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Transcriptional Profiles Underlying the Effects of Methyl Jasmonate on Apple Ripening

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Abstract Methyl jasmonate (MeJA) has significant effects on the apple ripening process, but little is known about the molecular mechanisms by which MeJA acts in the regulation of this complicated process. To address this question, transcriptome profiles of MeJA-treated apples and mock-treated controls were generated using RNA-sequencing technology and then compared. A total of 1092 transcripts changed significantly in response to MeJA, with 684 up-regulated and 408 down-regulated. In MeJA-treated apples, genes involved in anthocyanin biosynthesis and transport were commonly up-regulated. The up-regulated genes also included genes in the biosynthetic and signal transduction pathways for the hormones jasmonic acid and ethylene. In contrast, exogenous MeJA generally down-regulated genes associated with auxin signal transduction. Furthermore, the transcript levels of 10 jasmonic acid biosynthetic genes, 7 selected ethylene biosynthetic and signal transduction genes, 5 selected auxin signal transduction genes, and 8 selected anthocyanin biosynthetic and transport genes were confirmed via real-time qPCR. These results suggest that MeJA promotes apple ripening, likely by activating ethylene signaling and inhibiting auxin action. Because MeJA-treated apples have previously been documented to have high anthocyanin concentrations, the molecular mechanism underlying this phenomenon is also discussed.

Keywords MeJA · Apple · RNA-Seq · Fruit ripening

Introduction

Fruits are an important part of the human diet, providing vitamins, minerals, and 'bioactive' compounds (Miller and Rice-Evans 1997; Heim and others 2002; Briones-Labarca and others 2011). There is good evidence that fruits promote healthy aging by protecting against heart disease and cancer (Hertog and others 1993; Boyer and Liu 2004).

Ripening is the final stage of fruit development and is unique to plant species. Ripening is a complex developmental process involving a number of dramatic changes in color, texture, flavor, and aroma (Carrari and Fernie 2006). In general, fruits can be divided into two types based on ripening pattern: climacteric fruit and non-climacteric fruit (Yang and Hoffman 1984). Apples are economically important crop that are classified as climacteric fruits, in which ethylene is the main ripening trigger (Lay-Yee and others 1990; Schaffer and others 2007). In addition to ethylene, other hormones have recently been found to influence the apple ripening process (Romani and others 1989; Fan and others 1997; Kondo and others 2005; Li and others 2006). Amongst these hormones, jasmonic acid (JA) seems to play a major role.

The JA biosynthesis pathway has been well characterized. JAs are synthesized from α-linolenic acid in the octadecanoid pathway, and three key enzymes are involved in JA biosynthesis: lipoxygenase (LOX), allene oxide

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synthase (AOS), and 12-oxophytodienoate reductase 3 (OPR3) (Turner and others 2002). JA and its methyl ester (MeJA) are both involved in mediating fruit ripening (Concha and others 2013). For example, the levels of JA and the AOS gene in apples increase during ripening, suggesting their potential roles in the regulation of this process (Fan and others 1997; Lv and others 2015). Pre-climacteric applications of MeJA stimulate the fruit ripening process in apple; for example, MeJA stimulates ethylene and ester biosynthesis (Fan and others 1997; Kondo and others 2005), increases anthocyanin content as well as the accumulation of several phenolic compounds, and promotes red color (Pérez and others 1993; Fan and others 1998; Rudell and Mattheis 2008).

The physiological effects of JAs in the apple ripening process have been widely studied, but the precise mechanism by which JA contributes to this process remains elusive. In this study, high-throughput RNA-Seq was utilized to analyze the changes of transcription in apple with the pre-climacteric application of MeJA. This study may provide important information regarding the function of MeJA in apple ripening and may aid in the identification of genes involved in the MeJA-activated apple ripening process.

Materials and Methods

Plant Material

The fruits were collected from 'Taishan Zaoxia' plants in a commercial orchard, Liaocheng, Shandong, China, in June 2014, 6 days before ripening. The fruits were treated with 10^{-4}, 10^{-3}, and 10^{-2} M MeJA, and were then transferred to plant growth chambers at 25 °C and with 10/14 h light/dark cycle. The anthocyanin content was monitored daily for 6 days. Because 10^{-3} M MeJA concentration has beneficial effects on fruit ripening by accelerating anthocyanin synthesis (Fig. 1), this concentration was chosen for further RNA-Seq and qPCR studies. Ten fruits per time point were sampled at 2, 6, and 10 h after treatment. The peels of the fruits from each sampling time were frozen in liquid nitrogen and stored at −80 °C until use.

