Apple MdACS6 Regulates Ethylene Biosynthesis During Fruit Development Involving Ethylene-Responsive Factor

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Ethylene biosynthesis in plants involves different 1-aminocyclopropane-1-carboxylate synthase (ACS) genes. The regulation of each ACS gene during fruit development is unclear. Here, we characterized another apple (Malus × domestica) ACS gene, MdACS6. The transcript of MdACS6 was observed not only in fruits but also in other tissues. During fruit development, MdACS6 was initiated at a much earlier stage, whereas MdACS3a and MdACS1 began to be expressed at 35 d before harvest and immediately after harvest, respectively. Moreover, the enzyme activity of MdACS6 was significantly lower than that of MdACS3a and MdACS1, accounting for the low ethylene biosynthesis in young fruits. Overexpression of MdACS6 (MdACS6-OE) by transient assay in apple showed enhanced ethylene production, and MdACS3a was induced in MdACS6-OE fruits but not in control fruits. In MdACS6 apple fruits silenced by the virus-induced gene silencing (VIGS) system (MdACS6-AN), neither ethylene production nor MdACS3a transcript was detectable. In order to explore the mechanism through which MdACS3a was induced in MdACS6-AN fruits, we investigated the expression of apple ethylene-responsive factor (ERF) genes. The results showed that the expression of MdERF2 was induced in MdACS6-AN fruits and inhibited in MdACS6-AN fruits. Yeast one-hybrid assay showed that MdERF2 protein could bind to the promoter of MdACS3a. Moreover, down-regulation of MdERF2 in apple flesh callus led to a decrease of MdACS3a expression, demonstrating the regulation of MdERF2 on MdACS3a. The mechanism through which MdACS6 regulates the action of MdACS3a was discussed.

Keywords: ACS • Apple • Ethylene biosynthesis • Ethylene responsive factor • Fruit.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; CDS, coding sequence; CTR1, constitutive triple response 1; DAFB, days after full bloom; ERF, ethylene responsive factor; GD, Golden Delicious; 1-MCP, 1-methylcyclopropene; ORF, open reading frame; qRT–PCR, quantitative reverse transcription–PCR; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; UTR, untranslated region; VIGS, virus-induced gene silencing.

Introduction

Ethylene is the most important plant hormone controlling the ripening of climacteric fruits (Kende 1993). Ethylene biosynthesis and its signal transduction pathway have been studied extensively in many plant species including apple, a climacteric fruit (Yang and Hoffman 1984, Kende 1993, Seymour et al. 1993). In ethylene biosynthesis, ACC (1-aminocyclopropane-1-carboxylic acid) is formed from S-adenosylmethionine catalyzed by ACC synthase (ACS). EC 4.1.1.14) and converted into ethylene by ACC oxidase (ACO) (Yang and Hoffman 1984). The formation of ACC is generally considered to be the rate-limiting step in the biosynthesis of ethylene (Kende 1993). In the signal transduction pathway, ethylene is perceived by a family of receptors. The receptors physically interact with the Raf-like kinase constitutive triple response 1 (CTR1), activating a positive regulatory molecule, ethylene insensitive 2 (EIN2). A positive signal is then transferred to EIN3/EIL (EIN3 like). As transcription factors, EIN3/EIL proteins induce transcription of ethylene-responsive factor (ERF), and the expression of other ethylene-regulated genes is initiated (Alonso and Stepanova 2004, Klee and Giovannoni 2011).

The biosynthesis of ethylene is divided into two systems in higher plants (McMurche et al. 1972). System 1 is autoinhibitory and responsible for the basal level of ethylene production during normal vegetative growth, while System 2 is autostimulatory and operates for the burst of ethylene production during the ripening of climacteric fruit (Seymour et al. 1993, Lelièvre et al. 1997, Barry et al. 2000). The transition from System 1 to System 2 ethylene biosynthesis is an important step during fruit ripening and is developmentally regulated (Lelièvre et al. 1997). Ethylene biosynthesis is a complicated process that involves the co-operation of several ACS genes (Barry et al. 2000, Rodrigues et al. 2014). For example, in tomato (Solanum lycopersicum, formerly Lycopersicon esculentum), nine ACS genes have been reported: LeACS1A, LeACS1B and LeACS2–LeACS8 (Lin et al. 2009), of
which LeACS1A and LeACS6 operate in System 1 ethylene biosynthesis in green fruit, and LeACS2 operates in System 2 ethylene biosynthesis in ripening fruit, while LeACS4 works in the transition period (Nakatsuka et al. 1998, Barry et al. 2000). The differential expression of ACS genes might be an important way to adjust ethylene production in a particular developmental stage (Rodrigues et al. 2014).

