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ORIGINAL RESEARCH

Expression analysis of the cDNA for magnesium chelatase H subunit (CHLH) during sweet cherry fruit ripening and under stress conditions

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Abstract To understand details about the molecular mechanisms of abscisic acid (ABA) action in fruit maturation, one partial cDNA (*PaCHLH1*) encoding magnesium chelatase H subunit (CHLH) which was proven to be an ABA receptor in Arabidopsis was cloned from sweet cherry fruit. The sequence of *PacCHLH1* was 442 bp. The amino acid sequence of *PacCHLH1* shows high homology to other CHLHs. *PacCHLH1* expression dramatically declined after 25 days after full bloom (DAFB) which is the beginning of the ripening process, in contrast to the changes in ABA levels during this period. The application of ABA promoted endogenous ABA biosynthesis and fruit ripening but significantly decreased the expression of *PacCHLH1* in fruits. No significant correlation was observed between the *PacCHLH1* transcript and ABA accumulation in dehydrated fruits. In dehydrated leaves, the ABA and *PacCHLH1* transcript levels were both up-regulated.

Keywords Sweet cherry · Abscisic acid · Magnesium chelatase H subunit (CHLH) · Receptor · Ripening

Introduction

Abscisic acid (ABA) plays a key role in various aspects of plant growth and development, such as adaptation to environmental stress, seed maturation and germination, as well as fruit ripening including climacteric and non-climacteric fruits (Coombe 1976; Davies and Zhang 1991; Finkelstein et al. 2002). ABA has been indicated to trigger ethylene biosynthesis and ripening of tomato fruit (Zhang et al. 2009a). In the grape berry which is a non-climacteric fruit, the veraison of ripening is related to ABA (Coombe and Hale 1973; Zhang et al. 2009b). ABA may also play a role in sweet cherry fruit maturation. In non-climacteric sweet cherries, the level of ABA increases before maturation and thereafter decreases toward harvest (Kondo and Gemma 1993). Thus, ABA has been considered to play an important role in the regulation of fruit ripening. Although the conventional physiology of ABA action on fruit development and ripening has been well studied, the molecular mechanisms of ABA action remain unknown.

As a plant hormone signal, ABA should be firstly perceived by cells through the binding of ABA to an extracellular or intracellular specific site or receptor (Giraudat et al. 1994; Leung and Giraudat 1998; Michael and Valeria 1993), and then the ABA-receptor complex can trigger downstream signaling cascades to induce the final physiological effects (Wang and Zhang 2007). It has been confirmed that ABA binding proteins are present in flesh fruits such as grape berry (Zhang et al. 1999) and apple (Zhang et al. 2001). However, no significant progress has been made in the search for receptors that perceive ABA in flesh fruits. Recently, it has been proven that the Mg-chelatase H subunit (CHLH) is an ABA receptor in Arabidopsis (*Arabidopsis thaliana*) (Shen et al. 2006; Wu et al. 2009). CHLH specifically binds ABA and mediates ABA signaling as a positive regulator in seed germination, post-germination growth and stomatal movement, and it was indicated that CHLH might be able to perceive the ABA signal at the whole-plant level in Arabidopsis (Shen et al. 2006). However, there are no reports on the function of the *CHLH* gene in flesh fruit maturation.

In this study, we cloned one cDNA for CHLH from sweet cherry fruit and analyzed the expression of the cDNA and

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the relationship between CHLH cDNA and ABA during fruit ripening.

Materials and methods

Plant material

Fruits from *Prunus avium* L. cv. Hongdeng were collected from adult trees. Trees were grown at the Beijing Forest and Pomology Institute under field conditions. Fruits at different developmental and maturation stages were periodically harvested from April to June. The pulp was immediately separated using a knife and cut into small pieces. All samples were then frozen in liquid N_2 and stored at −80°C for subsequent analysis.

ABA field experiment

In 2008 season, straw-colored fruits 32 days after full bloom (DAFB) were harvested, divided into two groups and treated with water and 0.4 mmol l^{-1} ABA (containing 0.1% Tween-20), applied as a spray to runoff. At 5 days after the initiation of treatment, anthocyanin content and maturity index (SSC/TA) were analyzed.

In 2009 season, a 2-year-old shoot with fruit and leaves was cut at 29 DAFB, and the shoot was placed for 5 days at 24°C under a 16 h photoperiod in 1 mmol l^{-1} ABA solution and an untreated control containing only distilled water. At 5 days after the initiation of treatment, maturity index (SSC/TA), fruit firmness, and ABA concentrations were analyzed, and some samples were stored at −80°C before RNA extraction.

