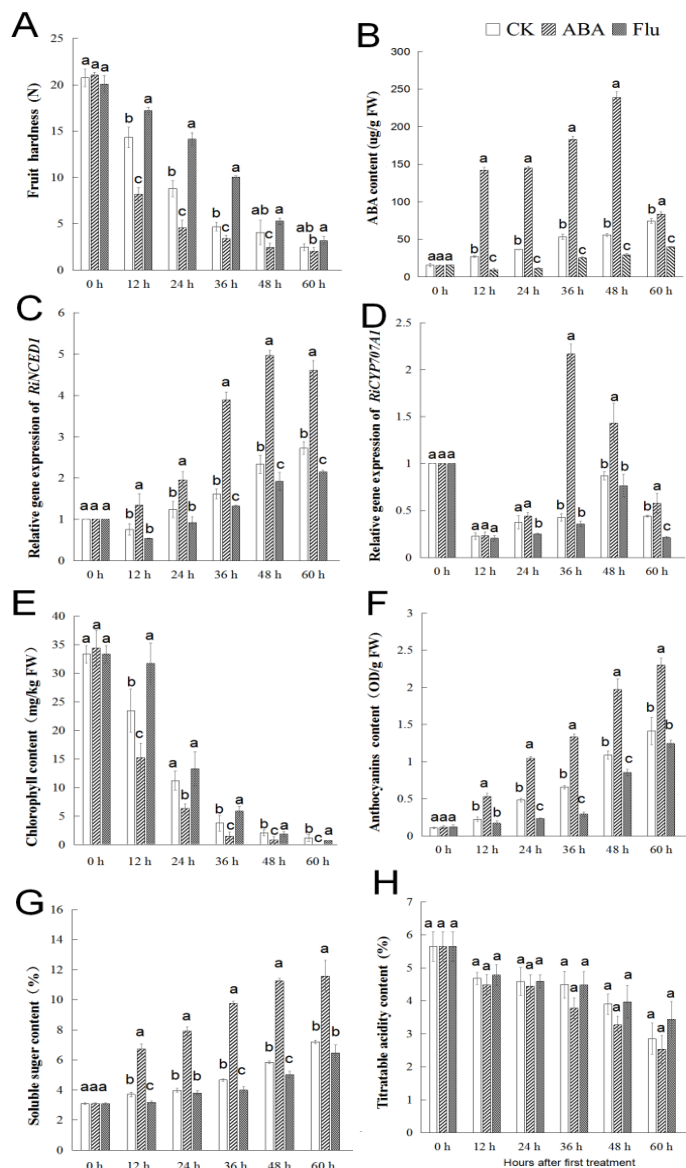
Fig. 5: Fruit hardness (**A**), ABA content (**B**), expression of *RiNCED1* (**C**) and *RiCYP707A1* (**D**), Chlorophyll (**E**), anthocyanins (**F**), soluble sugar (**G**) and titratable acidity (**H**) after 1 m*M* ABA and 0.6 m*M* fluridone applied. Each data (mean ± S.D., n = 3) represents the average of three independent plants, error bars indicate the percentage deviation. Error bars not visible fall within the symbol (*P* < 0.05).

content and titratable acidity content after treated is same to the fruit hardness (Fig. 5E and H). It indicates that ABA promotes a decrease of chlorophyll content, but ABA does not play a role to titratable acidity content in raspberry fruits. There is an increase in anthocyanins and soluble sugar content after treatment with different reagents (Fig. 5F and G). Anthocyanins and soluble sugar content after ABA treatment is further higher than the control from 12 h to 60 h, anthocyanins and soluble sugar content after fluridone treatment is little lower than the control from 12 h to 60 h and is no significant compared to control at 60 h. It indicates that ABA does play a role to increase the content of anthocyanins and soluble sugar in raspberry

fruits and fluridone only works in the early stages of processing in raspberry fruits.

