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Transcriptomic Analysis on the Regulation of Tomato Ripening by the Ethylene Inhibitor 1-methylcyclopropene

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Abstract

Tomato is a climacteric fruit whose ripening is regulated by the plant hormone ethylene. 1-methylcyclopropene (1-MCP) is a competitive ethylene inhibitor that can delay the fruit ripening process. To understand the molecular mechanism of how 1-MCP inhibits tomato fruit ripening, transcriptomics (RNA-Seq) was used to identify genes that were differentially expressed in 1-MCP-treated (Day 1) tomato fruits. Of the 35340 genes in the tomato genome, about 50% were expressed with 1-MCP treatment. There were 5683 genes identified as significantly differentially expressed. Quantitative reverse transcription PCR (qRT-PCR) assays were used to validate the RNA-Seq data. Our results showed that 1-MCP treatment resulted in the down-regulation of fruit ripening-related genes, including genes involved in ethylene synthesis, signal transduction and carotenoid biosynthesis. Our results provide insight at the whole genome level regarding gene regulation by 1-MCP during fruit ripening. Understanding the molecular basis of 1-MCP inhibition on tomato ripening may help farmers and food processors to better use 1-MCP in agriculture and food industry.

Keywords: 1-methylcyclopropene (1-MCP), tomato ripening, ethylene inhibitor, RNA-Seq analysis

1. Introduction

Fruit ripening is a complex developmental process that coincides with seed maturation. Fruit ripening includes changes in color (loss of green color and increase in non-photosynthetic pigments), texture (cell wall softening), taste (increase in sugar and decrease in organic acids), and flavor (production of volatile compounds). Ripened fruits with aromatic compounds and vivid pigmentation attract animal bites to facilitate their seed dispersal. Fruit ripening is regulated by temperature, gas content in the atmosphere, humidity, and plant hormones such as ethylene (Zhang et al., 2017).

Based on their respiration and ethylene biosynthetic rates, fruits are generally classified into two physiological classes: climacteric and non-climacteric (Osorio et al., 2013). Ethylene plays an important role in climacteric fruit ripening, including in tomatoes, and its function has been well-documented (Bapat et al., 2010). Ethylene is produced in climacteric fruit at the start of ripening. Elevated levels of ethylene bind to the ethylene receptors and activate signal transduction cascades, which lead to activation of a number of genes related to fruit ripening (Giovannoni et al., 2017; Alexander & Grierson, 2002). Application of exogenous ethylene to unripe tomatoes stimulated fruit ripening whereas inhibitors blocking ethylene synthesis and/or action delayed tomato ripening (Giovannoni, 2001).

It has been known that 1-MCP is an inhibitor of ethylene though blocking of ethylene receptors (Serek et al., 1995). 1-MCP can retard fruit ripening, and therefore, extend the shelf life of fruit (Zhang et al., 2017). Since

1-MCP is very effective and non-toxic, it has been used widely in the food industry to prolong the post-harvest shelf life of climacteric fruits (Zhang et al., 2017). EthyBloc® and SmartFresh® are the two commercially available products for 1-MCP for ornamentals and fruit and vegetables, respectively. However, the molecular mechanism of 1-MCP inhibition of fruit ripening remains unknown.

Tomato (*Solanum lycopersicum*) has been used as a good model system to study the mechanism of fruit ripening because it is an important fruit crop for humans, and the tomato genome has been fully sequenced (Sato et al., 2012; Giovannoni, 2001). In addition, tomato has a relatively small genome and well characterized mutants in fruit ripening. Moreover, the ease of transformation in tomato makes mechanistic hypotheses testing feasible using stable transgenic lines (Karlova et al., 2014; Giovannoni et al., 2017).

The advancement of next-generation sequencing (NGS) technologies has revolutionized functional genomic studies. One important application of NGS is to study the transcriptome; the resulting technique is named RNA sequencing (RNA-Seq). This technology has grown rapidly and is replacing microarrays for gene expression studies. RNA-Seq technique involves library construction, sequencing on a NGS platform, and statistical and computational analyses (Wang et al., 2009). RNA-Seq has been applied to differential gene expression (Bloom et al., 2009). RNA-Seq also allows quantification of the relative alterations of each transcript during defined developmental stages or under specific treatment conditions in plants. For example, RNA-Seq was used to study the regulation of auxin and gibberellin (GA), and the effect of abscisic acid (ABA) on fruit ripening (Mou et al., 2016; Tang et al., 2015; Li et al., 2016; Ye et al., 2015). In this study, RNA-Seq was used to study the transcriptome changes under 1-MCP treatment to understand the effect of 1-MCP on tomato fruit ripening at the genomic level. Our research provides insight on ethylene regulation in tomato fruit ripening.

2. Methods

2.1 Tomato Fruits and 1-MCP Treatments

Mature green tomatoes were purchased from East Coast Fresh Cuts (Savage, MD, USA), received just prior to performing the experiment and used as it is. Tomatoes were treated with SmartFresh™ (Agrofresh, Spring House, PA, USA) as described previously (Tassoni, et al., 2006), using airtight glass jars to create a "closed system" to generate 1-MCP at a fixed concentration. Each jar was equipped with a Petri dish in the bottom containing a stir bar and 1 g of the reagent, a tube with one side reaching the dish and another side connected with a 30 ml syringe was fixed with the lid through an airtight rubber horse. All jars were placed on stirrer plates at 22 ±1 ℃. After loading tomatoes, 5 or 6 (200-300 g per a tomato) in each, the jars were closed, 10 ml of water were injected to each Petri Dish, the stirrer was turned on simultaneously and continued for 24 hr. A control experiment was conducted simultaneously without SmartFresh™. After thus treatment, all tomatoes were removed from the jars, and placed in open space at 22 ±1 ℃. Pictures were taken at days 0, 1, 2, 3, 7, 10, 13, 14 and 20.