Anthocyanin Content Analysis

Anthocyanin content was measured using a previously described method (Wang and others 2004; Feng and others 2010). Anthocyanin was extracted with an HCl–methanol method, and was then monitored at 553 and 600 nm. Determinations were performed in triplicate. Statistical analyses were carried out using SAS software.

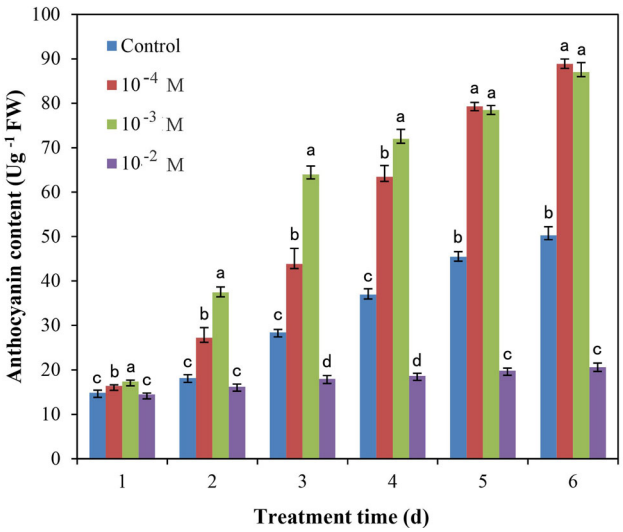

Fig. 1 Anthocyanin contents in MeJA-treated apples and mock-treated apples. The anthocyanin contents in apples after MeJA treatment for 1–6 days were compared. Data are the mean ± SE of three replicates. *a*, *b*, *c*, and *d* indicate statistical significance among four-treated groups at $P < 0.05$

RNA Isolation

Total RNA was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's recommendations. The integrity of RNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with an A260/280 ratio between 1.8 and 2.0, an A260/230 ratio greater than 1.7, and an RNA integrity number (RIN) above 8.0 were defined to be high-quality RNAs. High-quality total RNAs for each sampling time were equally mixed to construct two RNA-Seq libraries (MeJA-treated apple and mock-treated control) for sequencing.

cDNA Library Construction and Illumina Deep Sequencing

mRNA was purified from total RNA using oligo (dT) magnetic beads and then broken into short fragments in the fragmentation buffer. The mRNA fragments were used as templates to synthesize the first-strand cDNA. Then, the second strands were synthesized by adding cDNA, dNTPs, RNase H, buffer, and DNA polymerase I. The double-stranded cDNAs were purified using the Qiaquick PCR extraction kit (Qiagen, Hilden, Germany) and washed with EB buffer for end repair, poly(A) addition, and PCR amplification. Each cDNA library was sequenced using the Illumina sequencing system (HiSeq™ 2000, Illumina, San Diego, CA, USA).

Bioinformatic Analysis

Quality Control

Raw reads were initially processed using the NGS QC Toolkit (Patel and Jain 2012). In this step, low-quality reads and reads containing adapters and poly-N were removed from the raw data. All of the subsequent analyses were based on clean, high-quality data.

Mapping

Sequencing reads were mapped to the apple genome (http://genomics.research.iasma.it/) using bowtie2 (Langmead and Salzberg 2012). The FPKM and count value were calculated using eXpress (Mortazavi and others 2008).

Analysis of Differential Expression and of GO and KEGG Enrichment

Differential expression analysis was performed using the DESeq (2012) R package. $P < 0.05$ was set as the threshold for significant differential expression, and |log 2 fold change| ≥ 1 was used to identify the genes differentially expressed between the two libraries (MeJA-treated apple and mock-treated control). Gene Ontology (GO) and KEGG (Kyoto encyclopedia of Genes and Genomes) enrichment analyses of the DEGs were performed using R based on hypergeometric distribution.

qRT-PCR Analysis

qRT-PCR for validating RNA-Seq data was performed using the same RNA mixtures as for the RNA-Seq library construction. qRT-PCR for testing the expression patterns of phytohormone- and anthocyanin-related genes at different time points (2, 6, 10 h) was performed using the RNA for each sampling time. First-strand cDNAs were synthesized using the RevertAid^{TM} First-Strand cDNA Synthesis kit (Fermentas, USA) and used as templates for qPCR assays. qRT-PCR was conducted as described previously (Feng and others 2010). MdActin (XM_008384595.1) was used as a reference gene. The primers used in qPCR assays are listed in Supplementary Table 1. Three replications were conducted.