Water stress treatments

To evaluate the effect of water stress on fruit ABA content and CHLH cDNA expression, fruit 32 DAFB were used. The fruit was divided into 2 groups. The first group was covered with gauze, sprayed with water to keep humidified, and incubated in a storage room for 4 days at 25°C under high relative humidity (RH, 100%, control fruits). The second group was stored at the same temperature for 4 days under low relative humidity (45%, dehydrated fruits). Samples were collected every day at the same time and the pulp was separated. Each sample included 20 fruits. Some samples were quickly frozen in liquid nitrogen and stored at −80°C before RNA extraction.

Water stress experiments in leaves were performed using detached mature leaves. Leaves were collected, weighed and dehydrated to 70% of their initial fresh weight (FW) by a stream of warm air from a hair dryer in a few minutes.

RNA extraction, RT-PCR and sequencing

Total RNA was extracted from 1.0 g of pulp or leaves using the hot borate method (Wan and Wilkins 1994). Synthesis of the first-strand cDNA from 2 μg of total RNA isolated from sweet cherry fruit 30 days after full bloom was conducted using a Moloney murine leukemia virus reverse transcriptase (Takara, Beijing, China) and an oligo dT primer. The cDNA was used as a template for amplifying CHLH gene with degenerate primers (forward, 5′-ACNGCNYTNGARGARAAYTGG-3′; reverse, 5′-TRTANARNCCNGCRTTYTCNGC-3′) designed from the conserved regions of plant CHLHs (Z68495, FJ842115, GQ201451, XM_002284042, and DQ641092) in the database. RT conditions were: 65°C for 5 min, followed by 42°C for 30 min, followed by 5 min at 95°C. PCR was performed as follows: 3 min 94°C, 32 cycles of 94°C 30 s, 52°C for 1 min and 72°C for 1 min, and 72°C for 10 min. The PCR products were ligated into a pMD18-T vector (TaKaRa, Dalian, China) and subsequently transformed into *Escherichia coli* DH5α. Sequences encoding plant CHLHs were determined by a homology search of the NCBI databases using the BLAST program. Furthermore, using the same method as above mentioned, the desired genes from sweet cherry fruit were obtained using the primers PacACT1 (forward, 5′-ATGGTGAGGATATTCAACCC-3′; reverse, 5′-CTTCCTGTGGACAATGGATGG-3′).

RT-PCR analysis

Total RNA (1.0 μg) was reverse transcribed using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China), with oligo dT_{18} as the primer, according to the manufacturer's recommendations. Amplification of specific regions of targeted genes and real-time detection of amplicon production was conducted using a Rotor-Gene 3000 system (Corbett Research, Australia) with the primers: *PacCHLH1* (forward, 5′-TCCAATGAGGCTACTGTTCTCCAAA-3′; reverse, 5′-ATTCCAGTGAACCGTGAGTACCAAA-3′), *PacACT1* (forward, 5′-CTCCTCTCAACCCTAAGGCTAACAG-3′; reverse, 5′-CAGTTGTACGACCACTGGCATACAG-3′). Real Time PCR was performed using the SYBR Premix Ex Taq™ kit (TaKaRa, Dalian, China). Reactions contained 1 μl of primer mix (containing 5 μM of each forward and reverse primer), 2 μl cDNA template, 12.5 μl SYBR Premix Ex Taq™ (2×) mix and 9.5 μl water to make a total volume of 25 μl. Reactions were carried out under the following conditions: 95°C/30 s (1 cycle); 95°C/15 s, 60°C/20 s; 72°C/15 s (40 cycles).

PCR amplification of a single product of the correct size for CHLH was confirmed by agarose gel electrophoresis and double-strand sequencing. The amplified fragment of each gene was subcloned and used to generate efficiency

curves. Relative fold expression for *CHLH* gene was calculated using Rotor-Gene 6.1.81 software. The *PacACT1* transcript was used to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations.

Determination of ABA content

For ABA extraction, 1.0 g of pulp or leaves were ground in a mortar and homogenized in extraction solution (80% methanol, v/v). Extracts were centrifuged at 10,000*g* for 20 min. The supernatant liquid was eluted through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) to remove polar compounds, and then stored at −20°C for enzyme-linked immunosorbent assay (ELISA). The ELISA procedures were conducted according to the instructions provided by the manufacturer (China Agricultural University, Beijing, China). ABA was determined by Thermo Electron (labsystems) Multiskan MK3 (PIONEER Co., China).