2.2 RNA Isolation, Library Preparation, and Illumina Sequencing

Tomatoes from three groups: 1-MCP-treated (Day1) and untreated (Day 0, and Day 1) were sampled. Pericarps from six tomatoes (two biological replicates from each treatment) were prepared for transcriptomic analysis. Total RNA was extracted from tomato pericarps using the RNeasy Plant Mini Kit (Qiagen, Valacia, CA, USA, according to the manufacturer's instructions. RNA purity was evaluated using a Nanodrop Spectrophotometer (ThermoFisher, Foster City, CA, USA). RNA concentrations were measured using the Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorimeter (Life Technologies, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) with RIN numbers between 8.8 and 9.3. Library construction and genome sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). A total of 3 μg of RNA per sample was used as the initial input material for sequencing library preparation. Sequencing libraries were constructed using the NEBNext Ultra RNA library Prep Kit for Illumina following the manufacturer's instructions. Library concentrations were quantified using a Qubit® 2.0 fluorimeter. The insert sizes were evaluated using an Agilent 2100 Bioanalyzer. The libraries were sequenced using an Illumina HiSeq 2000 platform, and 150 bp paired-end reads were obtained.

2.3 Transcriptome Analysis

Clean reads generated by filtering low quality reads from the raw data were mapped to a reference tomato (*S. lycopersium*) genome (http://plants.ensembl.org/biomart/martview;
ftp://ftp.ensemblgenomes.org/pub/release-23/plants/fasta/solanum_lycopersicum/dna/

ftp://ftp.ensemblgenomes.org/pub/release-23/plants/gtf/solanum_lycopersicum/) using TopHat v2.0.12 software. Fragments per kilobase million (FPKM) mapped reads were used to estimate the expression levels (Trapnell,

2010). Differential expression analysis of two groups was performed using the DESeq R package (Anders et al., 2010). P-values were adjusted by Q-values using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) to control the false discovery rate. Genes with Q-values < 0.05 were defined as differentially expressed. KOBAS software was used to determine the statistical enrichment of DEGs in the GO terms and KEGG pathways as described previously (Mao et al., 2005). All of the raw sequence data were deposited at the NCBI BioProject database with Bio Project ID number PRJNA413422.

2.4 Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Assays

Synthesis of cDNA was carried out as described previously (Liu et al., 2011). Primers ordered from Integrated DNA Technologies (IDT) (www.idtdna.com) are listed in Table 1.

qRT-PCR was performed in a 96-well plate on a Quant Studio 6 Flex real-time PCR system (ThermoFisher, Foster City, CA). PCR reactions were carried out in a 50 µl total volume containing 25 µl Power SYBR Green PCR Master Mix (ThermoFisher, Catalog#4309155), 1.25 µl of each primer (forward and reverse) at 10 µM, 1 µl of cDNA and nuclease-free water (ThermoFisher, Catalog#AM9938). Thermal cycling parameters were as described previously (Liu et al., 2011). Results were visualized using the Quant Studio Real-Time PCR Software v1.3 provided with the Quant Studio 6 Flex Real-Time PCR System. To determine relative gene expression, the *Ct* value of the internal control gene (actin) was subtracted from the treated and untreated samples. The ΔCt, ΔΔCt, and the 2^{-fx} values were calculated as described previously (Pfaffl 2001).

Table 1. Primer sequences used in qRT-PCR assays

Genes	Forward primers	Reverse primers
Solyc04g051360.2.1	TGATTTCTAGGGGTGTTGGA	TTGAACCTTCACCACCTGAT
Solyc05g054890.2.1	TACGAAATGCTGCAAAAACA	CAAGACCTGAAGTTGGTTCG
Solyc11g072600.1.1	TTCCGATGGATAACAATGCT	GCACCTCTCGGAAACTAACA
Solyc07g053410.2.1	GAACCCCTTCAGTCCAATTT	GCTGGCACTTGAAAATCAGT
Solyc05g050830.1.1	CGGCTCACCTTAATTTTCCT	TTCCTCTTCCTCGTCTTCCT
Solyc03g095980.2.1	CAAAACAGCAGAAATCAGCA	ATCATCGCAACAAAAAGAGG
Solyc04g014400.2.1	CCTGAGCAAAATACGAGCAT	CTGTTGTGGGATAGGTCCAG
Solyc03g025350.2.1	GCTGGTGGTGTAGCAGTTCT	ACTTGCTTCGGATATTGCAC
Solyc09g089610.2.1	GTGATTGTCGGATTCCAAAG	AACCTCCACACCACGTAAAA
Solyc08g078180.1.1	TTGAGGATTAATTCCGGTGA	GAGCAACCTTCTTCCTCCTC
Solyc07g049550.2.1	ATGGGACTCGGATGTCACTA	TTTCGGATAAACTTGCTTGC
Solyc11g012980.1.1	AATGAGAAGCTGGGGATCAT	AAAAGTGCAGCATCATAGGC
Solyc11g010710.1.1	GCCATAAAGTTCAGGGGAGT	GCTCGATGATGATTTCTGCT
Solyc08g078170.1.1	TCGGATCCTGTTATTCCAAA	AACAGATTGTGCTGGTGGTT
Solyc06g053260.1.1	CCCTTGTCATGTCGAAGAAT	TCAGGCCTTATAGCACCAAG

2.5 Statistical Analysis

Student's t-test was used to determine the significant differences between samples.