Results

Effect of MeJA on Anthocyanin Accumulation in Apple Skin

We observed a dose-dependent effect of MeJA on anthocyanin accumulation in apple skin (Fig. 1). Treatment with MeJA at concentrations of 10^{-4} and 10^{-3} M generally promoted anthocyanin accumulation. The rate of anthocyanin accumulation increased as the MeJA concentration increased from 10^{-4} to 10^{-3} M during the first 4 days after treatment; however, MeJA concentration did not affect the final anthocyanin content at ripening. MeJA treatment at 10^{-2} M strongly inhibited anthocyanin production, which is consistent with See and others (2011), who reported that a higher concentration of MeJA caused a reduction in anthocyanin production and accumulation.

Transcriptome Sequencing and Sequence Alignment

A total of 8 Gb raw data were obtained in this study. There were 37,202,822 raw reads in the MeJA-treated apple library; the Q30 percentage (percentage of bases whose quality was greater than 30 in clean reads), valid ratio (base), and GC percentage are 87.11, 99.12, and 46.00 %, respectively. There were 48,438,800 raw reads in the mock-treated apple library; the Q30 percentage, valid ratio, and GC percentage are 87.56, 99.11, and 46.00 %, respectively. After removing the low-quality reads and reads with unknown nucleotides, 36,923,726 and 48,070,300 clean reads were obtained from the MeJA-treated apple library and the mock-treated apple library, respectively. Then, 86.43 % of the MeJA-treated apple reads and 86.42 % of the mock-treated apple reads were mapped to the reference apple genome sequence (Table 1).

Alterations in the Gene Expression Profiles Between MeJA-Treated Apple and Mock-Treated Control

After discarding the genes with small expression changes (absolute log 2 of the fold change <1), a total of 1092 differentially expressed genes (DEGs) in response to MeJA were obtained (Supplementary Table 2). The log 2 of the fold change of these DEGs ranged from −8.1 to 7.8. As shown in Fig. 2, 684 DEGs showed up-regulation, more than 86 % of the up-regulated genes showed changes in the range of 2–16-fold, and only a small proportion of the DEGs were up-regulated at least 16-fold. Compared with the up-regulated DEGs, a higher percentage of the down-regulated genes showed changes of at least 16-fold (17.6 % of the 408 down-regulated DEGs).

Annotation and Functional Classification of DEGs

To gain insights into the biological functions of the DEGs, we performed GO analyses by comparing each DEG with the GO database. A total of 1092 DEGs were annotated in 183 GO biological processes (Supplementary Table 3). There were 121 GO terms enriched among the up-regulated DEGs (Supplementary Table 4), and 130 GO terms were

Table 1 RNA-Seq data in the MeJA-treated and mock-treated apple libraries

Sample	Raw reads	Clean reads	Q30 (%)	GC content (%)	Percentage of mapped reads (%)
MeJA-treated	37,202,822	36,923,726	87.11	46.00	86.43
Mock-treated	48,438,800	48,070,300	87.56	46.00	86.42

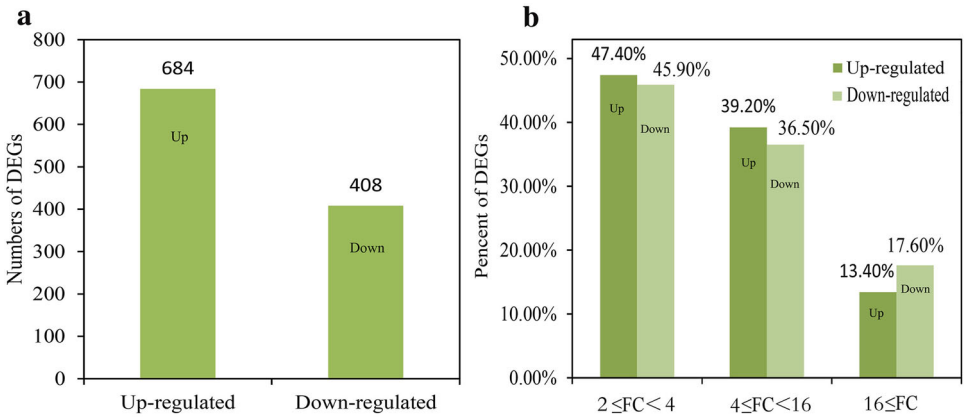

Fig. 2 Analysis of the DEGs in the apple response to MeJA treatment. **a** Numbers of the up- and down-regulated DEGs. **b** Percentages of the up- and down-regulated DEGs in the ranges of $2 \leq FC < 4$, $4 \leq FC < 16$, $16 \leq FC$. FC indicates fold change