Knowledge of the regulation of ACS gene expression is mainly acquired from their response to ethylene. In tomato, LeACS1A and LeACS6 are expressed in fruit before the onset of fruit ripening and inhibited by ethylene treatment, while LeACS2 is expressed in ripening fruit and strongly induced by ethylene (Nakatsuka et al. 1998, Barry et al. 2000). Moreover, the transcript of LeACS1A and LeACS6 is present and that of LeACS2 is absent in the fruit of the ripening inhibitor (rin) mutant (Barry et al. 2000). These results suggested that the initiation of LeACS1A and LeACS6 expression does not require the presence of ethylene, but that of LeACS2 does. In two plum cultivars, the expression of PsACS4 and PsACS5 is under positive and negative regulation, respectively, by ethylene in fruit (El-Sharkawy et al. 2008). Additionally, ACS genes are regulated transcriptionally by transcription factor genes such as ERF. For instance, tomato LeERF2 activates the expression of ACS genes in fruit ripening (Zhang et al. 2009). Chung et al. (2010) have reported that tomato SlAP2a, the ERF repressor, negatively regulates fruit ripening by suppressing the expression of ACS2 and ACS4. Although the regulation of ERFs in fruit ripening has been documented consistently by previous studies (El-Sharkawy et al. 2009, Zhang et al. 2009, Yin et al. 2010, Xiao et al. 2013), little information is available about the mechanism by which a particular ERF regulates its target genes during fruit development.

In apple, two ACS genes, MdACS1 and MdACS3a, have been studied intensively, and each of them is expressed in a unique pattern (Sunako et al. 1999, Wakasa et al. 2006, Wang et al. 2009, Varanasi et al. 2011). MdACS3a is expressed around 1 month before the onset of fruit ripening and its alleles are critical in determining the fruit shelf-life, while MdACS1 is expressed abundantly immediately after fruit ripening and is responsible for the burst of ethylene production in System 2 (Wang et al. 2009, Tan et al. 2013). Neither MdACS3a nor MdACS1 is expressed in the early stage of fruit development. How each ACS gene is initiated in different developmental stages remains unclear. In addition, two ERF genes (MdERF1 and MdERF2) have been reported to be involved in fruit ripening, and both of them are positively regulated by ethylene (Wang et al. 2007). More in-depth characterization of these two genes has not been conducted, and it is also unknown whether they are involved in the activation of apple ACS genes at different developmental stages.

In this study, we characterized another member of the apple ACS gene family, MdACS6. Based on our results, MdACS6 was expressed in the early stage of fruit development, which was prior to the initiation of MdACS3a and MdACS1 expression. Overexpressing and silencing of MdACS6 by transient assay revealed that MdACS6 could regulate ethylene biosynthesis through modification of the expression of MdACS3a, and the involvement of MdERF2 in this process was studied.

**Results**

**Cloning and structural analysis of MdACS6**

The coding region of MdACS6 was predicted by the apple genome database and named by Li et al. (2013). Its genomic DNA and cDNA of the coding region were PCR amplified using the primers MdACS6-full-F and MdACS6-full-R (Supplementary Table S1). The 3′-untranslated region (UTR) was obtained by 3′-RACE (rapid amplification of cDNA ends). Sequencing analysis showed that the cDNA of MdACS6 is 1,906 bp in length and includes 247 bp of 5′-UTR, 138 bp of 3′-UTR and 1,521 bp of open reading frame (ORF). The ORF encoded a predicted polypeptide containing 507 amino acid residues. The coding region of MdACS6 was compared with its genomic DNA and revealed four exons and three introns (Supplementary Fig. S1). The deduced amino acid sequence of MdACS6 shared 47% and 45% identity with MdACS3a and MdACS1, respectively.