3. Results

3.1 Effects of 1-MCP on Tomato Fruit Ripening

To test the effect of 1-MCP on tomato fruit ripening, 1-MCP was used to treat the mature green tomato fruits. As shown in Figure 1, a dramatic delay in color alteration was observed in 1-MCP-treated tomato fruits. Control tomatoes started to turn orange at Day 3, and turned completely red at Day 6. The 1-MCP-treated tomato fruits started to turn orange at Day 10, and turned completely red at Day 20 (Figure 1). Changes in fruit color were delayed by 14 days after 1-MCP treatment.

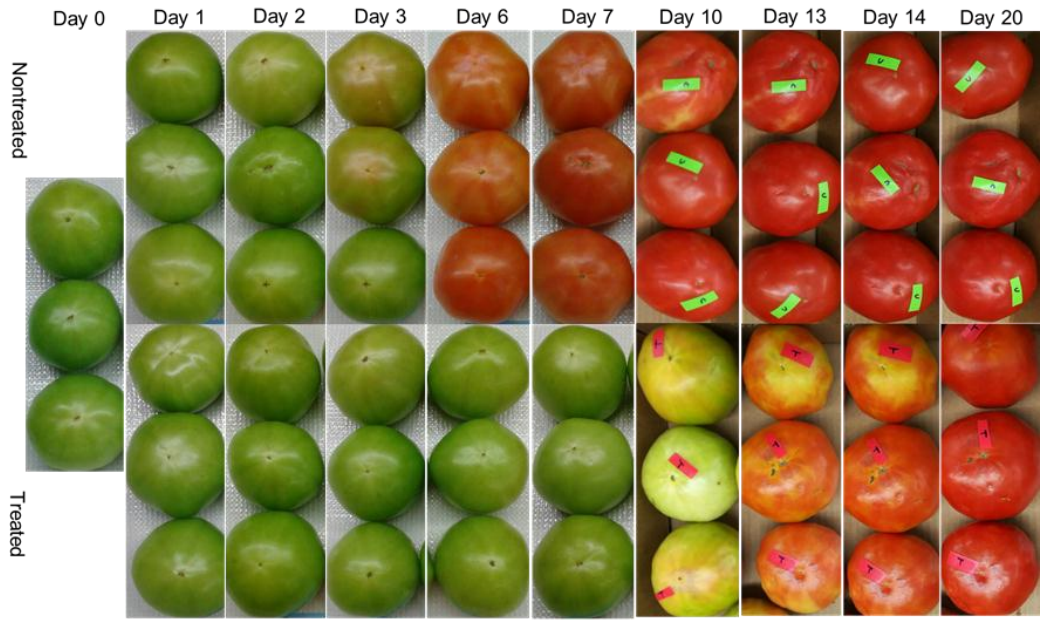

Figure 1. Color changes for 1-MCP treated and non-treated tomatoes. Mature green tomatoes were treated with 1-MCP for 24 hours and compared with untreated tomatoes. Pictures were taken at Day 1, 2, 3, 6, 7, 10, 13, 14, and 20

3.2 Summary of Transcriptomics Data

To investigate the effect of 1-MCP on tomato fruit ripening, pericarps from tomato fruits in both 1-MCP-treated and untreated groups were sampled at Day 1 after treatment. Pericarps from the control group before treatment (Day 0) were also collected. RNA was extracted and subjected to transcriptomic analysis. The raw sequence reads obtained by Illumina sequencing were from 73.7 to 123 million reads per sample, and over 97% had a quality score higher than 20. After data filtration, 71.7 to 120 million clean reads were gathered; roughly 50% of the clean reads were uniquely mapped to the tomato reference genome (Table 2).

Hierarchical cluster analysis was used to represent variances in DEGs (Figure 2A). Among more than 35340 genes with different reads detected, roughly 50% of them had a fragments per kilobase million (FPKM) >1. Of the 5683 differentially expressed genes (DEGs), 3066 genes were up-regulated and 2617 were down-regulated (Figure 2B). Of these DEGs, 2088 were significantly changed (more than 2-fold) compared to the untreated day 1 (Con-D1) samples. There were 1214 genes up-regulated whereas 874 genes were down-regulated in 1-MCP-treated samples (Tre-D1).

Table 2. Throughput and quality of RNA-Seq data

Sample name	Raw reads	Clean reads	Q20 (%)*	Total mapped	Multiple mapped	Uniquely mapped
U0-1	73720680	71720882	97.5	65851125 (91.82%)	1028752 (1.43%)	64822373 (90.38%)
U0-2	81324926	78662492	97.55	72002946 (91.53%)	1097549 (1.4%)	70905397 (90.14%)
U1-1	118771830	114590170	98.3	64748336 (56.5%)	1024390 (0.89%)	63723946 (55.61%)
U1-3	122902580	119541298	98.16	65657537 (54.92%)	1092604 (0.91%)	64564933 (54.01%)
T1-1r	123401776	119975950	97.95	60089836 (50.08%)	692821 (0.58%)	59397015 (49.51%)
T1-2r	113749964	110636956	98.28	56339418 (50.92%)	617191 (0.56%)	55722227 (50.36%)

*Q20 means that the percentages of bases whose correct base recognition rates are greater than 99% in total base. U0-1 and U0-2 represent untreated tomatoes (Day 0). U1-1 and U1-3 represent untreated tomatoes (Day1) whereas T1-1r and T1-2r represent 1-MCP treated tomatoes (Day1).