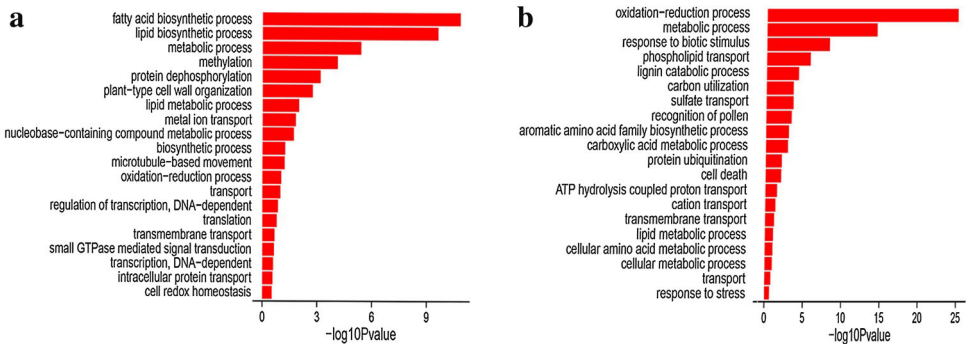

Fig. 3 The most enriched GO biological process terms analysis of the up- and down-regulated DEGs in the apple in response to MeJA treatment. **a** Top twenty GO biological process terms for the up-regulated DEGs in apples in response to MeJA treatment. **b** Top twenty GO biological process terms for the down-regulated DEGs in apples in response to MeJA treatment

enriched among the down-regulated DEGs (Supplementary Table 5). For the up-regulated DEGs, fatty acid biosynthetic process, lipid biosynthetic process, metabolic process, methylation, and protein dephosphorylation were the most enriched GO biological process terms (Fig. 3a). The most enriched GO biological process terms among the down-regulated DEGs were oxidation–reduction process, metabolic process, response to biotic stimulus, phospholipid transport, and lignin catabolic process (Fig. 3b).

To further understand the biological functions of the DEGs, we performed KEGG pathway enrichment analysis. In total, 72 and 87 regulated pathways were enriched among the down-regulated and up-regulated DEGs, respectively (Supplementary Tables 6, 7). The 20 KEGG pathways showing the strongest enrichment among the up-regulated and down-regulated DEGs are presented in Fig. 4a, b, respectively.

Impact of MeJA on Phytohormone Pathways

The enriched DEGs associated with phytohormone pathways were mainly involved in the biosynthesis and signal transduction of jasmonic acid, ethylene, and auxin. As shown in Supplementary Table 8, there are fifteen DEGs associated with JA biosynthesis and signal transduction. Among them, thirteen well-known genes in the JA biosynthesis pathway, including OPR2 (2 members), putative OPR11 (7 members), AOS (2 members), one allene oxide cyclase 4 (AOC4), one jasmonate *O*-methyltransferase (JMT), and two JA-receptor proteins (protein

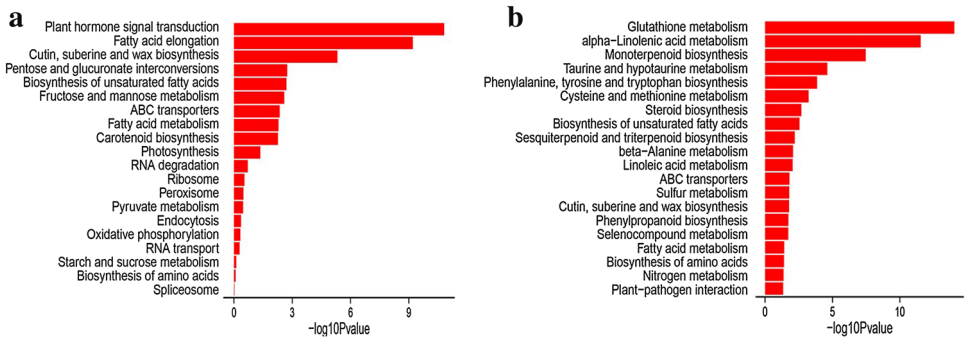

Fig. 4 The most enriched KEGG pathway analysis of the up- and down-regulated DEGs in apples in response to MeJA treatment. **a** Top twenty enriched KEGG pathways of the up-regulated DEGs in apples in response to MeJA treatment. **b** Top twenty enriched KEGG pathways of the down-regulated DEGs in apples in response to MeJA treatment

