Characterization and Expression of Genes Involved in the Ethylene Biosynthesis and Signal Transduction during Ripening of Mulberry Fruit

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Abstract

Although ethylene is well known as an essential regulator of fruit development, little work has examined the role ethylene plays in the development and maturation of mulberry (Morus L.) fruit. To study the mechanism of ethylene action during fruit development in this species, we measured the ethylene production, fruit firmness, and soluble solids content (SSC) during fruit development and harvest. By comparing the results with those from other climacteric fruit, we concluded that Morus fruit are probably climacteric. Genes associated with the ethylene signal transduction pathway of Morus were characterized from M. notabilis Genome Database, including four ethylene receptor genes, a EIN2-like gene, a CTR1-like gene, four EIN3-like genes, and a RTE1-like gene. The expression patterns of these genes were analyzed in the fruit of M. atropurpurea cv. Jialing No.40. During fruit development, transcript levels of MaETR2, MaERS, MaEIN4, MaRTE, and MaCTR1 were lower at the early stages and higher after 26 days after full bloom (DAF), while MaETR1, MaEIL1, MaEIL2, and MaEIL3 remained constant. In ripening fruit, the transcripts of MaACO1 and MaACS3 increased, while MaACS1 and MaACO2 decreased after harvest. The transcripts of MaACO1, MaACO2, and MaACS3 were inhibited by ethylene, and 1-MCP (1–methylcyclopropene) upregulated MaACS3. The transcripts of the MaETR-like genes, MaRTE, and MaCTR1 were inhibited by ethylene and 1-MCP, suggesting that ethylene may accelerate the decline of MaETR transcripts. No significant changes in the expression of MaEIN2, MaEIL1, and MaEIL3 were observed during ripening or in response to ethylene, while the expressions of MaEIL2 and MaEIL4 increased rapidly after 24 h after harvest (HAH) and were upregulated by ethylene. The present study provides insights into ethylene biosynthesis and signal transduction in Morus plants and lays a foundation for the further understanding of the mechanisms underlying Morus fruit development and ripening.
Introduction

In climacteric fruit, ethylene is necessary for the initiation of fruit ripening and senescence because it drives the majority of the ripening processes, such as fruit softening and cell wall disassembly [1–2]. The effects of ethylene in fruit mainly depend on its biosynthesis and signal transduction during fruit development.

The synthesis of ethylene begins with the production of S-adenosylmethionine (SAM) which is catalyzed by S-adenosylmethionine synthetase (SAM synthetase) from methionine. SAM is then metabolized to 5-methylthioadenine (MTA), which is incorporated into the methionine cycle to recover the sulfur atom and 1-aminocyclopropane-1-carboxylic acid (ACC); this reaction is catalyzed by ACC synthase (ACS). Finally, in the presence of oxygen, ACC is oxidized by ACC oxidase (ACO) to yield ethylene and CO₂ [3].

ACS and ACO are the key enzymes in ethylene production and are encoded by the ACS and ACO gene families, respectively. The expression profiles and regulation mechanisms of ACS and ACO genes in fruit have been investigated in plants. In tomato (Solanum lycopersicum L.), nine ACS genes and five ACO genes have been characterized and shown to be differentially expressed during fruit ripening [4]. During development (System I), lower and auto-inhibitory levels of ethylene are synthesized by LeACS1A and 6 and in conjunction with LeACO1, 3, and 4. At the transition stage, LeACS4 is induced and a large increase in auto-catalytic ethylene is initiated. LeACS2 and 4, in conjunction with LeACO1 and 4 are then responsible for the high ethylene production throughout System II [4]. ACS and ACO genes have previously been used as targets to suppress the production of ethylene and delay the postharvest ripening and senescence of fruit [5–7].

The signal transduction of ethylene is well understood in higher plants. Ethylene signals are transported via five elements: ethylene receptor (ETR), constitutive triple response 1 (CTR1), ethylene insensitive 2 (EIN2), ethylene insensitive 3/EIN3-like protein (EIN3/EILs), and ethylene response factor (ERF). Ethylene is perceived by a family of endoplasmic reticulum (ER)–localized receptors (ETR) that are similar in sequence and structure to bacterial two-component histidine kinases [8]. These ETR then activate CTR1, a Raf-like serine-threonine kinase that acts as a negative regulator of ethylene responses, which directly interacts with the ETR and transmit the signal to EIN2 [9]. EIN2 is a central regulator of the signaling pathway located in the ER. In the presence of ethylene, CTR1 is suppressed, and leading to the dephosphorylation of EIN2 and the slicing of its C-terminal end (CEND), which enters into the nucleus, and activates EIN3/EILs. EIN3/EILs eventually stimulate the expression of ERF and other responsive genes [10–11].