**Expression profile of MdACS6**

The expression of MdACS6 was first examined in different tissues including roots, leaves, flowers, flower buds and fruits of Golden Delicious (GD) apple. MdACS6 was expressed not only in fruits but also in roots, leaves and flowers (Fig. 1).

We then investigated the expression of MdACS6 in different stages of GD apple fruit development and ripening. The expression of MdACS3a and MdACS1 was also investigated for comparison. MdACS6 was highly expressed at 70 and 85 DAFB (days after full bloom) when ethylene production was at a very low level and MdACS3a and MdACS1 had not been expressed (Fig. 2A, B). These results suggested that the ethylene produced in apple fruits at these two stages was mainly from the catalysis by MdACS6. The expression of MdACS6 turned out to be much lower from 105 DAFB when ethylene production slightly increased and MdACS3a started to be expressed, and this
trend was continuously maintained until 140 DAFB (H, commercial harvest day) when fruits were harvested (Fig. 2B). During the period of storage at room temperature after harvest (ripening stage), the apple fruits produced a large amount of ethylene (Fig. 2A), and the expression of *MdACS6* slightly increased and then decreased during the end of storage (Fig. 2B). Meanwhile, *MdACS3a* and *MdACS1* were highly transcribed during this period; *MdACS1* in particular showed a large amount of transcription (Fig. 2B). These results indicated that *MdACS6* only contributed a small part to ethylene production compared with *MdACS3a* and *MdACS1* during apple fruit ripening, while in the early stage of fruit development, the ethylene was mainly from the contribution of *MdACS6*.

The effect of ethylene and 1-MCP treatment on *MdACS6*

In order to understand if the expression of *MdACS6* is under the control of ethylene, we treated apple fruits with Ethephon and 1-methylcyclopropene (1-MCP). As the expression of *MdACS6* was detected from 70 to 140 DAFB and during the storage period (Fig. 2), we used two groups of fruits collected at 70 and 140 DAFB, respectively, for the treatments. Ethephon greatly induced the ethylene production and loss of firmness, and 1-MCP greatly inhibited them in both groups of treatments (Supplementary Fig. S2). In the fruits treated at 70 DAFB, Ethephon significantly increased the expression of *MdACS6* at all sampling points (Supplementary Fig. S2A), whereas it only increased *MdACS6* expression at 15 and 20 d after harvest in the fruits treated at 140 DAFB (Supplementary Fig. S2B). In both groups of fruits, 1-MCP had no significant effect on the expression of *MdACS6* (Supplementary Fig. S2A, B).

**Enzyme activity of *MdACS6***

In order to compare the enzyme activity of *MdACS6* with that of *MdACS3a* and *MdACS1*, we purified their protein and measured their enzyme activity in vitro. The result showed that the activity of *MdACS6* was significantly lower than that of *MdACS3a* and *MdACS1* (Fig. 3), suggesting that *MdACS6* has a much smaller role in the formation of ethylene compared with *MdACS3a* and *MdACS1*.

**Overexpression and silencing of *MdACS6* by *Agrobacterium* infiltration**

In order to characterize *MdACS6* further, we constructed a pRI101-MdACS6 recombinant plasmid to overexpress it and a pTRV2-MdACS6 plasmid to silence it by *Agrobacterium* infiltration in ‘Hanfu’ apple fruits which is a low ethylene producing
variety. Fruits infiltrated with empty vector and non-infiltrated fruits were used as controls. After infiltration, we examined the expression of MdACS6 in fruits at 25, 50 and 70 DAFB (10, 35 and 55 d after infiltration, respectively). The expression of MdACS6 was significantly enhanced in MdACS6-overexpressing (MdACS6-OE) fruits (Fig. 4A) and significantly declined in MdACS6-silenced (MdACS6-AN) fruits (Fig. 4B) compared with that in control fruits. We also detected the protein level of MdACS6, and the result showed that MdACS6 protein was increased in MdACS6-OE fruits and decreased in MdACS6-AN fruits (Fig. 4C). These results suggested that the transient expression system worked well in our study. Moreover, the ethylene production was significantly enhanced in MdACS6-OE fruits (Fig. 4D) and it was not detectable in MdACS6-AN fruits (data not shown).