TIFY 5A and protein TIFY 10B), were up-regulated in response to exogenous MeJA.

In ethylene biosynthesis and signal transduction, there are thirteen up-regulated DEGs and six down-regulated DEGs (Supplementary Table 9). The up-regulated DEGs include six ethylene biosynthetic genes (four 1-aminocyclopropane-1-carboxylate oxidase ACO genes, and two 1-aminocyclopropane-1-carboxylate synthase (ACS) genes) and seven ethylene signal transduction genes (one ethylene-responsive transcription factor 1B gene (ERF1), two ethylene-responsive transcription factor ERF073 genes (ERF073), one ethylene-responsive transcription factor ERF011 gene (ERF011), one ethylene-responsive transcription factor ERF071 gene (ERF071), and two ethylene-responsive transcription factor ABR1 genes (ABR1)). In contrast, ethylene signal transduction elements such as ethylene-insensitive protein 2 like (EIN2), ethylene-insensitive protein 3 like (EIN3), the ethylene-responsive transcription factor RAP2-13 like (RAP2-13), and the ethylene-responsive transcription factor ERF003 like (ERF003), as well as the AP2-like ethylene-responsive transcription factor At1g16060, were down-regulated by MeJA.

The DEGs involved in the auxin signal transduction pathway are listed in Supplementary Table 10. Among these DEGs, 18 genes were down-regulated. The down-regulated DEGs mainly function as auxin-induced proteins, auxin-responsive proteins, auxin receptors, and IAA-conjugating enzymes. The two up-regulated DEGs were ABC transporter B family member 15-like (XM_008370208.1) and IAA-amino acid hydrolase ILR1-like 4 (XM_008391362.1), which are involved in auxin transport and IAA amidohydrolysis, respectively.

Effects of MeJA on Anthocyanin Biosynthesis and Transport

Seventeen DEGs for anthocyanin biosynthesis were identified in our RNA-Seq dataset, and all of them showed up-regulated expression in response to MeJA (Supplementary Table 11). These up-regulated DEGs included key anthocyanin biosynthetic enzymes: flavonol synthase (FLS), anthocyanidin 3-*O*-glucosyltransferase 2 (UFGT2), putative UDP-glucose flavonoid 3-*O*-glucosyltransferase 3 (UFGT3), and UDP-glucose flavonoid 3-*O*-glucosyltransferase 7 (UFGT7). In addition, DEGs involved in anthocyanin transport were also found in the dataset, including nine glutathione *S*-transferase (GST), two glutathione transferase GST23-like genes, and four ABC transporter C family (ABCC) members. The expression levels of genes encoding anthocyanin transporters were all up-regulated by MeJA.

qRT-PCR Analysis

The expression patterns of a subset of the phytohormone- and anthocyanin-related genes were detected by qPCR. We determined that the expression levels changed significantly in MeJA-treated apple after 2, 6, and 10 h of treatment. Especially during the first 6 h, most genes were strongly up- or down-regulated by MeJA treatment. However, the expression levels of the majority of genes were not changed (fold change <2) in non-treated controls (Fig. 5; Supplementary Table 12). These data suggest that MeJA is an important factor in gene regulation. All the tested genes were then used to validate and verify the RNA-Seq data (Fig. 6). We observed that the qRT-PCR results showed trends similar to those for the RNA-Seq data, suggesting that the RNA-Seq data are reliable.