These elements of ethylene signaling have been isolated from several fruit plants to date, and their expression and function have been investigated. The transcript levels of ETR are correlated with the accumulation of ethylene during fruit development, while the concentration of ETR protein decreases gradually [12–13]. ETR protein probably degrades rapidly in the presence of ethylene and this changeable concentration may modulate the onset of fruit ripening by measuring cumulative ethylene exposure, as the expression of ETR transcripts causes the production of new ETR protein [13–14]. The expression of CTR1 tends to increase during fruit maturation in kiwifruit, pear, and plums and responds to ethylene and 1–methylcyclopropene (1-MCP), but expression of this gene is steady in peach and apple [15–18]. EIN2 is also closely related to the regulation of the fruit ripening process, the silencing of EIN2 will delays fruit development and ripening and greatly reduces the expression levels of ethylene–related and ripening–related genes compared to the control fruits [19–20]. EIN3/EILs are expressed differentially during the development of fruit, and the reduction in tomato LeEIL gene expression using antisense technology causes ripening–impaired phenotypes [21–22]. EIN3/EILs...
family proteins regulate the expression of responsive genes in fruit, including ERF, ACO, xylol

glucan endotransglycosylase gene (XET), and cell wall-modifying genes [23–24].

Mulberry (Morus L.) provides the optimal food (leaves) for the domesticated silkworm (Bombbyx mori L.). Morus also has multiple uses in ecology, pharmaceuticals, and traditional Chinese medicine, and Morus fruit is very popular due to its unique flavor [25]. However, ethylene biosynthesis and signaling perception in Morus remain relatively unexplored. In 2008, Pan and Lou [26] became the first to characterize an ACO gene from Morus, which may be associated with tissue aging or senescence and stress responses. In our previous study, five MaACS and two MaACO genes were screened from the M. notabilis Genome Database, and their expression profiles were determined to be related to fruit development, and response to abscisic acid and ethephon [27]. These studies were insufficient to clarify the functions of ethylene during development of Morus fruit. In this study, 11 putative genes associated with the ethylene signal transduction pathway were identified in the M. notabilis genome. The expression patterns of these genes during M. atropurpurea cv. Jialing No.40 fruit development were studied using real-time PCR. These expression patterns were also examined in fruit treated with ethylene and the receptor inhibitor 1-MCP.

Materials and Methods
Screening and identification of the genes involved in signal transduction

The protein sequences of the genes involved in the signal transduction from other plant species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and were used as queries to blast against M. notabilis Genome Database (http://morus.swu.edu.cn/morusdb/). This yielded non-redundant full mRNA sequences. The candidate genes were identified using BLASTN and SMART (http://smart.embl-heidelberg.de/).

The amino acid sequences involved in the ethylene signaling pathway were downloaded from NCBI and used for alignment with putative protein sequences of Morus signal transduction elements given by MUSCLE 3.6, and the alignment was corrected manually using BioEdit software. And a phylogenetic tree was constructed by MEGA 5.0.

Plant material and treatments

The mulberry tree M. notabilis, which is used for genome sequencing, is an isolated wild mulberry species with a chromosome number of 14 [25, 28]. The fruits of M. atropurpurea cv. Jialing No.40, a new cultivated variety for obtaining mulberry fruits, were used for fruit evaluation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in this study. Three different batches of M. atropurpurea cv. Jialing No.40 fruit were collected in the Morus garden at Southwest University, Chongqing, China. The batch 1 fruits were collected at 2, 8, 14, 21, 26, 31, 34 and 37 days after full-bloom (DAF), with the last sampling date set at full maturity, and were used for genes cloning and qRT-PCR. The batch 2 fruits were collected at 34 DAF, the fruit were at early ripening stage for this batch, and were incubated in a container with C2H4 (20 ppm) and 1-MCP (0.5 μL L−1 SmartFresh™) at 23°C for 12 h. Then the containers were opened to release the remaining C2H4 and 1-MCP. Finally, the treated fruits were incubated continually at 23°C for 0, 12, 24, and 36 h. The batch 3 fruits were collected at 21, 26, 31, 34 and 37 DAF, with the last sampling date set at full maturity, and their respiration rates were measured.
Measurements of ethylene production, fruit firmness, soluble solids content (SSC) and respiration rate

Eight headspace bottles (20 ml), each containing one fruit, were used to evaluate the fruit ethylene production rate. The sealed bottles were held at 23°C for 2 h, and then 1 ml of headspace gas was removed using an airtight syringe. The ethylene was measured using a gas chromatograph (GC2010; Shimadzu, Japan) fitted with a flame ionization detector. The temperatures of the oven, injector and detector were 110°C, 130°C, and 200°C, respectively, and nitrogen was used as the carrier gas at 40 ml min⁻¹.