We then investigated the expression of MdACS3a in MdACS6-OE and MdACS6-AN fruits. Surprisingly, MdACS3a was expressed in MdACS6-OE fruits at 50 and 70 DAFB but not in control fruits (Fig. 5A), or in MdACS6-AN fruits (data not shown). The protein level of MdACS3a was also enhanced in MdACS6-OE fruits (Fig. 5B). The expression of MdACS1 was not detectable in these samples (data not shown). These results suggested that MdACS6 could regulate the expression of MdACS3a during fruit development.

Expression of MdERF2 and its interaction with MdACS3a

In order to explore the mechanism through which MdACS3a was induced in MdACS6-OE fruits, we investigated the transcription of MdERF1 and MdERF2 which were previously characterized by Wang et al. (2007). MdERF1 did not show any difference in transcription between MdACS6-OE and control fruits (data not shown), while the expression of MdERF2 was increased in MdACS6-OE fruits (Fig. 5C) and decreased in MdACS6-AN fruits (Fig. 5D). We then investigated the expression profile of MdERF2 in apple fruit development and ripening. MdERF2 was expressed at very low levels at 70 and 85 DAFB and started to increase at 105 DAFB (Supplementary Fig. S3A) when the expression of MdACS3a was increased (Fig. 2B). During the storage period, the expression of MdERF2 was markedly increased (Supplementary Fig. S3A). In order to understand if MdERF2 is regulated by ethylene, we checked its expression in Ethephon- and 1-MCP-treated fruits. In fruits treated with both 70 and 140 DAFB, the expression of MdERF2 was enhanced by Ethephon and inhibited by 1-MCP (Supplementary Fig. S3B, C), indicating that MdERF2 is positively regulated by ethylene in both young and mature fruits.

Because ERF is a transcription factor gene, MdERF2 might bind to the promoter of MdACS3a and regulate its expression. Based on this speculation, we conducted a yeast one-hybrid assay. The promoter of MdACS3a was divided into six parts and assayed with MdERF2 protein. The result showed that MdERF2 was able to bind to the region containing a DRE (dehydration-responsive element) domain in the promoter of MdACS3a (Fig. 6). In order to confirm whether MdACS3a was under the regulation of MdERF2, we silenced MdERF2 by a virus-induced gene silencing (VIGS) system in apple flesh callus. The expression of MdERF2 was decreased significantly, and the expression of MdACS3a was also down-regulated (Fig. 7). This result suggested that MdERF2 directly regulates the expression of MdACS3a in apple fruit.

Discussion

Regulation of ethylene biosynthesis is very important in plant development and in response to stress (Zhang et al. 2009, Klee and Giovannoni 2011), but little is known about how ethylene biosynthesis and its synthesis genes are regulated during fruit development. Thus it is essential to characterize the ethylene biosynthesis genes and identify the elements involved in their regulation in fruit development. The formation of ACC catalyzed by ACS is the rate-limiting step in ethylene biosynthesis and its signal transduction pathway (Kende 1993). MdACS3a and MdACS1 have been studied intensively in apple fruit ripening (Sunako et al. 1999, Wang et al. 2009). In this study, we characterized another member of the apple ACS gene family, MdACS6, and highlighted its role in ethylene biosynthesis of fruits, providing new insights into regulation of ethylene biosynthesis during apple fruit development.

MdACS6 was expressed not only in vegetative tissues but also in reproductive tissues (Fig. 1). This is different from the expression of MdACS1 and MdACS3a which are specifically expressed in fruits (Sunako et al. 1999, Wang et al. 2009), suggesting that MdACS6 is also responsible for the ethylene biosynthesis in vegetative tissues in addition to fruits.