Anthocyanin analysis

Anthocyanin concentration was measured by extracting peels of equal weight (five replications) with 1% HCl-methanol and determining absorbance at 530 and 657 nm. The formula $A = A_{530} - 0.25A_{657}$ was used to compensate for the contribution of chlorophyll and its degradation products to the absorption at 530 nm (Rabino and Mancinelli 1986). Anthocyanin concentration was a relative value, and we set $A = 0.01$ equal to 1 unit (U).

Measurement of fruit firmness, diameter, and SSC/TA

Fruit firmness and diameter were measured to observe the stages of fruit development. The firmness of 15 fruits was determined after the removal of fruit skin on each sides of the fruit suture using a KM model fruit hardness tester (FUJIHARA Co., Japan). The strength of flesh firmness was recorded as *N*. Thirty fruits were labeled and fruit diameter was measured 3–7 days intervals from 11 to 50 DAFB. Fifteen randomly selected fruits per treatment were juiced to determine soluble solids content (SSC) and titratable acidity (TA). The mesocarp was squeezed and SSC was determined with a Pocket Refractometer Pal-1 (ATAGO, TOKYO), with units of ^{0}Brix. The acidity of the juice was determined by titration with 0.1 mol l^{-1} NaOH, and the malic acid content estimated. The maturity index is expressed as the ratio of SSC/TA.

GenBank accession numbers of the sweet cherry genes used in this study are: *PacCHLH1* (GQ913651) and *PacACT1* (FJ560908).

Results

Cloning and sequencing of the partial cDNA for CHLH from sweet cherry fruit

Using cDNA of pulp along with degenerated primers, a 442 bp fragment was amplified. The sequence showed high homology to other plant CHLHs and was designated as *PacCHLH1* (accession number: GQ913651). A BLAST homology search revealed that the deduced amino acid sequence of *PacCHLH1* shared 96.60% identity with *A. thaliana* CHLH (Z68495, located at 587–733 of the *A. thaliana* CHLH amino acid sequence), 99.32% identity with the *Prunus persica* (peach) CHLH (FJ842115, located at 588–734 of the *P. persica* CHLH amino acid sequence), 98.64% identity with the *Fragaria x ananassa* (strawberry) CHLH (GQ201451, located at 588–734 of the *Fragaria x ananassa* CHLH amino acid sequence), and 96.60% identity with the *Vitis vinifera* (grape) CHLH (XM_002284042, located at 587–733 of the *Vitis vinifera* CHLH amino acid sequence), and 94.56% identity with *Cucumis sativus* (cucumber) CHLH (DQ641092, located at 1–147 of the *C. sativus* CHLH amino acid sequence. Fig. 1).

Changes in ABA content in relation to sweet cherry fruit ripening

By measuring changes in fruit maximum diameter and firmness, a profile of berry development was produced for 'Hongdeng' sweet cherry fruit grown in the 2007 season (Fig. 2). ABA levels were also measured 3–4 days intervals throughout development, beginning at 14 DAFB. 'Hongdeng' sweet cherry fruit development lasts about 40 days from full bloom to maturation, and about 45 days to full ripe. Like other stone fruit, sweet cherry fruit exhibits a biphasic growth pattern. The first 14 DAFB is the first rapid growth period during which cells proliferate and differentiate. Subsequently, following a period of 15–25 DAFB is the pit hardening period during which the fruit exhibits only slight enlargement and the stony endocarp (pit) undergoes hardening. When the pit hardening period ends, a second rapid growth period occurs after 25 DAFB (Fig. 2). This second growth phase is initiated synchronously with ripening processes such as sharply declining of firmness (Fig. 2). Fruit growth ceases at 40 DAFB and there are no further increases in fruit volume and weight, and the fruit comes into the full ripe and senescence period. The ABA level of pulp at 14 DAFB was relatively high, but this decreased until 25 DAFB, and then the ABA content began to increase after 25 DAFB when the pit hardening period ended (Fig. 2, arrow), coincident with the decrease in firmness. After peaking at 36 DAFB, 4 days before harvest, the level of ABA declined (Fig. 2).