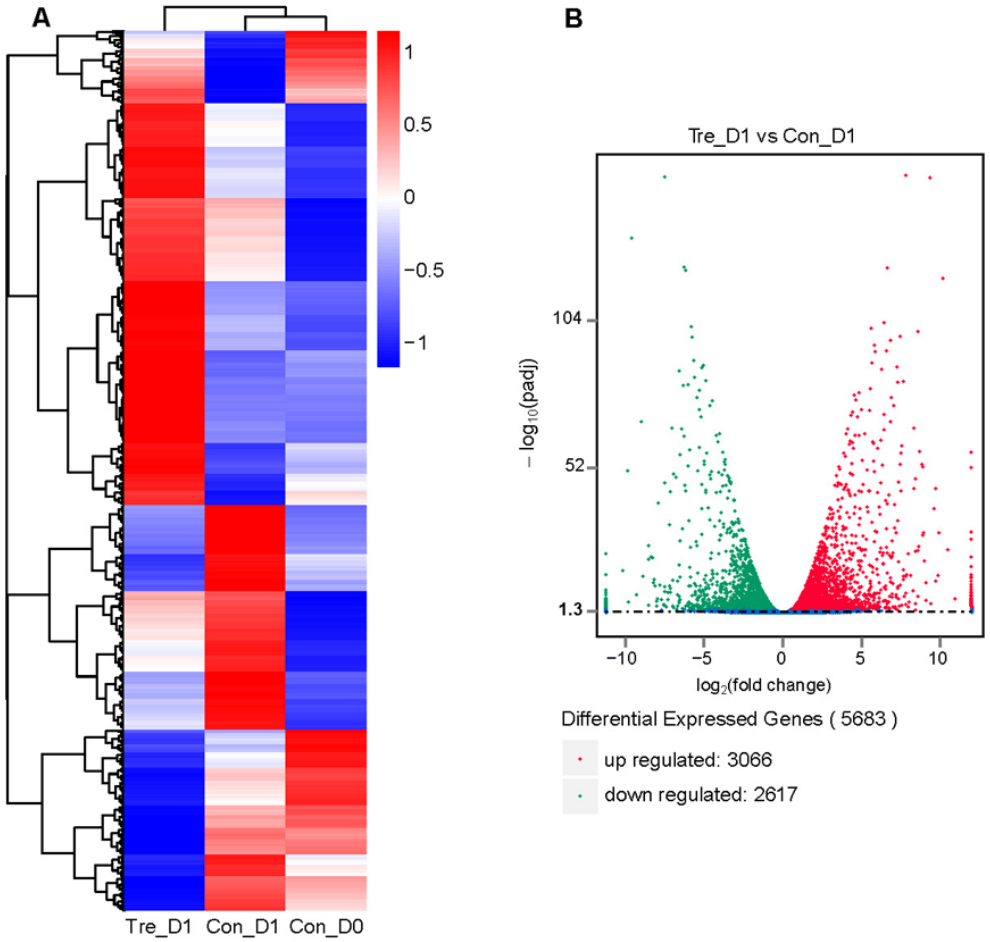

Figure 2. Differentially expressed genes (DEGs) in the MCP-treated samples (Day1). (A) Hierarchical clustering and heat map of differentially expressed genes based on the expression levels (RPKM) in Con-D1, Tre-D1 and Con-D1 samples. Con-D0 represents untreated tomatoes before 1-MCP treatment. Con-D1 represents untreated tomatoes (Day1), whereas Tre-D1 represents 1-MCP treated tomatoes (Day 1). Red and blue colors represent high and low expressed genes, respectively. (B) Volcano plot showing the numbers of significantly differentially expressed genes in 1-MCP treated (Day1) and untreated (Day1) tomatoes. Red and Green colors represent up- and down-regulated genes, respectively. Data presented here are averages of two biological replicates

3.3 Gene Ontology (GO) Enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis of DEGs

GO enrichment analysis was used to study the distribution of DEGs for gene function in terms of biological process (BP), cellular component (GC) and molecular function (MF). There were 148 GO categories that were differentially enriched (corrected p-value <0.05). Of the thirty GO terms (BP: 16 terms, CC: 12 terms, MF: 3 terms) significantly enriched in 1-MCP-treated (Day1) compared with untreated (Day1) shown in Figure 3A, the most enriched terms were DNA packaging complex (GO: 0044815, 44 DEGs), photosynthesis, and light harvesting (GO: 0009765, 30 DEGs) in CC and BP. All DEGs were also aligned to KEGG pathways to identify major pathways (Figure 3B). In the 1-MCP-treated (TreD1) samples, DEGs were matched in 120 KEGG metabolic pathways. The top four enriched pathways including the highest numbers of DGEs were: Metabolic pathways (586 DEGs), Biosynthesis of secondary metabolites (320 DGEs), Carbon metabolism (90 DGEs) and Plant hormone signal transduction (86 DGEs). There were thirteen pathways that had p-values <0.05.