The expression of MdACS6 started from the early stage in fruit tissues of GD apple (before 85 DAFB) when MdACS3a and MdACS1 were not yet expressed (Fig. 2B), implying that the ethylene produced in the early stage of fruit is mainly due to the catalysis by MdACS6. In fruit of ‘Hanfu’ apple, the expression of MdACS6 showed the same pattern as in GD apple (Supplementary Fig. S4A, B), indicating its similar role in different apple varieties. Considering the gene structure, MdACS1...
possessed an 'RLSF' motif and a long C-terminal tail which is essential for CDPK and MPK6 phosphorylation, whereas MdACS6 and MdACS3a do not have this motif (Li et al. 2013). This might be one reason why MdACS1 is expressed specifically during fruit ripening, and MdACS6 and MdACS3 are expressed earlier. The transcription level of MdACS6 decreased in apple fruits from 105 to 140 DAFB (Fig. 2B). At these stages, MdACS3a is already expressed (Fig. 2B) and it may take the place of MdACS6 to meet the ethylene requirement of fruit. At the ripening stage (after harvest), the expression of MdACS6 returned to being slightly higher (Fig. 2B). This may be caused by the induction of ethylene because a huge amount of ethylene is evolved during ripening (Fig. 2A), and this was also confirmed by the ethylene treatment assay in which the expression of MdACS6 was promoted by Ethephon treatment (Supplementary Fig. S2). Both Nakatsu et al. (1998) and Barry et al. (2000) have found that LeACS6, a homolog of MdACS6, is inhibited by treatment with ethylene or its homologous compound in tomato fruits, and its transcript disappears during fruit ripening; whereas, in our study, MdACS6

Fig. 4 Overexpression and silencing of MdACS6 by Agrobacterium infiltration in apple fruit. Young fruit of ‘Hanfu’ apple was used to overexpress and silence MdACS6. The change in the transcription and protein level of MdACS6 was investigated and the ethylene production was measured. (A) Expression of MdACS6 by qRT–PCR after being overexpressed. MdACS6-OE, fruits infiltrated with the plasmid for overexpression of MdACS6; pRI101, fruits infiltrated with an empty pRI101 vector; non-infiltration, intact fruits. (B) Expression of MdACS6 by qRT-PCR after being silenced. MdACS6-AN, fruits infiltrated with the plasmid for silencing MdACS6; pTRV2, fruits infiltrated with an empty pTRV2 vector; non-infiltration, intact fruits. (C) Level of MdACS6 protein in MdACS6-OE and MdACS6-AN fruit by Western blot. Coomassie Brilliant Blue-stained protein was used as loading control. Brightness, the strips were quantified by Image J. (D) The ethylene production of MdACS6-OE fruits. DAFB, days after full bloom; DAI, days after infiltration. Double asterisks indicate a significant difference at $P < 0.01$ by t-test.
was expressed continuously during fruit ripening and its transcription was induced by ethylene treatment (Fig. 2B; Supplementary Fig. S2). This difference indicated that MdACS6 and LeACS6 may have the same function in the early stage of fruit development, for example being responsible for ethylene biosynthesis in young fruits, but differ in function during fruit ripening. Moreover, the promoter region of MdACS6 contains an ERE motif (Supplementary Fig. S1A), which might be the reason why it was induced by Ethephon treatment.