Fig. 1 Alignment of the deduced amino acid sequences of *PacCHLH1* (accession number: GQ913651) from sweet cherry (*Prunus avium* L. cv. Hongdeng) with *Arabidopsis thaliana* CHLH (*AtCHLH*, Z68495), *Prunus persica* CHLH (*PpCHLH*, FJ842115), *Fragaria x ananassa* CHLH (*FaCHLH*, GQ201451), *Vitis vinifera* CHLH (*VvCHLH*, XM_002284042) and *Cucumis sativus* CHLH (*CsCHLH*, DQ641092). Identical amino acids and conservative regions are shown on a *black or grey background*, respectively

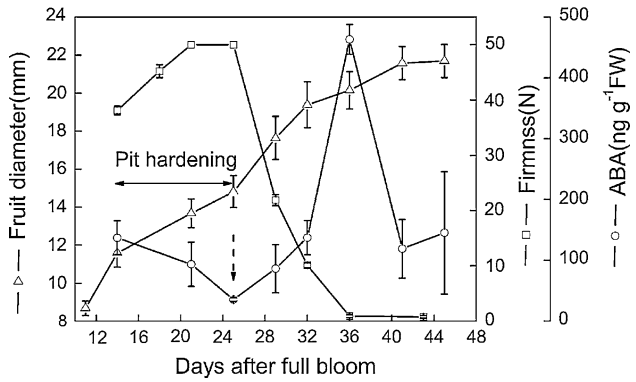

Fig. 2 Changes in fruit maximum diameter, firmness and ABA concentration during 'Hongdeng' sweet cherry fruit development from the 2007 season. Standard errors are indicated

Changes in ABA content and *PacCHLH1* expression during sweet cherry fruit development

Changes in endogenous ABA content during fruit maturation in 2008 and 2009 seasons were also determined. The timing and pattern of ABA accumulation was remarkably stable over the 3 years (Figs. 2, 3). The results suggest that ABA may trigger the sweet cherry fruit maturation (Kondo and Inoue 1997). In order to research the molecular mechanism of ABA action on fruit maturation, *PacCHLH1*, which was the Arabidopsis homologue of CHLH that was an ABA receptor (Shen et al. 2006; Wu et al. 2009), was cloned from sweet cherry fruit. RT-PCR was performed to analyze the relative-fold expression of *PacCHLH1* during fruit maturation in the 2008 and 2009 seasons. The results showed that the transcript of *PacCHLH1* was detected during the whole stage of sweet cherry fruit maturation.

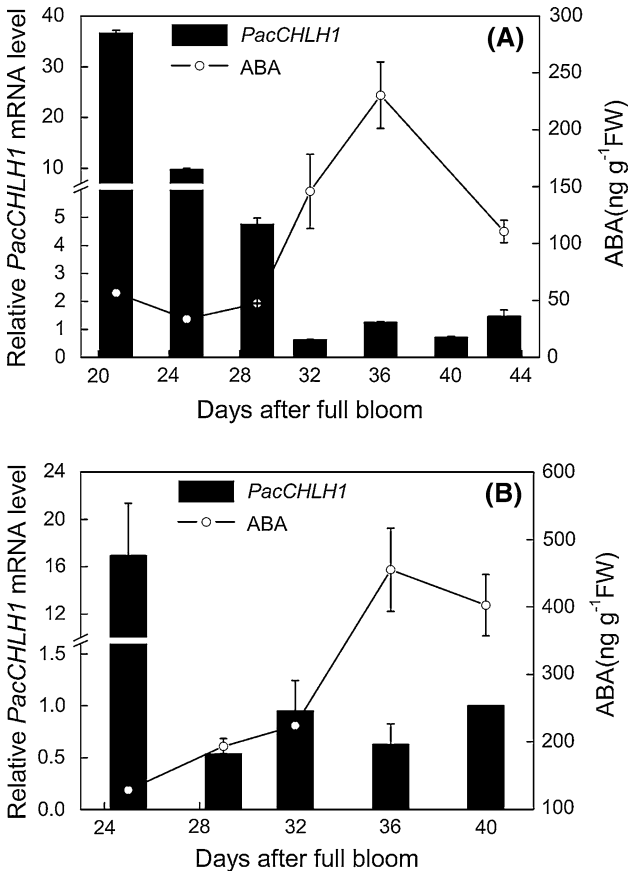

Fig. 3 Changes in ABA content and quantitative expression analysis of *PacCHLH1* of 'Hongdeng' sweet cherry fruit during fruit growth. RT-PCR was performed on total RNA isolated from pulp of 2008 (**a**) and 2009 seasons (**b**). *PacACT1* was used to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations. Expression of each gene is presented as relative fold change. Values presented are means ± SE ($n \geq 3$)