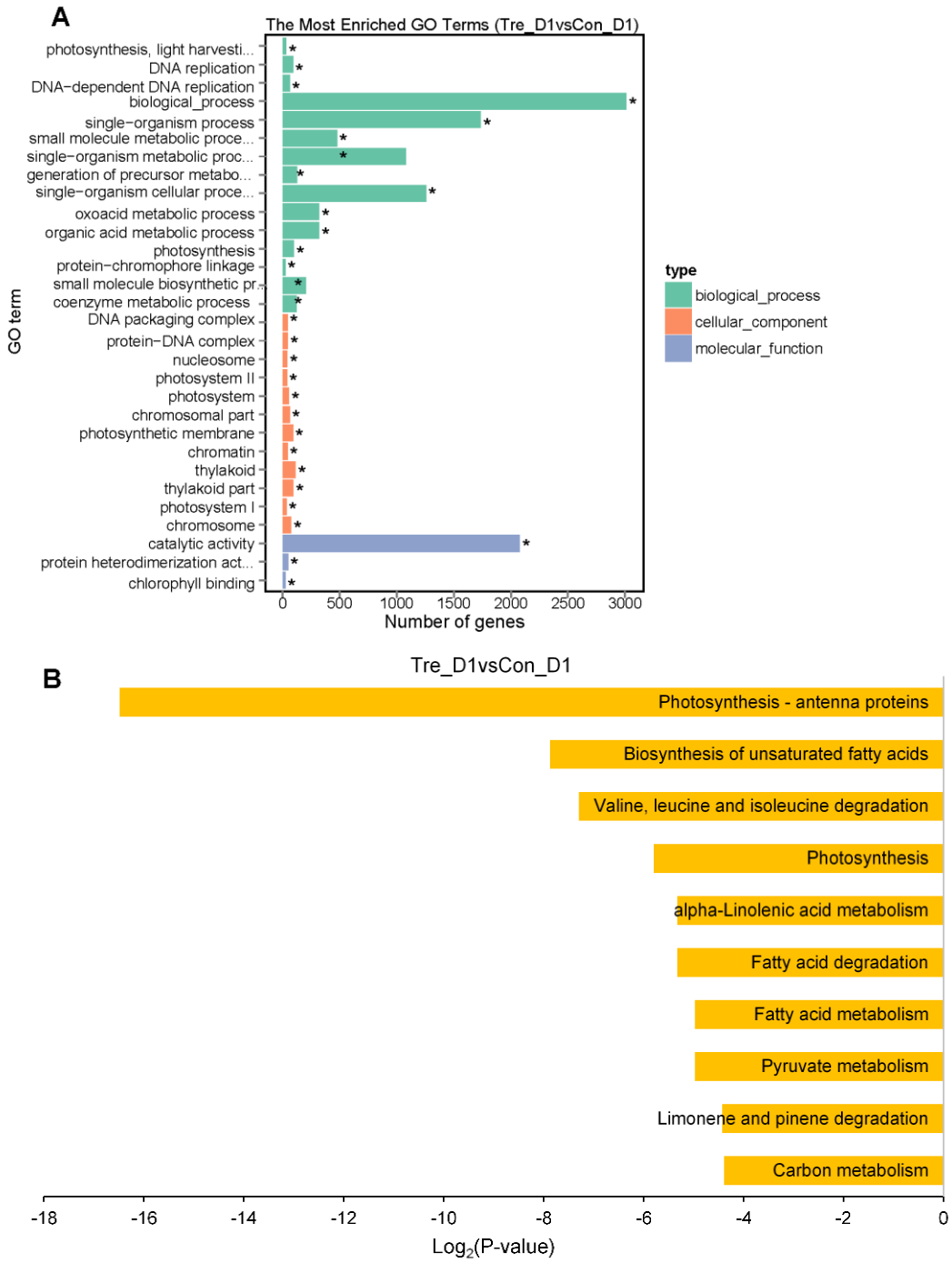

Figure 3. GO and KEGG enrichment analysis of DEGs in 1-MCP-treated tomatoes. (A) The top thirty most enriched GO terms in 1-MCP treated (Tre-D1) compared to Con-D1. The x-axis is the number of differentially expressed genes and the y-axis is GO terms enriched. Asterisks (*) indicate significantly enriched GO terms. Different colors are used to identify biological process. (B) The top ten most enriched KEGG pathways in 1-MCP treated (Tre-D1) compared to untreated (Con-D1) tomatoes

3.4 Analysis of Genes Involved in Ethylene Biosynthesis and Signal Transduction Pathways

To investigate whether 1-MCP inhibited ethylene synthesis, genes involved in ethylene pathways were extracted from transcriptomic data (with adjusted p value <0.05) and compared (Figure 4 and Table 3). The expression level of two S-adenosylmethionine synthase genes (Solyc10g083970 and Solyc09g008280) was 3.14 and 7.04-fold elevated, respectively, whereas the expression level of the S-adenosylmethionine synthase 1 gene

(*Solyc01g101060*) was not altered significantly. Three genes (*Solyc01g095080*, *Solyc08g081550*, and *Solyc08g081540*) encoding for 1-aminocyclopropane-1-carboxylate synthase were all up-regulated 6 to 8-fold whereas one gene was down-regulated significantly. Of the 5 genes encoding for 1-aminocyclopropane-1-carboxylate oxidase, the expression level of one gene (*Solyc02g081190*) encoding for 1-aminocyclopropane-1-carboxylate oxidase 4 was 5.6-fold up-regulated whereas the expression levels of other three genes (*Solyc09g089580*, *Solyc07g049550* and *Solyc07g026650*) were significantly down-regulated (0.02-0.1 fold).