Ethylene biosynthesis in plants, especially in fruits, requires the co-operation of several ACS genes, and each gene is initiated at a different developmental stage (Barry et al. 2000, Rodrigues et al. 2014). In tomato, for example, LeACS6 is expressed in the early stage of fruit development and is mostly related to maintenance of basal ethylene production, while LeACS2 and LeACS4 are expressed at a later stage and are mainly associated with autocatalytic ethylene production during fruit ripening (Nakatsu et al. 1998, Barry et al. 2000, Cara and Giovannoni, 2008). In our data, the expression of MdACS6, MdACS3a and MdACS1 was initiated at different developmental stages in fruit (Fig. 2B). Additionally, their enzyme activity differed significantly (Fig. 3). These results indicated that fruit requires different ACS genes to form ethylene at distinct developmental stages. Among these genes, MdACS3a plays important roles in determining the shelf-life of apple fruits (Wang et al. 2009). It will be interesting to determine how its expression is initiated. In this study, we overexpressed MdACS6 by Agrobacterium infiltration in ‘Hanfu’ apple fruit. The ethylene production was significantly increased in MdACS6-OE fruits compared with control fruits (Fig. 4D). Surprisingly, MdACS3a was expressed in MdACS6-OE fruits but not in control fruits (Fig. 5A). This result suggested that MdACS6 can regulate the expression of MdACS3a. More interestingly, the expression of MdERF2 was also increased in MdACS6-OE fruits (Fig. 5C) and decreased in MdACS6-AN fruits (Fig. 5D).
for the activation of AtACS2 and MdACS3a activated the expression of pathogen-induced ethylene production in Arabidopsis. On the other hand, the expression of MdACS6 is naturally up-regulated by fruit ripening in both GD and ‘Hanfu’ apple (Supplementary Figs. S3A, S4C) and induced by ethylene (Supplementary Fig. S3B, C). Based on the above results, we can explain the initiation of MdACS3a expression in apple fruits: expression of MdACS6 led to accumulation of ethylene production in fruits; when the ethylene was accumulated to a certain level, the expression of MdERF2 was induced and it activated the expression of MdACS3a.

These findings led us to consider whether MdERF2 was involved in the initiation of MdACS3a expression in apple fruits since ERF is the last transcription factor gene in ethylene signal transduction and controls the transcription of ethylene-responsive genes (El-Sharkawy et al. 2009, Klee and Giovannoni 2011). Yeast one-hybrid assay showed that MdERF2 protein could bind to the DRE domain of the MdACS3a promoter (Fig. 6) in our study. Moreover, when MdERF2 was silenced in apple flesh callus, the expression of MdACS3a was also decreased (Fig. 7), suggesting that MdERF2 could directly regulate the expression of MdACS3a as a transcription factor. Although Xiao et al. (2013) have reported that a banana MaERF9 is able to bind to the promoter of MaACS1 to regulate its promoter activity, the authors failed to provide direct evidence of whether MaERF9 can regulate the expression of MaACS1 in banana fruit. In this study, we performed an in vivo experiment by silencing MdERF2 in apple flesh callus, and demonstrated that MdERF2 can directly regulate the expression of MdACS3a, providing deeper insights into the role of the ERF transcription factor gene in regulating ethylene biosynthesis during fruit development. On the other hand, the expression of MdERF2 was naturally up-regulated by fruit ripening in both GD and ‘Hanfu’ apple (Supplementary Figs. S3A, S4C) and induced by ethylene (Supplementary Fig. S3B, C). Based on the above results, we can explain the initiation of MdACS3a expression in apple fruits: expression of MdACS6 led to accumulation of ethylene production in fruits; when the ethylene was accumulated to a certain level, the expression of MdERF2 was induced and it activated the expression of MdACS3a.

Fig. 6 Interaction of MdERF2 and the promoter of MdACS3a by yeast one-hybrid assay. The transformed yeast cells were grown on SD/-Leu media with (+) or without (−) adding AbA. The growth of yeast cells on SD/-Leu media with addition of AbA indicates the interaction between the MdERF2 protein and the fragments of the MdACS3a promoter.

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Li et al. (2012) have reported that AtWRKY33 is responsible for the activation of AtACS2 and AtACS6 which are involved in pathogen-induced ethylene production in Arabidopsis. On the other hand, MdMADS9 acts as a transcriptional activator of MdACS1 in apple fruit ripening (Ireland et al. 2013). Therefore, the mechanism of activation of ACS genes differs in different species and in different developmental stages of the same species. Different transcription factors may have overlapping roles in this process (Rodrigues et al. 2014). In this sense, it would be attractive to screen a wider range of the important transcription factors and study their roles and possible cross-talk mechanism in activation of ACS genes.

In conclusion, ethylene in young apple fruits is mainly produced by catalysis by MdACS6. MdACS6 can regulate ethylene biosynthesis during fruit development and the ERF MdERF2 is involved in this process.