Fruit firmness was measured using a CT3 texture analyzer (Brookfield, USA) fitted with a 2 mm diameter probe. The probe was pressed into eight different fruits to a depth of 3 mm in a single smooth motion. Juice samples of 200 μl pressed from Morus fruit were used to measure SSC by using a PAL-1 refractometer (Atago, Japan).

Respiration rate was measured as CO₂ production. Three replicates of Morus fruit were weighed before being sealed in a 5 L container at 25°C. Increased CO₂ concentration in the container was monitored using a CO₂ monitoring instrument (RD-7AG; Nanjing Analytical Instrument Factory Co., Ltd. China). The results were calculated as mg CO₂ kg FW⁻¹ h⁻¹.

Isolation of RNA, and synthesis of the first strands of cDNA

Total RNA of fruit was extracted using the RNA Extraction Kit Transzol Plant (TransGen Bio-tech, China). DNase I (Takara, Japan) was used for removing contaminating DNA. The cDNA was synthesized from DNA-free RNA with the Reverse Transcriptional M-MLV (Promega, USA). Ten-fold diluted cDNA was used in qRT-PCR.

Quantitative RT-PCR expression analysis

The primers (listed in Table 1) were used to isolate the genes involved in ethylene signal transduction in Morus atropurpurea cv. Jialing No.40. The purified PCR products were inserted into the pMD19-T simple vector (Takara, Japan) and the sequences were confirmed by sequencing.

The primers (listed in Table 2) used for qRT-PCR were designed based on the gene sequences obtained from Morus atropurpurea cv. Jialing No.40. The qPCR mixture (20 μl total reaction volume) composed of 10μl of 2× SYBR® Premix Ex Taq II ™ (Takara, Japan), 0.8 μl of each primer (10 μM), 0.4 μl of 50× ROX Reference Dye, 2 μl of cDNA, and 6 μl of ddH₂O. The thermal parameters for qRT-PCR were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and

Table 1. Primers used for cloning the genes involved in the ethylene signal transduction in Morus atropurpurea cv. Jialing No.40.

| Genes   | Primers (forward/ reverse) |
|---------|----------------------------|
| MaETR1  | 5'-TGAGTGTTAAGCAGGGAGCT-3 5'-GAAATCATTCGCGACAGAATA-3 |
| MaETR2  | 5'-GCTCTGGAGCAGATCGAGAT-3 5'-CAACCTGTTATATCGACATC-3' |
| MaERS   | 5'-GGCATGTTAGAATGCTGACTCA-3' 5'-AGCAAGGACATATGCAAGG-3' |
| MaEIN4  | 5'-CGGCAATTCGGATGCAAATGC-3' 5'-ACCACTCTCTAGAAACCAG-3' |
| MaCTR1  | 5'-ATGGAAATGCGCGTGAGGAT-3' 5'-TCAGCCTGATAGACATGATGG-3' |
| MaEIL1  | 5'-ATGGGCCCTTTCGAAGAGCT-3' 5'-TCAGATGTACCGAGGAAGA-3' |
| MaEIL2  | 5'-ATGGGCTTTCTGGAAT-3' 5'-TCAGAGGTACGAGCGAG-3' |
| MaEIL3  | 5'-ATGGCTCTAATGCTTGTG-3' 5'-TTATGCTCAGAAATGAAATC-3' |
| MaEIL4  | 5'-ATGTGGAAGCTTCATGAAAGA-3' 5'-TCACCTATATCAGAATCC-3' |
| MaRTE   | 5'-ATGGATTCTGATGCAGACACTG-3' 5'-CTACAATGATTAATTCCTTG-3' |

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60°C for 30 s, with a final extension at 72°C for 10 min. MaACTIN3 (HQ163775.1) gene was used as an internal control. All data was analyzed using 2^−ΔCt method.

**Results**

Identification of genes involved in ethylene signaling in the *M. notabilis* genome

Five *MaACS* and two *MaACO* genes involved in ethylene biosynthesis in *Morus* have been reported by our previous research [27]. In this study, we identified 11 putative ethylene signaling genes in *M. notabilis* using bioinformatic methods. These were four ethylene receptor genes (*MnETR1*, *MnETR2*, *MnERS*, and *MnEIN4*), an EIN2-like gene, a CTR1-like gene, four EIN3-like genes (*MnEIL1*, *MnEIL2*, *MnEIL3*, and *MnEIL4*), and a Reversion-to-ethylene sensitivity 1-like gene (*MnRTE*) (Table 3; Table 4).