In our study, the expression levels of two ethylene receptors, ethylene receptor homolog (ETR4) (*Solyc06g053710*) and Never ripe (*Solyc09g075440*) were significantly down regulated (0.07 and 0.15, respectively) in 1-MCP-treated tomato fruits. Our data indicate that 1-MCP binds to specific ethylene receptors, possibly ETR4 and Never ripe, therefore reducing their production.

The ethylene signal transduction pathway ultimately leads to the activation of transcriptional regulators belonging to the Ethylene Response Factor (ERF) family of transcription factors. In our study, of the 9 ERFs, 5 of them (*Solyc07g008250*, *Solyc12g009560*, *Solyc01g065980*, *Solyc02g077370* and *Solyc09g066360*) were significantly down-regulated (0.02 to 0.15) whereas two of them (*Solyc04g051360* and *Solyc04g014530*) were significantly up-regulated (350 and 72-fold, respectively) in 1-MCP-treated tomato fruits. Our data suggest that 1-MCP delayed ripening by inhibiting ERFs, therefore inhibiting ripening-related gene expression.

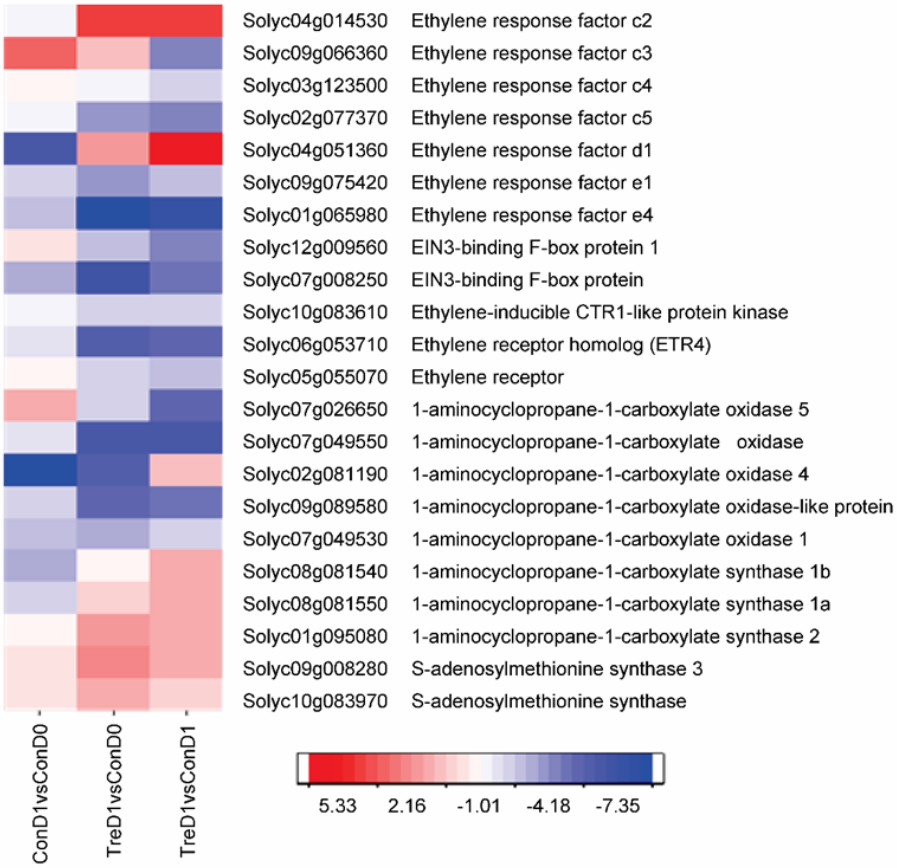

Figure 4. Heat map of the differentially expressed genes (DEGs) in ethylene synthesis and signal transduction after 1-MCP treatment. The RPKM values were normalized and converted to Z-scores to scale the gene expression levels. Red and blue colors indicate high and low expression, respectively