Discussion

RNA-Seq is a high-throughput method that has been widely utilized to reveal information on the transcript profiles of model organisms, especially for discovering and identifying genes involved in the biosynthesis of various secondary metabolites and the formation of special architecture (Zhang and others 2015). In this work, we studied

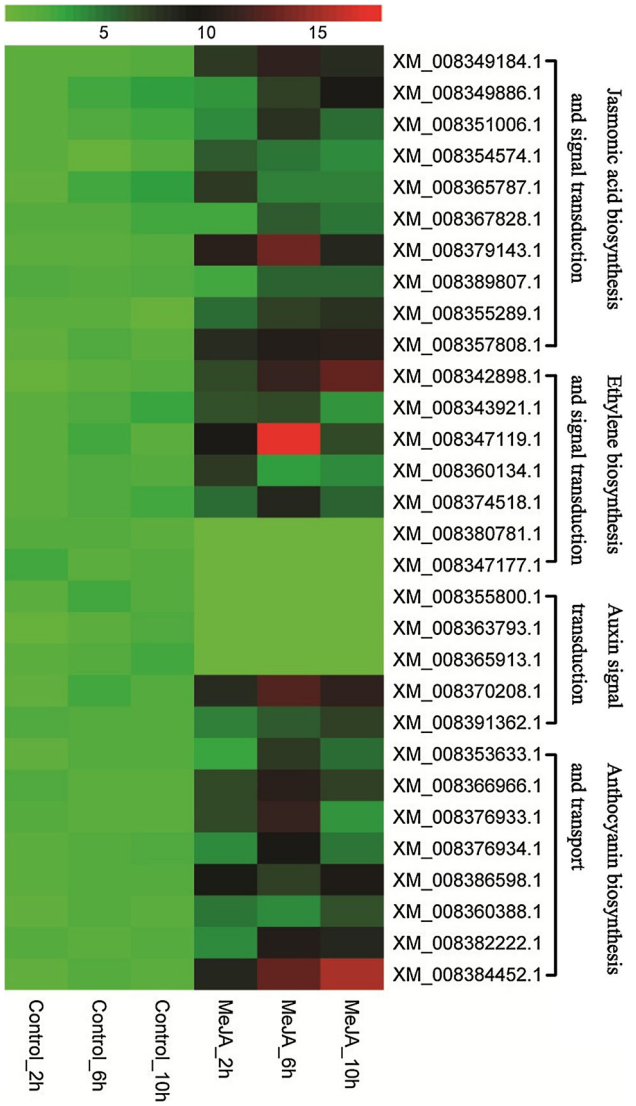

Fig. 5 qPCR analysis of a subset of phytohormone- and anthocyanin-related genes at 2, 6, 10 h after MeJA treatment. Heat map showing changes in the expression levels of phytohormone- and anthocyanin-related genes in controls and MeJA-treated samples at 2, 6, and 10 h compared to 0 h after MeJA treatment. Genes in *red* and *green* represent highly and lowly expressed genes, respectively. The qPCR data are provided in Supplementary Table 12

the transcript information of apple in response to MeJA treatment at the early stage using RNA-Seq technology. Test tissues of MeJA-treated apple and a mock-treated control at various time points were mixed into MeJA and control libraries, respectively. Finally, a total of 1092 DEGs were detected and annotated. The changes in relative expression of DEGs were further confirmed by qPCR analysis using a subset of regulated genes, indicating that the RNA-Seq data are high in quality.

Previous studies suggested that phytohormones play important roles in the apple ripening process. Among these phytohormones, ethylene is known to function as a pivotal regulation factor for fruit ripening (Giovannoni 2001; Liu and others 2016). However, the complex phenotypes related to ripening cannot be explained only by ethylene-mediated effects. Other phytohormones including JA and auxin have recently been implicated in controlling this biological process (Ohmiya 2000; Trainotti and others 2007; Li and others 2006; Schaffer and others 2013). Usually, these phytohormones function as a complex network rather than as independent linear pathways (Lv and others 2015). In this study, 55 out of 1092 DEGs were annotated for jasmonic acid, ethylene and auxin biosynthesis, and signal transduction pathways, suggesting that the promotion of fruit ripening by MeJA may be mediated directly or indirectly via regulating the transcripts of these phytohormone-related genes.