The ABA accumulation of the control rise from the beginning until 60 h gradually and it reaches a maximum of 76.21 *µ*g/g FW in 60 h (Fig. 5B). However, the content of ABA in raspberry fruit after ABA treatment has an effective increase, which is significantly higher than the control, and reaches the peak in advance, which is 673.98 *µ*g/g FW in 48 h, and then it decreases from 48 h to 60 h, yet it is still higher than the control (Fig. 5B). While the trend of ABA accumulation in raspberry fruit after fluridone treatment is different, which has a sudden down from the beginning until 12 h, then it has a gradual rise from 12 h until 60 h,

afterwards it increases and reaches a maximum of 39.72 μg/g FW in 60 h, yet it is always lower than the control from 12 h to 60 h, and there is an significant difference compared to the control (Fig. 5B). Interestingly, the trend of *RiNCED1* expression is similar to the trend of the ABA accumulation. It showed that *RiNCED1* expression is closely related to the level of ABA, and it regulates the synthesis of ABA in raspberry fruits (Fig. 5C). The trend of *RiCYP707A1* expression after different treatments is similar, which drops significantly from the beginning until 12 h, then rises to the peak, and yet down (Fig. 5D). Exogenous ABA make *RiCYP707A1* expression reach the peak in advance. The transcript level of *RiCYP707A1* after ABA treatment is always higher than the control at same processing time, at the same time, the transcript level of *RiCYP707A1* after fluridone treatment is always lower than the control. It suggested that exogenous ABA also could promotes the expression of *RiCYP707A1*.

Discussion

'Heritage' raspberry can be classified as a non-climacteric fruit, along with others like strawberry, grape and citrus, in which fruit ABA plays a vital effect in the fruit natural ripening. ABA content is found to have increased from the green stage until ripe stage with the decrease of fruit firmness, chlorophyll content and titratable acidity content as well as the increase of soluble sugar content and anthocyanins content in the raspberry fruit. An elevation of ABA content was accompanied by a rise of *RiNCED1* transcription level at the ripening of raspberry fruits, which indicating that an effect of ABA in regulating the ripening of raspberry fruits. In grapes (Zhang *et al.* 2009), endogenous ABA increases rapidly to a peak around the first 20 days of harvest and quickly declining before the harvest stage. The whole growth, development, maturity and senescence of the fruit is accompanied by the increase of ssc/ta and the decrease of hardness. At the onset of ripening of grape, the *VvNCED1* genes were expressed only by Northern analysis when ABA accumulation was high. In sweet cherry fruit, the trend of *PacNCED1* expression and ABA accumulation was coincident in time, which was related to the ripening of fruit. In strawberry (Jia *et al.* 2011), chlorophyll declined continually after the BG stage yet the anthocyanins content increased rapidly at the late ripening stage, soluble sugars content showed the same trend with ABA during fruit development.

In raspberry, the accumulation of anthocyanins and soluble sugar is a significant signal of fruit ripening. In the present study, do anthocyanins and soluble sugar sharply increase only in the late ripening stage of raspberry fruits, meanwhile, ABA accumulation is also rise significantly. Exogenous ABA applied to the raspberry fruits in white stage advanced colouration, anthocyanin accumulation and increase of soluble sugar. Besides, fluridone, a ABA biosynthesis inhibitor, applied to the raspberry fruits in

white stage postharvest treatment delayed fruit colouration, also reduced the accumulation of anthocyanin and soluble sugar in raspberry fruits. It has been shown that exogenous ABA could modify anthocyanin profile in bilberry fruits (Ju *et al.* 2016; Oh *et al.* 2018). Also ABA accelerated sugar and anthocyanin accumulation at véraison in grape berries (Villalobos-González *et al.* 2016). S-ABA also could promote the fruit colouration from yellow to deep orange in orange (Wang *et al.* 2016; Rehman *et al.* 2018). The key anthocyanin biosynthetic genes expression was down regulated at once silencing of *VmNCED1* in bilberry fruit (Karppinen *et al.* 2018). Silencing *FaNCED1,* which causes a notable decrease in ABA accumulation in strawberry fruit and the inability to turn red colourless fruits, however, the colourless phenotype fruit can be converted into normal red fruit by exogenous ABA (Jia *et al.* 2011).