Table 1 Effect of ABA treatment on the maturation of sweet cherry fruit

Year	Treatment	Firmness/*N*	Maturity index (SSC/TA)	Anthocyanin (U g^{-1})
2008	Control	19.4	17.2	37.9
	ABA	11.7*	20.8*	208.5*
2009	Control	20.3	14.4	13.4
	ABA	11.9*	16.8*	23.8*

Data shown are the mean of three replicates of 10 fruits. Mean separation according to the *t*-test, 5% level

After being relatively high in young fruit, the transcript levels decreased until 32 DAFB and then remained at low levels until commercial harvest in the 2008 season (Fig. 3a). The expression patterns of *PacCHLH1* of pulp in the 2009 season were quite similar to that in the 2008 season (Fig. 3b).

Effects of ABA application on sweet cherry fruit ripening

The effects of ABA application on sweet cherry fruit ripening were investigated in the 2008 and 2009 seasons. In the 2008 season, colored fruits at 32 DAFB were harvested and treated with water and 0.4 mmol l^{-1}ABA respectively, applied as a spray to runoff. In the 2009 season, a 2-year-old shoot with fruits and leaves was cut at 29 DAFB and the shoot was placed for 5 days at 24°C under a 16 h photoperiod in 1 mmol l^{-1} ABA solution and distilled water as untreated control. Results of 2 years showed that ABA treatment for 5 days significantly enhanced fruit softening and increased the maturation index and anthocyanin levels compared with the control treatment (Table 1).

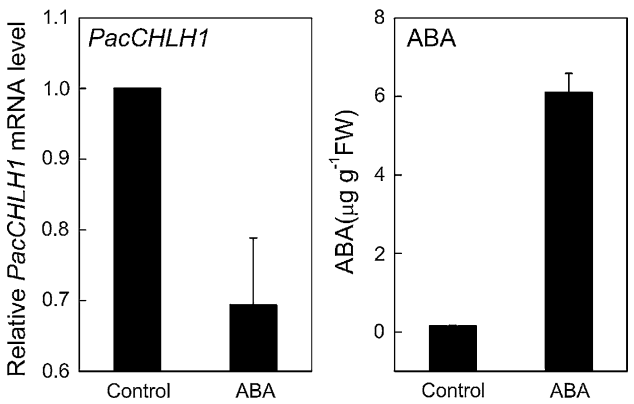

Fig. 4 Effects of ABA on ABA content and accumulation of *PacCHLH1* transcripts in pulp from sweet cherry fruits. Fruits of 29 DAFB were used. Data shown are the means ± SE ($n = 3$)

ABA accumulation in ABA-treated fruit and its effect on the expression of *PacCHLH1*

To investigate effects of ABA application on ABA content and expression of *PacCHLH1*, a 2-year-old shoot with fruit and leaves was cut at 29 DAFB, and the shoot was placed for 5 days at 24°C under a 16 h photoperiod in 1 mmol l^{-1} ABA solution and distilled water as untreated control. Five days after treatment, there was a significant increase in ABA levels in the ABA treated tissues compared with the levels in the control tissue samples (Fig. 4). Real-Time PCR analysis revealed that *PacCHLH1* transcript levels were reduced in the pulp of ABA-treated samples.

Changes in content of ABA and accumulation of *PacCHLH1* transcript in response to dehydration in fruits stored under low humidity conditions

To test the expression of *PacCHLH1* during fruit dehydration, a comparative analysis of transcript accumulation and ABA content in the pulp of fruit 32 DAFB stored for 4 days under low (45%) or high RH (100%) was performed (Fig. 5). During a dehydration experiment lasting 4 days, detached fruits lost 39% of their water content. Figure 5a shows that ABA concentration significantly increased after water stress and by 4 days it was 3.6 times higher than that at the beginning. No significant differences in ABA content were detected in the pulp of control fruits (stored under 100% RH) stored for 2 days, but there was a significant increase 3 days after treatment and then declined (Fig. 5b). Expression of *PacCHLH1* in dehydrated fruits stored for 1 day was significantly up-regulated. This then declined to original levels, but continued to increase from 2 days after treatment (Fig. 5a). In control fruits, *PacCHLH1* expression increased 1 day after treatment as well as that of dehydrated fruit, reached peak value 2 days after treatment, and then declined to levels at 1 day (Fig. 5b). There were no significant differences between control and dehydrated fruits except 2 and 4 days after treatment.