**Table 2. Primers used for qRT-PCR in *Morus atropurpurea* cv. Jialing No.40.**

| Genes  | Primers (forward/ reverse) |
|--------|-----------------------------|
| MaACO1 | 5'-AAGTGATGGGGAATTGC-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaACO2 | 5'-TTTGAGGGAATTGC-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaACS1 | 5'-GACACCTGACCTCATCCAC-3' / 5'-TGCGAGGATACTAATCCGAA-3' |
| MaACS3 | 5'-TGCGAGGATACTAATCCGAA-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaETR1 | 5'-GCCAGACTACGGCCTCTTAG-3' / 5'-TGGCCAGTCATTTATTTGGA-3' |
| MaETR2 | 5'-GCCAGACTACGGCCTCTTAG-3' / 5'-TGGCCAGTCATTTATTTGGA-3' |
| MaERS | 5'-AGGTGGAGGGAATTGC-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaEIN4 | 5'-TTTGAGGGAATTGC-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaEIN2 | 5'-TGATATCCCGGAGGCG-3' / 5'-ATTCCCGGAGGCG-3' |
| MaCTR1 | 5'-GCAGACCGGAGGAGGTTACC-3' / 5'-GTGGAGTGGGATATCTG-3' |
| MaEIL1 | 5'-AAGTGATGGGGAATTGC-3' / 5'-GTCCTTTGGAGGATGAT-3' |
| MaEIL2 | 5'-GACACCTGACCTCATCCAC-3' / 5'-TGCGAGGATACTAATCCGAA-3' |
| MaEIL3 | 5'-GACACCTGACCTCATCCAC-3' / 5'-TGCGAGGATACTAATCCGAA-3' |
| MaEIL4 | 5'-GACACCTGACCTCATCCAC-3' / 5'-TGCGAGGATACTAATCCGAA-3' |
| MaRTE | 5'-GACACCTGACCTCATCCAC-3' / 5'-TGCGAGGATACTAATCCGAA-3' |

**Table 3. Genes involved in ethylene signaling in *Morus notabilis* genome.**

| Gene family                  | Number | Gene Names | Accession No.* | Scaffold(strand): start-end |
|-----------------------------|--------|------------|----------------|-----------------------------|
| Ethylene receptor           | 4      | *MnETR1*   | Morus018344    | scaffold521(-):266232–271313 |
|                             |        | *MnETR2*   | Morus008145    | scaffold606(-):247113–250061 |
| Ethylene insensitive 2      | 1      | *MnERS*    | Morus007485    | scaffold1526(+):209537–212408 |
| Ethylene insensitive 3      | 4      | *MnEIL1*   | Morus024538    | scaffold205(+):594177–596734 |
| Constitutive triple response | 1      | *MnCTR1*   | Morus003569    | scaffold548(+):106459–113027 |
| Ethylene insensitive 3      | 4      | *MnEIL2*   | Morus002491    | scaffold3281(-):31544–33397 |
| Reversion-to-ethylene sensitivity 1 | 1    | *MnRTE*    | Morus026815    | scaffold184(+):1246802–1247817 |

*Accession No. are from [http://Morus.swu.edu.cn/Morusdb/](http://Morus.swu.edu.cn/Morusdb/)

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![Table 2](http://Morus.swu.edu.cn/Morusdb/)

![Table 3](http://Morus.swu.edu.cn/Morusdb/)
Structure and phylogenetic analysis

His kinase (HisKA), GAF and receiver domains (REC) are the key domains of ETR-like proteins; all four of the ETR-like proteins detected in the present study possessed transmembrane domains: MnETR1 and MnERS each had three transmembrane domains, in contrast with the four domains of both MnETR2 and MnEIN4 (Fig. 1). Within the ETR-like family, MnETR1 and MnERS were clustered into the ETR1 subfamily, while MnETR2 and MnEIN4 belonged to the ETR2 subfamily in the phylogenetic tree (Fig. 2A).

The CTR1-like gene (\textit{MnCTR1}) isolated from \textit{M. notabilis}, has a genomic sequence length of 6569 bp and a 17 exon/16 intron structure. The coding sequence (CDS) of \textit{MnCTR1} is 2592 bp, encoding 863 amino acids with a predicted molecular mass of 93.95 kDa (Table 4). The amino acid sequence from 589 to 843 contains the serine/threonine protein kinase domain (Fig. 1).