Table 3. DEGs involved in ethylene synthesis and signal transduction

Gene ID	Annotation	TreD1vsConD1	
		Fold change	P value
Solyc10g083970	S-adenosylmethionine synthase	3.14	0.001
Solyc09g008280	S-adenosylmethionine synthase 3	7.04	1.70E-08
Solyc01g101060	S-adenosylmethionine synthase 1	1.37	0.05
Solyc01g095080	1-aminocyclopropane-1-carboxylate synthase 2	6.99	2.43E-25
Solyc08g081550	1-aminocyclopropane-1-carboxylate synthase 1a	8.16	0.05
Solyc08g081540	1-aminocyclopropane-1-carboxylate synthase 1b	6.69	8.77E-10
Solyc07g049530	1-aminocyclopropane-1-carboxylate oxidase 1	0.54	0.05
Solyc09g089580	1-aminocyclopropane-1-carboxylate oxidase-like protein	0.10	4.89E-49
Solyc02g081190	1-aminocyclopropane-1-carboxylate oxidase 4	5.58	0.0001
Solyc07g049550	1-aminocyclopropane-1-carboxylate oxidase	0.03	1.62E-91
Solyc07g026650	1-aminocyclopropane-1-carboxylate oxidase 5	0.07	4.30E-48
Solyc05g055070	Ethylene receptor	0.38	1.48E-10
Solyc06g053710	Ethylene receptor homolog (ETR4)	0.07	2.83E-60
Solyc09g075440	Never ripe	0.15	2.38E-12
Solyc10g083610	Ethylene-inducible CTR1-like protein kinase	0.55	2.22E-05
Solyc07g008250	EIN3-binding F-box protein	0.10	2.26E-46
Solyc12g009560	EIN3-binding F-box protein 1	0.15	2.78E-05
Solyc01g065980	Ethylene response factor e4	0.02	1.78E-105
Solyc09g075420	Ethylene response factor e1	0.42	3.07E-08
Solyc04g051360	Ethylene response factor d1	350.08	1.84E-05
Solyc02g077370	Ethylene response factor c5	0.15	0.0003
Solyc03g123500	Ethylene response factor c4	0.59	0.0002
Solyc09g066360	Ethylene response factor c3	0.13	0.008
Solyc04g014530	Ethylene response factor c2	71.82	1.06E-07

3.5 Analysis of Genes Involved in Carotenoid Biosynthesis in 1-MCP-treated Tomato Fruits

The expression levels of the genes encoding the enzymes associated with carotenoid metabolism were investigated (Table 4). The expression level of the gene encoding for phytoene synthase 1 (*psy1*) (*Solyc03g031860*) was significantly inhibited (only 8% compared to untreated) in 1-MCP treated tomato fruits. PSY1 catalyzes the first critical step in the carotenoid biosynthetic pathway, and down-regulation of this gene abolishes normal carotenoid accumulation (Bartley et al., 1992; Fray and Grierson, 1993). On the other hand, the expression level of the gene (Solyc03g007960) encoding for Beta-carotene hydroxylase-2 was 13-fold elevated (Table 4).

Table 4. DEGs associated with carotenoid metabolism

Gene ID	Annotation	TreD1vsConD1	
		Fold change	P value
Solyc03g031860	Phytoene synthase 1	0.08	7.27E-57
Solyc04g040190	Lycopene beta-cyclase	1.81	0.01
Solyc03g007960	Beta-carotene hydroxylase-2	13.03	3.70E-41

3.6 Analysis of Genes Involved in Cell Wall Degradation

The expression levels of genes involved in cell wall degradation were investigated in the RNA-Seq experiments (Table 5). Of the 8 genes related with cell wall metabolism, *Solyc07g017600* and *Solyc09g010210* encoding for pectinesterase and Endo-1, 4-beta-glucanase precursor were highly expressed in 1-MCP-treated tomato fruits.

Table 5. DEGs associated with cell wall degradation

Gene ID	Annotation	TreD1vsConD1	
		Fold change	P value
Solyc07g017600	Pectinesterase	38.468	9.03E-24
Solyc09g010210	Endo-1,4-beta-glucanase precursor	60.597	8.32E-05
Solyc02g091680	Probable beta-D-xylosidase 6-like	0.620	0.01
Solyc01g104950	Beta-xylosidase	0.245	0.01
Solyc10g047030	Beta-D-xylosidase 1 precursor	0.109	0.04
Solyc09g005850	Probable pectate lyase 4-like	2.170	1.86E-05
Solyc03g031840	Expansin precursor	1.635	0.02
Solyc06g051800	Expansin 1	2.453	0.02

3.7 Analysis of Genes Involved in Cell Wall Degradation

DNA methylation has been shown to be involved in fruit ripening (Gallusci et al., 2016). Genomic DNA cytosine methylation from tomato pericarps was decreased prior to ripening initiation (Cao et al., 2014). The expression level of *Solyc12g100330.1* was 17-fold elevated in 1-MCP treated tomato fruits (Table 6). *Solyc12g100330.1* encodes for cytosine-5 DNA methyltransferase (C5-MTase) in tomato. High expression of this gene may contribute to maintaining of high DNA methylation levels in the nucleus, which delayed fruit ripening.

Table 6. DEGs associated with DNA methylation

Gene ID	Annotation	TreD1vsConD1	
		Fold change	P value
Solyc12g100330.1	Cytosine-specific methyltransferase	1.82	0.0002
Solyc01g006100.2	Cytosine-specific methyltransferase	17.17	3.43E-08
Solyc08g005400.2	Cytosine-specific methyltransferase	2.18	3.74E-05
Solyc02g062740.2	DNA (Cytosine-5-)-methyltransferase 3	0.67	0.008
Solyc05g053260.2	DNA methyltransferase	0.68	0.03
Solyc09g009080.2	Repressor of silencing 1	2.91	4.04E-09
Solyc11g007580.1	HhH-GPD family protein	1.54	0.006

3.8 Validation of Selected DEGs by qRT-PCR

RT-PCR was used to validate the gene expression data obtained from RNA-Seq experiments. Fifteen genes with different expression levels were randomly selected from transcriptomic data and tested by qRT-PCR assays. The expression levels of all tested genes in 1-MCP treatments showed similar expression tendency between RNA-Seq and qRT-PCR, and there was a high correlation (R^2=0.91) between qRT-PCR and RNA-Seq data (Figure 5). Our data suggest that the transcriptomic data obtained from RNA-Seq experiments were very reliable.