MeJA treatment of apple produced significant changes in JA-related gene expression. The JA biosynthetic genes AOC, AOS, and OPR, as well as the MeJA biosynthetic gene JMT (Turner and others 2002) were all strongly activated by MeJA treatment. This is consistent with results in *Arabidopsis* (Kubigsteltig and others 1999) and tomato (Wasternack 2014), in which JA biosynthesis is transcriptionally regulated by a JA-mediated positive feedback loop. According to a previous study, the levels of JAs and JA biosynthetic genes were dramatically up-regulated prior to apple ripening (Lv and others 2015), and the application of exogenous MeJA may have initiated this biological process. In addition, the JA signal receptor gene JAZ is also up-regulated in response to JA treatment. Similarly, JA induction of the expression of JAZ genes has also been observed in *Arabidopsis* (Yan and others 2007), poplar (Major and Constabel 2006), and tomato (Chung and others 2008), indicating that this phenomenon is conserved among plants. Because the JAZ proteins are known as repressors of JA signaling, the rapid synthesis of new JAZ proteins was suggested to attenuate the transcriptional response soon after it is initiated.

It has previously been shown that transient increases in endogenous JA concentrations occurred prior to the stage of apple ripening during which ethylene production increases rapidly. The increase in endogenous JAs was suggested to be responsible for the ethylene climacteric peak, which is a hallmark of fruit ripening (Fan and others 1998). In this study, MeJA strongly up-regulated two ethylene biosynthetic genes, ACO1 and ACS1, which have been reported to increase before and during ripening (Tan and others 2013; Bulens and others 2014). The rise of transcripts required for the ethylene climacteric likely accelerates the progression of apple ripening. A similar ethylene response to JAs has also been reported in pear and tomato, in which the expression of ACS and ACO was enhanced in pre-climacteric fruit under treatment with

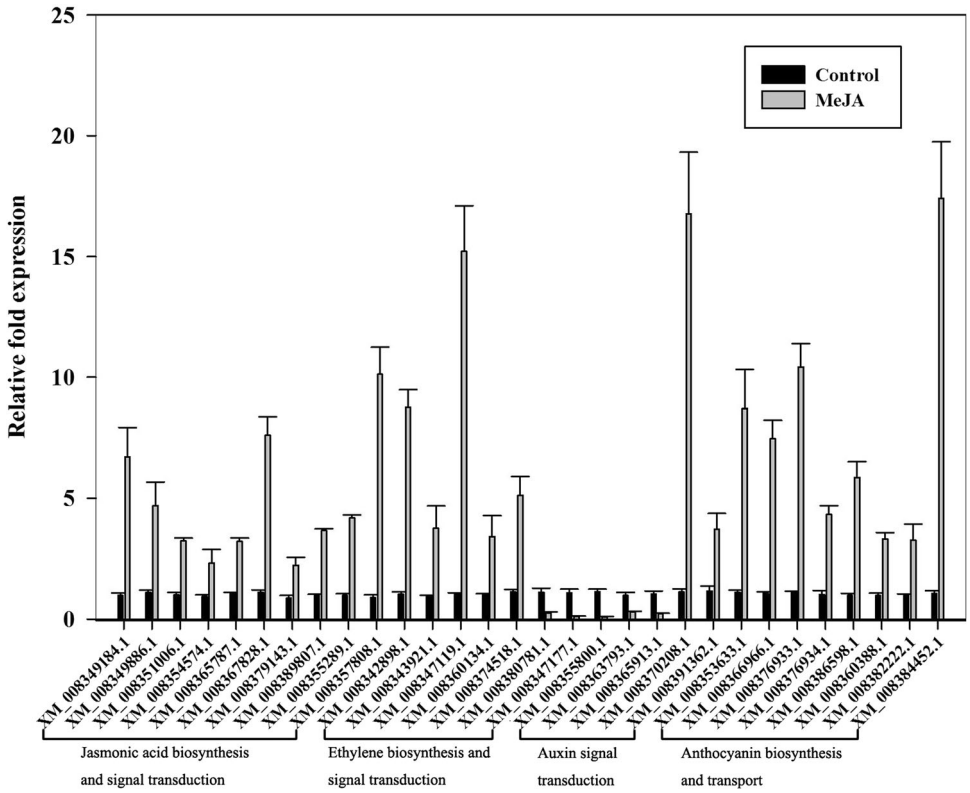

Fig. 6 qPCR validation of a subset of phytohormone- and anthocyanin-regulated genes. *MdActin* was used as an internal control to normalize gene expression. Data are the mean ± SE of three replicates