Materials and Methods

Plant materials and treatments

Young leaves, flowers, flower buds and fruits were collected from mature GD apples (Malus ×domestica) trees growing in an orchard at the experimental farm of Liaoning Pomology Institute (Xiongyue, China). Roots were collected from tissue-cultured GD apple. The fruits of GD were collected at 70, 85, 105, 125 and 140 DAFB during development. GD fruits collected at 140 DAFB (commercial maturity, September 26, 2012) were kept at room temperature (24°C) for 20 d and sampled every 5 d. In addition, four other groups of GD fruits collected at 70 and 140 DAFB were subjected to 1-MCP (an ethylene inhibitor) and Ethephon (an ethylene producer) treatments according to Tan et al. (2013).

After treatment, the fruits were held at room temperature (24°C) for 20 d and sampled every 5 d for RNA extraction. At each of the above sampling points, five fruits were sampled for measurements of ethylene production and flesh firmness, and then fruits were sliced, frozen quickly in liquid N2 and stored at –80°C for RNA extraction.

The fruits from mature ‘Hanfu’ apple (‘Dongguan’×’Fuji’) trees were used for overexpressing or silencing MdACS6 in this study because it is a low ethylene producing variety and easy to reach in our experimental farm. ‘Hanfu’ apple trees were growing in the experimental farm of Shenyang Agricultural University (Shenyang, China). It is difficult to perform infiltration assays in fruits when MdACS6a is expressed due to the high firmness of fruits; we used apple flesh callus to silence MdERF2 due to the ease with which it can be infected by Agrobacterium. The callus was made from GD fruit according to Alayón-Luaces et al. (2008) and maintained in our lab.

Measurements of flesh firmness and ethylene production rates

Flesh firmness was measured with a portable pressure tester (FT-327; Facchina) fitted with an 11 mm diameter probe. Four skin discs (approximately 2.5 cm in diameter) were removed from opposite sides of each fruit. The probe was pressed into the tissue of the cut surface to a depth of 8–9 mm in a single smooth motion. Five fruits per sample were measured.

For ethylene production measurement, intact fruits were enclosed in a gas-tight container (0.86 liter, 24°C) equipped with septa, and 1 ml of headspace gas was sampled by means of a syringe. The ethylene concentration was measured with a gas chromatograph (Agilent 7890A) equipped with a flame ionization detector according to Tan et al. (2013). Five fruits per sample were measured.

Cloning of MdACS6

Information from the coding region of MdACS6 (MDP0000133334) was obtained from the apple genome database in our previous report (Li et al. 2013). The full genomic DNA and cDNA sequences were obtained by PCR amplification from GD genomic DNA and cDNA, respectively, using primers MdACS6-full-F and MdACS6-full-R (Supplementary Table S1). The PCR products were cloned into pGM-T vector and sequenced. The 3′-UTR was obtained by 3′-RACE. The outer and inner primers for 3′-RACE were MdACS6-GSP-1F and MdACS6-GSP-2F, respectively (Supplementary Table S1).
Materials and Methods, and the expression of MdACS3a was investigated by semi-qRT–PCR and qRT–PCR. (A) Expression of MdACS3a and MdERF2 by semi-qRT–PCR. Control, intact fruits; pTRV2, fruits infiltrated with an empty pTRV2 vector; MdERF2-AN, fruits infiltrated with the plasmid for silencing MdERF2. (B) Expression of MdACS3a and MdERF2 by qRT–PCR.

Quantitative reverse transcription–PCR

Total RNAs were extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Gasic et al. 2004). A 1 μg aliquot of total RNAs was used to synthesize first-strand cDNA using a PrimeScript First Strand cDNA Synthesis Kit (TAKARA).

qRT–PCR was conducted as described in Tan et al. (2013). Specific primers for each ACS gene were designed by Primer3 (http://frodo.wi.mit.edu/) and are listed in Supplementary Table S1. The apple Actin gene (EB136338) was used as an internal control. Three replications were conducted.