Within the EIN3-like family, the sequence similarity matrix analysis of MnEIL amino acids revealed that sequence identity ranged from 40% to 82% (Table 5). MnEIL1 and MnEIL2 were most similar to each other (82% amino acid identity) and were located near each other on scaffold3287. Phylogenetic analysis of the deduced amino acid sequences suggested that MnEIL1 and 2, MnEIL3, and MnEIL4 could be divided into three groups (Fig. 2D).

The RTE-like gene (\textit{MnRTE}) isolated from \textit{M. notabilis} has a genomic sequence length of 1016 bp and a 2 exon/1 intron structure. The CDS is 693 bp, encoding 230 amino acids with a predicted molecular mass of 25.96 kDa and an isoelectric point (pI) of 6.64 (Table 4). MnRTE is most closely related with AtRTE2 in the phylogenetic tree (Fig. 2C). MnRTE has four transmembrane domains, two of which are located in its N-terminus and another two near its C-terminus (Fig. 1).

Tissue specific expression

Reads per kilobase of exon model per million mapped reads (RPKM) was used to normalize the expression levels of the ethylene signal-related genes using \textit{M. notabilis} RNA sequencing data. Expression of the 11 genes involved in ethylene signal transduction was detected in five tissues: root, bark, bud, flower, and leaf (Fig. 3).

| Gene Names | Genome length | CDS length\textsuperscript{a} | Exon Number\textsuperscript{b} | Proteins Size | MW (Da) | PI  |
|------------|---------------|----------------|----------------|--------------|--------|-----|
| MnETR1     | 5082          | 2340           | 7              | 779          | 87392.90 | 7.93 |
| MnETR2     | 2949          | 2382           | 2              | 793          | 88251.87 | 6.62 |
| MnERS      | 2872          | 1853           | 5              | 617          | 68930.31 | 5.84 |
| MnEIN4     | 2558          | 2295           | 2              | 764          | 86210.43 | 6.95 |
| MnEIN2     | 5614          | 3921           | 7              | 1306         | 142614.61 | 5.72 |
| MnCTR1     | 6569          | 2592           | 17             | 863          | 99948.76 | 6.26 |
| MnEIL1     | 1854          | 1854           | 1              | 617          | 69703.57 | 5.74 |
| MnEIL2     | 1824          | 1824           | 1              | 607          | 68945.70 | 5.46 |
| MnEIL3     | 1836          | 1836           | 1              | 611          | 68835.59 | 5.66 |
| MnEIL4     | 1632          | 1632           | 1              | 643          | 62078.05 | 4.74 |
| MnRTE      | 1016          | 693            | 2              | 230          | 25964.05 | 6.64 |

\textsuperscript{a} The CDS length was predicted according to the predicted \textit{Morus} genes.
\textsuperscript{b} The number of exon was predicted based on the \textit{Morus} genomic data.

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Fig. 3 shows that the ethylene signal-related genes had similar expression patterns, except for MnEIL4, which had significantly lower expression levels in the root and flower (0.05 and 0.037 RPKM, respectively). MnETR1, MnETR2, MnERS, MnEIN4, MnRTE, MnEIL1, MnEIL2, and MnEIL3 were expressed primarily in the bark and more weakly in the root, bud, flower, and leaf. However, MnEIN2 and MnCTR1 were more highly expressed in the root than in other tissues.

Changes in fruit firmness, SSC and ethylene production during fruit development and harvest

The ethylene production, SSC, and firmness were detected over the development of Morus fruit (Fig. 4B). Ethylene production and SSC were relatively stable before 26 DAF and then increased rapidly to peak at 34 DAF, while fruit firmness declined rapidly after 26 DAF. Thus, 26 DAF may represent the transition stage of Morus fruit, and Morus fruit are probably climacteric.