Changes in content of ABA and accumulation of *PacCHLH1* transcripts in response to dehydration in leaves

To examine whether the expression of *PacCHLH1* is induced by environmental stress in vegetative tissues, expression of *PacCHLH1* gene was analyzed in water-stressed leaves and related to the changes in ABA content. During a dehydration experiment where leaves were exposed to a stream of warm air from a hair dryer for a few minutes, detached mature leaves lost 30% of their water content. After water stress, a noticeable increase in ABA and transcript accumulation was clearly detected (Fig. 6).

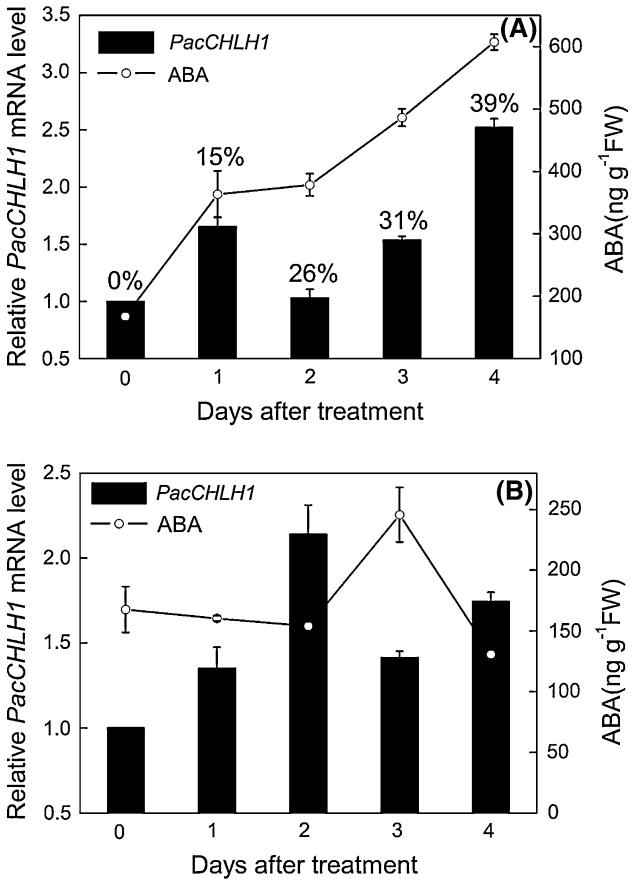

Fig. 5 Changes in content of ABA and accumulation of *PacCHLH1* transcripts in response to dehydration in fruits from sweet cherry. **a** Colored fruits stored at 25°C under low (45%) RH for 0, 1, 2, 3 and 4 days and the numbers at the top of the *bars* indicate the percentage of water loss. **b** Colored fruits stored at 25°C under high (100%) RH for 0, 1, 2, 3 and 4 days. Data are the means ± standard deviation of three measurements

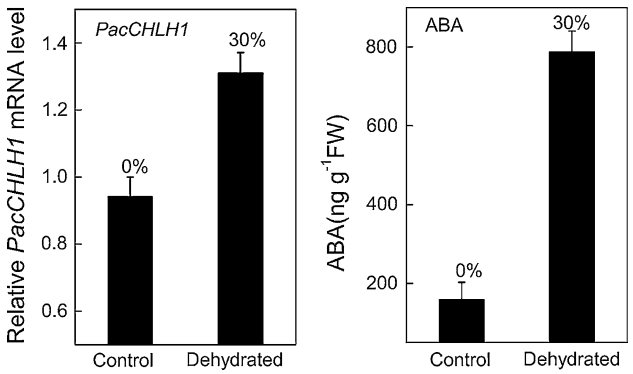

Fig. 6 Changes in content of ABA and accumulation of *PacCHLH1* transcripts in response to dehydration in leaves from sweet cherry. The numbers at the top of the *bars* indicate the percentage of water loss. Data are the means ± standard deviation of three measurements

RT-PCR revealed that the relative expression of *PacCHLH1* in dehydrated leaves was 1.3 times higher than that in control leaves (Fig. 6).