Besides fruit colouration, fruit softening is related to the ripening of fruit. The increase of the ABA content after exogenous ABA treatment accelerated the softening of the flesh and the ripening and senescence of the fruits, while the fluridone-treated raspberry fruit has lower endogenous ABA and also the softening of the fruit is delayed. More research showed that the decrease of fruit hardness is controlled by ABA and exogenous ABA could promote fruit softening in non-climacteric fruit, such as strawberry (Li *et al.* 2014), northern highbush blueberry (Oh *et al.* 2018). Some reseachers found that

hydrolytic enzymes and expansions could make the structural changes in cell wall polysaccharides pectin, hemicellulose and cellulose, which lead an decrease of fruit hardness with the fruit ripening (Posé *et al.* 2018; Zhi *et al.* 2018). Silencing of *SlNCED1* leads a higher pectin content, a loss of hardness and a down-regulation expression of genes encoding cell wall degrading enzymes in tomato (Sun *et al.* 2012). Therefore, ABA is believed to be involved in the ripening process of raspberry and many changes in the growth and development of the fruit berry.

The expression of *RiNCED1* were similar to ABA accumulation during raspberry fruit development, which shows that *RiNCED1* plays a key effect in ABA content and it was considered as a primary gene involved in the ABA biosynthesis in raspberry fruit. The data that ABA treatment could promote the expression of *RiNCED1* indicates that ABA could regulate its own biosynthesis in raspberry fruit. Similar discoveries were found grapes (Wheeler *et al.* 2009) and sweet cherry (Luo *et al.* 2014). In the ripening of strawberry fruits, up-regulated of *FaNCED1* expression by ABA was believed that the autocatalytic biosynthesis of ABA induces mass increase in ABA accumulation (Chen *et al.* 2016; Medina-Puche *et al.* 2016).

The transcription level of *RiCYP707A1*, which act as ABA degradation enzyme genes, also influenced ABA accumulation apart from the regulation of *RiNCED1*. The relative expression of the *RiCYP707A1* gene is higher than the *RiNCED1* at the green stage, which may be the reason of the low accumulation of ABA at green stage of raspberry

fruit. Moreover, the relative expression of the *RiCYP707A1* gene is very high on the overripe stage of fruit development, which may be material cause of the decrease of ABA in the late stage. The relative expression of the *RiCYP707A1* gene is relatively low from white stage to ripe stage. It seems that the *RiCYP707A1* gene has no effect on accumulation of ABA that the accumulation of ABA keeps going up, though the relative expression of the *RiCYP707A1* gene fluctuate up and down during the middle stage. The inverse relationship between the expression of *RiCYP707A1* and ABA accumulation indicates that anabolic enzymes and catabolic enzymes play a significant effect on the regulation of the ABA content in raspberry fruit. ABA treatment enhanced the expression of *PacCYP707A1-PacCYP707A3* and *PacNCED1* in sweet cherry fruits, but down-regulated the transcription level of *PacCYP707A4* (Ren *et al*. 2010). Therefore, the key synthetase gene *RiNCED1* and degradation gene *RiCYP707A1* regulated the content of ABA together in raspberry fruits.

More and more evidence proves the view that the ripening of fruit is not regulated by simplex hormones at all, but by a complex feedback and crosstalk networks among different plant hormones (McAtee *et al*. 2013; Kumar *et al*. 2014). In citrus, epicarp carotenoid content was increased and ABA synthesis stimulated by exogenous ethylene treatment, though minute amounts of ethylene produced during maturation (Rodrigo and Zacarias 2007; Wang *et al*. 2016). In grape fruits, it was suggested that the trace endogenous ethylene induced the expression of *VvNCED1* and then the accumulation of ABA followed (Sun *et al*. 2010). While in cherry fruit, it was found that postharvest fruit senescence was induced by ABA through stimulation of ethylene release (Luo *et al*. 2014). Therefore, how does ethylene and ABA interact in raspberry fruit need further research.

Conclusion

ABA accumulation of raspberry fruits reaches its maximum in the ripe stage. Exogenous ABA application to raspberry fruits significantly promote ABA, soluble sugar and anthocyanins contents, fruit softening, *RiNCED1* and *RiCYP707A1*expression. While fluridone, as an ABA biosynthesis inhibitor, could inhibit increase of ABA, soluble sugar and anthocyanins contents, delay softening of fruit and decrease of chlorophyll in raspberry fruits. The results above indicated that exogenous ABA could promote the synthesis of endogenous ABA and it has an affirmative effect on softening and ripening of raspberry fruits.

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