To investigate the response of fruit to exogenous ethylene and 1-MCP, the batch 2 fruits at 34 DAF were used. It is noteworthy to indicate that the days of growth required for fruit ripening varied between batches. The batch 2 fruit used here were at early ripening stage as revealed by SSC, ethylene production as well as fruit firmness (Fig. 5). Clearly, the production of ethylene decreased after harvest, and this decline was accelerated by 1-MCP (Fig. 5). Besides, slightly higher firmness and lower SSC were maintained in 1-MCP treated fruit (Fig. 5).
Fig 2. Phylogenetic analysis of MnETRs (A), MnCTR1 (B), MnRTE (C), and MnEILs(D). The amino acid sequences were analyzed with MUSCLE 3.6 and the phylogenetic tree constructed with MEGA 5.0 using a bootstrap test of phylogeny with minimum evolution test and default parameters. AtETR1 (AAA70047.1), AtERS1 (NP_181626.1), AtETR2 (NP_188956.1), AtERS2 (AAC22209.1), AtEIN4 (AAD02485.1), AtCTR1 (AAA32780.1), AIE1R (AAG31143.1), AtETR1 (F4I16L6.1), AtETR2 (Q9SD42.1), AtEIN3 (NP_188713.1), AtEIL1 (NP_180273.1), AtEIL2 (NP_197611.1), AtEIL3 (NP_177514.1) and AtEIL4 (NP_196574.1) in Arabidopsis; LeETR1 (AAC02213.1), LeETR2 (AAC02214.1), LeETR3 (AAC49124.1), LeETR4 (AAU34076.1), LeETR5 (AAD31397.1), LeETR6 (AAL86614.1), LeCTR1 (AAL87456.1), TCTR2 (CAAO6334.1), LeCTR3 (AAR89820.1), LeCTR4 (AAR89822.1), LeETR1 (AAM58587.1), LeETR2 (AAM58585.1), LeETR3 (AAM58589.1) and LeETR4 (BAC99307.1) in Solanum lycopersicum; PmRTE (XP_008220470.1) in Prunus mume; ObRTE (XP_006654719.1) in Oryza brachyantha; CsRTE (XP_00647936.1) in Citrus sinensis; SIRTE (XP_006439939.1) in Solanum tuberosum; SiRTE (XP_004961370.1) in Setaria italic; CaRTE (XP_004498900.1) in Cicer arietinum; FaRTE (XP_004291036.1) in Fragaria vesca subsp. Vesca; CsRTE1 (XP_004157357.1) in Cucumis sativus; CsRTE2 (XP_004141314.1) in Cucumis sativus; DcRTE1 (ADW80941.1) in Dianthus caryophyllus.

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Table 5. Sequence similarity matrix of MnEIN3-like proteins.

| Gene name | MnEIL1 | MnEIL2 | MnEIL3 | MnEIL4 |
|-----------|--------|--------|--------|--------|
| MnEIL1    | 100%   |        |        |        |
| MnEIL2    | 82%    | 100%   |        |        |
| MnEIL3    | 59%    | 59%    | 100%   |        |
| MnEIL4    | 40%    | 51%    | 50%    | 100%   |

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Expression patterns during fruit development

During development from 2 DAF through 37 DAF (to maturity), MaETR-like genes showed two expression patterns. MaETR2, MaERS, and MaEIN4 had relatively low abundance in fruit at the early stages of development and higher abundance at 26 DAF (Fig. 6). Conversely, MaETR1 was constantly expressed throughout fruit development, although it exhibited a strong expression peak at 2 DAF.

The expression patterns of MaRTE and MaCTR1 were similar throughout fruit development, with relatively lower expression levels before 8 DAF and relatively higher levels at 14 DAF. The transcript levels from 31 DAF to 37 DAF were relatively constant and lower than those at 14 DAF and 26 DAF (Fig. 6).

Different patterns were observed among the MaEIN3-like family genes. MaEIL1, MaEIL2, and MaEIL3 were constantly expressed during development, although the three genes exhibited a strong expression peak at 14 DAF, lower levels at 2 DAF and 8 DAF, and lower levels at 21 DAF, respectively. MaEIL4 showed a different expression pattern, with much higher expression levels at 8 DAF and 31 DAF but lower levels at other stages (Fig. 6).

Expression in response to ethylene and 1-MCP

The transcript accumulations for two MaACS and two MaACO genes were investigated in the present study. During fruit storage, the transcript abundances of MaACO1 and MaACS3 increased while those of MaACS1 and MaACO2 decreased. The transcripts of MaACO1, MaACO2, and MaACS3 were inhibited by ethylene, but those of MaACS1 were upregulated during 12 to 36 hours after treatment. 1-MCP could downregulate the expression levels of MaACO1, MaACO2, MaACS1 and MaACS3 (Fig. 7).
Fig 4. Changes in ethylene production, SSC and firmness during fruit development of *Morus atropurpurea* cv. *Jialing* No.40. (A) *Morus* fruit during development. (B) The ethylene production, SSC and firmness. The batch 1 fruits were used for these experiments. Error bars on each column indicate SDs from eight replicates.

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The expression levels of all four MaETR-like genes increased transiently 12 h after harvest (HAH) and then decreased rapidly. The expression levels of MaETR-like genes were
upregulated before 24 HAH and inhibited after 24 HAH by ethylene; expression was also significantly inhibited by 1-MCP during storage (Fig. 7).

The transcript abundances of MaRTE increased gradually during storage, but were inhibited by ethylene and 1-MCP. MaCTR1 transcripts decreased during storage and were inhibited by ethylene and 1-MCP. MaEIN2 was transiently upregulated by ethylene at 12 HAH but was not affected significantly by ethylene and 1-MCP (Fig. 7).