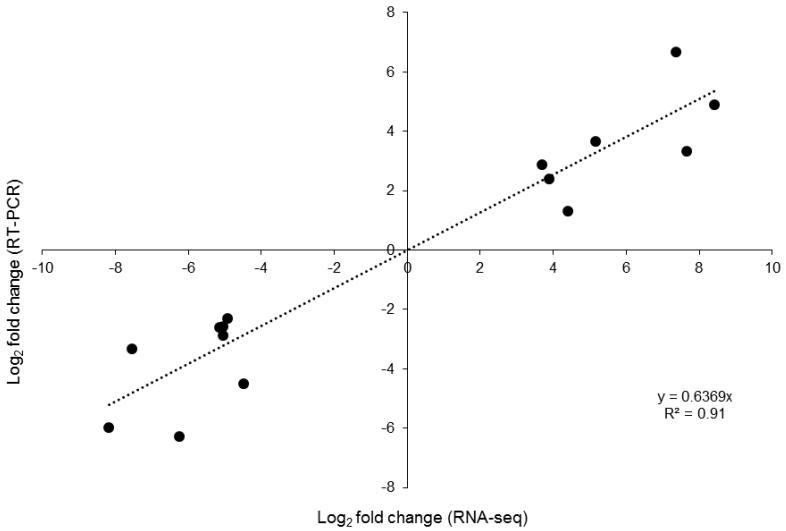

Figure 5. Correlation of gene expression data from RNA-Seq and qRT-PCR

4. Discussion

The ethylene biosynthesis pathway is well studied in higher plants (Giovannoni et al., 2017). S-adenosylmethinin synthase, aminoclyclopr opane-1-carboxylate synthase, and 1-aminocyclopropane-1-carboxylate oxidase are the three key enzymes involved in ethylene synthesis. Although the expression levels of genes encoding for S-adenosylmethionine synthase and 1-aminocyclopropane-1-carboxylate synthase were higher in 1-MCP treated fruits, the expression levels of three genes encoding for 1-aminocyclopropane-1-carboxylate oxidase were inhibited significantly. The down-regulation of the gene encoding for 1-aminocyclopropane-1-carboxylate oxidase may influence the low production of ethylene. Our data were consistent with previous data showing that the production of ethylene was inhibited by 1-MCP in tomato fruits (Tassoni et al., 2006).

Ethylene regulates fruit ripening through binding to ethylene receptors and activating signal transduction pathways (Alexander & Grierson, 2002). The ethylene signal transduction pathway is negatively regulated by ethylene receptors (Hua & Meyerowitz, 1998). In the absence of ethylene, ethylene receptors actively inhibit ethylene responses. After ethylene binds to receptors, the receptor undergoes a conformational change from the active to inactive state, suppression is removed, and the ethylene response occurs. There are six known receptors in tomato (Klee & Tieman, 2002). Previous studies suggested that 1-MCP inhibited ethylene function by competing with ethylene receptors (Sisler, 2006; Serek et al., 1995). A model was proposed for 1-MCP action. In this model, 1-MCP binds irreversibly to ethylene receptors, preventing ethylene binding and keeping the receptor in the active signaling state, therefore, inhibiting ripening (Binder & Bleecker, 2003). This model was supported by the fact that 1-MCP inhibited gene expression of ethylene receptors.

The color of the pigment in tomato fruits is determined mainly by chlorophyll and carotenoids. In tomato ripening, the color of tomatoes changes from green to red with pigmentation containing carotenoids and lycopene. Tomato ripening is followed by elevated transcription of several genes encoding enzymes involved in the biosynthesis of carotenoids (Bramley, 2002). Although there was no color difference between 1-MCP treated (Day 1) and untreated tomatoes (Day 1) (Figure 1), the reduced expression of *Solyc03g031860* correlated with the significant color change delay in 1-MCP-treated tomato fruits in later stages (Figure 1). In addition, 1-MCP has been shown to inhibit carotenoid biosynthesis in papaya (Fabi et al., 2007; Shen et al., 2017). 1-MCP also inhibited the expression of the *psy* gene and carotenoid accumulation in apricot (Kita et al., 2007). β-carotene hydroxylase-2 catalyzes hydroxylation reactions that convert beta-carotene to zeaxanthin. Although majority of carotenoid biosynthesis is regulated at the transcriptional level, post-transcriptional regulation, including feedback inhibition has also been reported (Bramley, 2002). The lack of correlation at the gene expression level may indicate some post-transcriptional regulation.

Fruit ripening is also associated with cell wall softening. Pectinesterase is an important enzyme responsible for pectin demethylation that is involved in cell-wall softening. Silencing of this gene in tomato has been shown to enhance softening in fruit ripening (Phan et al., 2007). Since 1-MCP inhibited fruit ripening, its expression was also elevated. In agreement with our gene expression data, the protein level of pectinesterase was also elevated after 1-MCP treatment in papaya fruit (Huerta-Ocampo et al., 2012).

Our results showed that 1-MCP treatment results in the down-regulation of a number of fruit ripening related genes, such as those involved in ethylene synthesis and signal transduction, cell wall synthesis, and pigment synthesis. This represents the first transcriptomic study of the effect of 1-MCP in tomato fruits. Our results also provide insight at the genome level on gene regulation by 1-MCP during fruit ripening and provide a better understanding of the role of ethylene in tomato ripening. Understanding the molecular basis of MCP action can help farmers and food processors to better use 1-MCP in the food industry.

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