Measurement of ACS activity

The coding regions of MdACS1 (AB91156), MdACS3a (AB243060) and MdACS6 were each ligated into the pGEX-4T-1 vector and transformed into Escherichia coli strain BL21 (DE3) to express their protein. The protein was purified according to Yuan et al. (2014). The purified MdACS1, MdACS3a and MdACS6 proteins were used to measure their activity according to Wang et al. (2009).

Agrobacterium infiltration assay

In order to overexpress MdACS6, its coding sequence (CDS) was PCR amplified from cDNA of GD apple fruits using primers pRI101-MdACS6-F and pRI101-MdACS6-R with EcoRI and NdeI sites attached (Supplementary Table S1). The PCR product was cloned into the plant transformation vector pRI101 downstream of the Cauliflower mosaic virus (CaMV) 35S promoter. To silence MdACS6 or MdERF2 by the VIGS system, the partial CDSs of MdACS6 or MdERF2 (AB288348) were ligated into the pTRV2 vector using primers pTRV2-MdACS6-F/R or pTRV2-MdERF2-F/R, respectively (Supplementary Table S1) and confirmed by sequencing.

The resulting recombinant plasmids were transformed into Agrobacterium strain EHA105. One single separated colony containing the target gene was inoculated to 10 ml of YEP medium containing 50 mg ml−1 kanamycin and 50 mg ml−1 rifampicin and grown at 28°C overnight with shaking at 160 r.p.m. Then, 200 μl of the overnight culture was added to 50 ml of fresh YEP containing 50 mg ml−1 kanamycin, 50 mg ml−1 rifampicin and 20 μM acetoxyringone, mixed thoroughly and centrifuged at 10,000 g for 1 min at 24°C. The supernatant was discarded. The pellet was then resuspended in 10 ml of infiltration buffer containing 10 mM MgCl2, 10 mM MES (pH 5.6) and 100 μM acetoxyringone, and the OD600 was adjusted to 1.0. The suspension was left on the bench for 3 h at room temperature and immediately used for infiltration. To overexpress or silence MdACS6, 100 μl of the suspension was injected at a depth of approximately 0.5 cm into young fruits of ‘Hanfu’ apple at 15 DAFB (the fruits at this stage are not as hard as at later stages and thus it is easy to perform the infiltration assay). Each fruit was used as one biological replicate and 10 fruits were infiltrated for each construct. At each sampling point, five fruits were collected for ethylene measurement and RNA extraction. The silencing of MdACS6 was conducted according to Wang et al. (2013).

In order to silence MdERF2, the callus was placed into the prepared suspension containing pTRV2-MdERF2 for 20 min. The infected callus was cultured in the dark at 24°C for 4 d, and then used for RNA extraction.

Yeast one-hybrid assay

Yeast one-hybrid assay was performed by using the ‘Matchmaker™ Gold Yeast One-Hybrid Library Screening System’ (Clontech, 630491) according to the manufacturer’s instructions. The CDS of MdERF2 was ligated into the pGADT7 vector. Each fragment of the MdACS3a promoter was ligated into the pAbAi vector. The vectors containing the MdERF2 CDS and a fragment of the MdACS3a promoter were co-transformed into yeast strain Y1HGold and the transformed cells were grown on SD/-Leu with or without the addition of ABA (aureobasidin A) which is a growth inhibitor of yeast cells.

Protein extraction from fruit and Western blot

Protein was extracted from ground tissue of fruit by using Plant protein extraction reagent (Cat#CW08858, Beijing ComWin Biotech). The protein extracts were separated in SDS–polyacrylamide gels and transferred to a nitrocellulose membrane using standard procedures. The purified protein of MdACS6 and MdACS3a was sent to Beijing ComWin Biotech for injection into rabbits to raise antibodies. Western blot analysis was performed using the rabbit polyclonal antibodies at a 1:1,000 dilution for MdACS6, a 1:3,000 dilution for MdACS3a and 1:10,000 anti-rabbit horseradish peroxidase secondary antibodies (Beijing ComWin Biotech). Coomassie Brilliant Blue-stained protein was used as loading control. The strips were quantified by Image J (version 3.0).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