Among the MaEIN3-like genes, MaEIL1 and MaEIL3 were expressed relatively constantly, with little increase after harvest, while MaEIL2 and MaEIL4 expression increased rapidly after 24 HAH. All four genes were inhibited by 1-MCP and showed little response to ethylene, except for MaEIL2 (Fig. 7).

Discussion

Morus fruit is valued worldwide for its rich nutrient contents and unique flavor. However, the short maturity stages and shelf-life of Morus complicates efforts to popularize the fruit. The control of ethylene production may delay the ripening of Morus fruit and prolong its shelf life.

However, no previous work has determined the respiratory type of Morus fruit, which is vital...
for establishing the mechanisms controlled by ethylene in *Morus* fruit development and ripening. In this study, ethylene production, firmness, and SSC were measured during the development of *Morus* fruit. These factors were relatively constant before 26 DAF but exhibited remarkable changes after this point. Ethylene production and SSC increased rapidly at 26 DAF, while fruit firmness decreased (Fig. 4). Our previous study suggested that *MaACO2* and *MaACS3* showed rapid increases in expression throughout fruit development and were upregulated by ethephon in the fruit at 20 DAF, which may be attributed to the production of ethylene during the fruit transitional period [27]. Therefore, fruit at 26 DAF may represent the transition stage from system I ethylene production to system II ethylene production, and the

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Fig 7. Expression of the genes involved in ethylene biosynthesis and signaling pathway during fruit ripening of *Morus atropurpurea* cv. Jialing No.40. The batch 2 fruits were used for qRT-PCR. Error bars on each column indicate SDs from three replicates. Significant differences (*P* < 0.05) among treatment are marked with different letters above bars.

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respiration rate during fruit development also supports this suggestion (S1 Fig.). In addition, ethylene production, fruit firmness, and SSC responded to ethylene and 1-MCP in the fruit harvested at 34 DAF. 1-MCP acted as a negative factor by retarding fruit softening, while ethylene accelerated fruit maturation (Fig. 5). However, unlike typical climacteric fruits, exogenous ethylene slightly decreased, rather than stimulated, endogenous ethylene production as well as temporarily inhibited expression of MaACO1, MaACO2, and MaACS3 in Morus fruit after harvest. Why this occurred remains unclear and more studies are needed in the future.

Morus fruit at 34 DAF were harvested for treatment, and the production of ethylene decreased during storage in both treated and untreated fruit. MaACO1 and MaACS3 expression increased after harvest, while MaACS1 and MaACO2 decreased. The transcript levels of MaACO1, MaACO2, and MaACS3 were inhibited by ethylene and 1-MCP, while that of MaACS1 upregulated (Fig. 7). These results are similar to those reported in fig fruit (Ficus carica L.). In the fig fruit harvested at the post-climacteric stage, which had higher ethylene contents, ethylene production fell during storage and was inhibited by propylene; 1-MCP inhibited the accumulation of Fc-ACS1, Fc-ACS3, and Fc-ACO1 transcripts, but induced the accumulation of Fc-ACS2 transcripts. Propylene induced the accumulation of Fc-ACS1, Fc-ACS3, and Fc-ACO1 transcripts, but inhibited Fc-ACS2 expression in the propylene treated fruit [29]. Additionally, as both fig and Morus fruits are collective, their mechanisms of ethylene biosynthesis and fruit maturation may differ from those of other fruits.

In the present study, 11 putative genes were identified in the M. notabilis genome as associated with ethylene signal transduction using bioinformatics tools. These were four ETR1-like proteins, an RTE-like protein, a CTR1-like protein, an EIN2-like protein, and four EIN3-like proteins (Table 3; Table 4). Their expression patterns were studied during the development and ripening of M. atropurpurea cv. Jialing No.40 fruit.

Among the ETR-like genes, the expression levels of MaETR2, MaERS, and MaEIN4 increased during development and were associated with the production of ethylene (Fig. 6), which was similar to the genes found in plum (Pc-ETR1a) [16], tomato (NR, LeETR4, LeETR5 and LeETR6) [12], and strawberry (FaETR2) [30]. Notably, the expression of MaETR1 differed from that of other ETR-like genes; the gene was constantly expressed during fruit development and showed higher expression at the early stage of fruit development, as was observed for similar genes in tomato (LeETR1 and LeETR2) [12], strawberry (FaETR1 and FaERS1) [30] and persimmon (DkERS1) [31]. MaETR2, MaERS, and MaEIN4 may thus be associated with the production of ethylene during the maturation of Morus fruit, while MaETR1 additionally contributes to the growth of immature fruit.