Discussion

The pit hardening period was over at 25 DAFB, accompanied by the beginning of fruit maturation, and the fruit exhibited ripening phenomena in terms of sharp decline in firmness (Fig. 2). In accordance with this, the endogenous ABA of pulp began to accumulate at the same time in sweet cherry fruit, which peaked at 36 DAFB (4 days before commercial harvest), and then decreased (Fig. 2). ABA, which accumulated at the beginning of fruit maturation, has been demonstrated to trigger fruit ripening (Coombe and Hale 1973; Kondo and Gemma 1993; Kondo and Inoue 1997; Zhang et al. 2009a). In order to elucidate the mechanism by which ABA triggers fruit maturation, *PacCHLH1*, the homologue of Arabidopsis CHLH, was cloned from sweet cherry fruit (Fig. 1). The data collected over 2 years showed that *PacCHLH1* expression dramatically declined after 25 DAFB, when the ripening process begins, in contrast to the changes of ABA levels in this period (Fig. 3). These results were inconsistent with CHLH expression in Arabidopsis. In Arabidopsis, CHLH mediates ABA signalling as a positive regulator. Transgenic downregulation of CHLH expression results in a decline in the number of CHLH-binding sites and leads to ABA insensitive phenotypes, and CHLH-overexpressing plants have ABA-hypersensitive phenotypes with an elevated number of ABA binding sites (Shen et al. 2006). Moreover, exogenous ABA promoted endogenous ABA content (Fig. 4) and fruit ripening (Table 1), but there was a significant decrease in *PacCHLH1* transcript level in the ABA-treated fruits. This negative regulation of *PacCHLH1* by ABA was inconsistent with the observed response of *CHLH* expression to ABA in Arabidopsis. In Arabidopsis, exogenous ABA significantly stimulated *CHLH* expression (Shen et al. 2006).

Mg-chelatase, which is composed of three subunits, namely CHLD, CHLI and CHLH, catalyses the insertion of Mg^{2+} into protoporphyrin-IX (Proto) to form Mg-protoporphyrin-IX (MgProto), the first step unique to chlorophyll synthesis (Walker and Willows 1997). CHLH has a central function as a monomeric Proto-binding protein (Karger et al. 2001; Walker and Willows 1997). In sweet cherry, the total fruit chlorophyll content sharply decreased from immature green stage (0.20 mg g^{-1}) to turning (0.038 mg g^{-1}) and red stages (0.018 mg g^{-1}) during ripening (data not shown), coincident with the changes of *PacCHLH1* transcript level (Fig. 3). Moreover, ABA treatment decreased the chlorophyll content (Shen et al. 2006) and *PacCHLH1* transcript level (Fig. 4), suggesting that *PacCHLH1* may be involved in chlorophyll biosynthesis in sweet cherry fruit.

During a dehydration experiment lasting 1 day, detached fruits lost 15% of their water content, and the ABA level

and *PacCHLH1* expression were up-regulated. However, the expression increase was not due to water stress, as *PacCHLH1* expression in control fruits was also up-regulated 1 day after treatment. There was also an unexpected decrease and a continuous increase in *PacCHLH1* expression in dehydrated and control fruits, respectively, 2 days after treatment in contrast to the changes in ABA levels (Fig. 5). The expression of *PacCHLH1* was higher 3 and 4 days after treatment than the beginning in dehydrated fruits as well as in control fruits. These results suggest that *PacCHLH1* may not respond to water stress in fruits. However, in dehydrated leaves, the ABA and *PacCHLH1* transcript levels were both up-regulated (Fig. 6), coinciding with the result that the plants overexpressing CHLH in Arabidopsis were more resistant to dehydration (Shen et al. 2006).

Additionally, other studies reported opposing results for CHLH than those reported in Arabidopsis. More recently, it was reported that ABA had no effect on magnesium chelatase activity in barley and the barley chlorophyll-deficient mutants with mutations in the XanF gene (the same large subunit of Mgchelatase as the CHLH in Arabidopsis) showed no ABA-related phenotypes in seed germination, postgermination growth, and stomatal movement. These results question the function of the large magnesium chelatase subunit as an ABA receptor (Müller and Hansson 2009).

Our results, however, did not necessarily exclude an interaction between PacCHLH1 and ABA. It is difficult to clearly explain the differences between sweet cherry fruit and Arabidopsis according to the current research on mRNA level. Therefore, the binding of ABA to PacCHLH1 protein needs to be further studied.

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