propyl dihydrojasmonate (PDJ) or MeJA (Yu and others 2009). These results are in agreement with the observations that suggest an acceleration of fruit ripening by JAs (Kondo and others 2009; Mukkun and Singh 2009; Yu and others 2011). In addition, MeJA treatment also up-regulated the expression of ethylene signal transduction genes, such as ERF1, ERF011, ERF071, and ERF073. Accumulating evidence suggests that ERF1 is an upstream component in both the JA and ET signaling pathways and is involved in pathogen resistance and the responses to salt and drought stress (Lorenzo and others 2003). The induction of ERF1in our study suggested that ERF1 may accelerate the fruit ripening process via ripening-specific gene regulation that integrates JA and ET signals. Unlike ERF1, EIN3 was down-regulated in MeJA-treated apple, which is consistent with the reports that EIN3 transcription is repressed by JAZ (Zhu and others 2011).

The effects of auxin on fruit ripening have been reported in several species, including peach (Ohmiya 2000), tomato (Buta and Spaulding 1994; Su and others 2015), grape (Böttcher and others 2010), and strawberry (Symons and others 2012). In peaches, increased levels of IAA are required for fruit ripening (Trainotti and others 2007). MeJA treatment strongly inhibits auxin-related genes and delays fruit ripening (Ziosi and others 2008; Soto and others 2012). GH3 inactivates IAA via conjugation, and it is considered a marker of free auxin (Staswick and others 2005). In peaches, MeJA treatment led to a decrease of GH3 gene expression. This behavior would explain the lower levels of free IAA in MeJA-treated fruits. In addition, the auxin receptor TIR1 (Dharmasiri and others 2005) was also down-regulated. This result reflected the changes of auxin reception and transport response to MeJA treatment, which further support the notion that auxin availability was low in treated peaches. The down-regulation of GH3 and TIR1 by MeJA is in accord with the ripening delay. In the present study, a similar auxin response to JAs was also found in apples. GH3, TIR1, and other auxin-responsive genes were down-regulated by MeJA, which suggest a possible decrease in free auxin availability. Unlike in peaches, decreases in IAA prior to fruit ripening have been reported in apples (Schaffer and others 2013), which is also consistent with the results in tomatoes and strawberries (Buta and Spaulding 1994; Srivastava and Handa 2005). Therefore, we propose that the positive role of MeJA in promoting apple ripening likely occurs via inhibition of auxin signaling. Surprisingly, the auxin transport-related gene ABCB15 (Matsuda and others 2011) and the IAA-amino acid hydrolase ILR1 (LeClere and others 2002) were up-regulated in treated apples. This suggests that auxin signaling is partially but not completely inhibited by MeJA. Exogenous JAs can induce a variety of phenotypical effects during fruit ripening. For example, MeJA promotes degreening of apple peel (Fan and Mattheis 1999) as well as anthocyanin accumulation (Rudell and others 2002). Anthocyanins are biosynthesized through

the flavonoid pathway. Two types of genes are required for anthocyanin synthesis: biosynthetic structural genes and regulatory genes (Li 2014). Previous studies illustrated that JAs can induce both types of anthocyanin-related genes (Dombrecht and others 2007; Qi and others 2011). In our study, anthocyanin accumulation is greatly promoted, and the anthocyanin biosynthetic structural genes F3Hs and UFGTs were also up-regulated in MeJA-treated apples. However, no regulatory genes were identified among the DEGs. A recent study in apple has documented that JAs could directly mediate anthocyanin biosynthesis by removal of the JAZ repression on anthocyanin regulatory factors (An and others 2015). Considering the up-regulation of JAZ genes at this stage, the activation of regulatory genes probably occurred at the later stage. Anthocyanins are biosynthesized in the cytosol and transported into the vacuole by putative anthocyanin transporters. ABCC transporters and GSTs have been suggested to be involved in anthocyanin transport (Zhao 2015). The up-regulation of ABCC transporters and GSTs indicated that JAs also mediate anthocyanin accumulation by transcriptionally regulating anthocyanin transporters.

Conclusions

Using RNA-Seq technology, we investigated the differences in transcription between MeJA-treated and mock-treated apples. In total, 1092 DEGs were identified as showing significant responses to MeJA. The reliability of the RNA-Seq data was confirmed by qPCR. Furthermore, DEGs related to phytohormone pathways (ethylene, auxin, and JA), anthocyanin biosynthesis, and transport were identified. This study may provide important information for the identification of genes involved in MeJA-meditated apple ripening and may aid in understanding the molecular mechanisms underlying this complex biological process.

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Compliance of Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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