During fruit ripening, the expression levels of all four MaETR-like genes transiently increased at 12 HAH and then decreased rapidly, in conjunction with the trends of SSC and ethylene production after harvest (Figs. 5B and 7). This result suggested that MaETR genes are closely related with the maturation of Morus fruit. The expression levels of MaETR-like genes were upregulated before 24 HAH and inhibited after 24 HAH in the ethylene treated fruit (Fig. 7). Ethylene thus likely accelerates the decrease of MaETR transcripts at post-climacteric stage.

RTE1 is a membrane protein located at the Golgi and endoplasmic reticulum (ER) that acts as a cofactor coordinated with ETR protein to negatively regulate the plant response to ethylene through a conformational effect on the ethylene-binding domain [32–34]. We are the first to report the expression pattern of the RTE gene during fruit development and maturation. MaRTE had relatively lower transcript abundance early in fruit development and higher abundance after 14 DAF (Fig. 6), which was similar to the expression patterns of MaETRs. We hypothesized that MaRTE regulates Morus fruit maturation in cooperation with MaETR2, MaERS and MaEIN4. MaCTR1 expression was higher in ripe fruits, as has been reported in
plum and pear [15–16], and showed a relatively similar pattern to that of MaRTE. The transcript abundance of MaRTE, unlike those of the MaETR genes, increased gradually after harvest, while that of MaCTR1 decreased after harvest. However, MaRTE and MaCTR1 expression levels were inhibited by ethylene and 1-MCP (Figs. 6 and 7).

The functions of EIL genes have been studied in tomato [22, 35], banana [36], melon [23], and kiwifruit [18, 24]. Typically, EIL genes are constitutively expressed during fruit development, but MA-EIL2 expression increases with the development of fruit and is induced by ethylene [36]. In our results, MaEIL1, MaEIL2 and MaEIL3 (but not MaEIL4) were constitutively expressed during fruit development, with slightly higher transcript abundances of MaEIL1 and MaEIL2 observed early in fruit development and a lower abundance of MaEIL3 observed at 21 DAF. However, MaEIL4 showed a different pattern with much higher expression levels early in development and at 31 DAF but lower levels at other stages (Fig. 6). In postharvest fruit, MaEIL1 and MaEIL3 were relatively constantly expressed, but were inhibited by 1-MCP, and showed little response to ethylene. Conversely, MaEIL2 and MaEIL4 increased rapidly after 24 HAH and were upregulated by ethylene (Fig. 7). The functions of MaEIL2 and MaEIL4 during fruit ripening require further research.

In our previous study, the expression pattern of MaEIN2 was determined and MaEIN2 was showed to be involved in the ripening and senescence of Morus organs [37]. However, no significant change in the expression of MaEIN2 was found during ripening or in response to ethylene and 1-MCP treatment in the present study (Fig. 7). The function of MaEIN2 in fruit development and maturation requires further investigation.

In conclusion, we measured the ethylene production, fruit firmness and SSC during Morus fruit development and harvest, concluding that these fruit are probably climacteric. Analysis of Morus genome data characterized 11 elements associated with ethylene perception, and the genes involved in ethylene biosynthesis and signal transduction showed different expression patterns during Morus fruit development as well as different responses to ethylene and 1-MCP after harvest. The present study provides preliminary insights into the roles of ethylene and the expression profiles of ethylene-related genes during Morus fruit development, laying a foundation for a further understanding of the mechanisms of Morus fruit development and ripening. It may be possible to control Morus fruit ripening and artificially prolong its shelf life by manipulating ethylene production using transgenic approaches.

Supporting Information

S1 Fig. Changes in respiration rate during fruit development of Morus atropurpurea cv. Jialing No.40. The batch 3 fruits were used for measuring respiration rate. Error bars on each column indicate SDs from three replicates.

(SDOCX)

S1 File. Multiple sequence alignment of deduced Morus notabilis ethylene signaling proteins. The alignment was performed using CLUSTALX and the results were displayed using Genetyx 7.

(SDOCX)

S2 File. Multiple sequence alignment of ethylene signaling genes between Morus notabilis and M. atropurpurea cv. Jialing No.40.

(SDOCX)
Characterization and Expression of Mulberry Ethylene Related Genes

Author Contributions
Conceived and designed the experiments: CLiu MY AZ JL XW RL CW. Performed the experiments: CLiu PZ MY WF LH CW. Analyzed the data: CLiu AZ MY PZ LH RL. Contributed reagents/materials/analysis tools: MY AZ LH XW ZL CLu. Wrote the paper: CLiu AZ MY.

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