

Article

Gene Expression in 1-Methylcyclopropene (1-MCP) Treated Tomatoes during Pre-Climacteric Ripening Suggests Shared Regulation of Methionine Biosynthesis, Ethylene Production and Respiration

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Abstract: The physiology of fruit ripening is defined as either ‘climacteric’ or ‘non-climacteric’. In climacteric fruit respiration during ripening increases until it reaches a peak, which is accompanied by an increase in autocatalytic ethylene production, whereas the respiration of non-climacteric fruit does not increase and they have no requirement for ethylene to complete their ripening. In an attempt to gain further insight into the involvement of autocatalytic ethylene production with the climacteric rise in respiration, tomato fruit were harvested at three defined stages of maturity prior to the climacteric peak (mature green, breaker, and early orange) and immediately exposed to the gaseous molecule 1-methylcyclopropene (1-MCP). The gene expression profile at each of these stages was monitored after 24 h, using an Affymetrix tomato microarray chip. This approach enabled us to identify ethylene responsive genes that are commonly regulated at early stages of ripening, as well as new candidate genes. In addition, 1-MCP treatment affected the levels of metabolites related to methionine biosynthesis. Methionine feeds climacteric ethylene production and we found that promoters of the genes of enzymes that catalyze the production of homoserine and homocysteine (aspartokinase/homoserine dehydrogenases and cystathionine beta lyase, respectively), precursors in the methionine pathway, contain the AtSR1 binding motif. This binding motif is recognized by ethylene activated transcription factors, hence indicating a role for ethylene in methionine synthesis during early ripening, explaining the autocatalytic ethylene production during subsequent ripening stages.

Keywords: tomato; ethylene; climacteric ripening; 1-methylcyclopropene

1. Introduction

The physiology of fruit ripening is defined as either ‘climacteric’ or ‘non-climacteric’. In climacteric ripening, respiration reaches a peak, which is accompanied by an autocatalytic increase in ethylene production, whereas the respiration of non-climacteric fruit does not increase and only minimal quantities of ethylene are commonly produced [1,2]. For example, tomato, banana, avocado, apple, pear are climacteric fruit and citrus, grape, strawberry, pepper are non-climacteric fruit. Application of exogenous ethylene to mature climacteric fruit stimulates ethylene biosynthesis and induces the onset

of the rise in respiration, which accompanies fruit ripening, while non-climacteric fruit at any stage of maturity respond with a transient increase in respiration, often without any obvious change in ripening, apart from chlorophyll degradation, as observed in citrus fruit [3]. Climacteric fruit are commercially harvested at completion of their growth and ethylene plays a major role in the ripening process during subsequent storage and marketing. Ethylene is required not only for the initiation of ripening and softening but also for their advancement [4]. As non-climacteric fruit do not continue to ripen after detachment from the plant, the changes occurring can be described as senescence. The ethylene biosynthetic pathway is well understood; however, there is still a lot to be disclosed and understood regarding its role and regulation during climacteric ripening.

Tomato fruit serve as a model for climacteric fruit ripening and many orthologous genes related to tomato ripening have been identified in various climacteric and non-climacteric fruit [5–7]. The conservation of ripening mechanisms is impressive, considering the significant differences between fruits in their morphological, physiological, and biochemical characteristics. Discovery of the ripening control mechanisms has disclosed primary ripening agents, including transcription factors, such as those encoded by RIN (ripening-inhibitor), a MADS-box gene, and CNR (colourless non-ripening), a SPB-box gene, which are necessary for the progress of virtually all ripening processes and downstream signal transduction components that impact the hormonal and environmental stimuli leading to coordination and modulation of different ripening phenotypes [8].

The gaseous molecule 1-MCP (1-methylcyclopropene) binds to the ethylene receptors and effectively inhibits ethylene action. In the postharvest context, 1-MCP retards fruit respiration, inhibits ethylene production, and delays ripening [9]. Accordingly, application of 1-MCP to pears improved their keeping quality during storage [10]. Tiwari and Paliyath [11] analyzed gene expression of MG tomatoes ten days after 1-MCP treatment, using the TOM2 tomato oligo-array. Yan et al. [12] studied the effect of 1-MCP on gene expression in detached, ripening-impaired *rin* and *nor* tomato mutants, when applied at the BR stage and sampled three days after treatment. Another experiment in which tomatoes were exposed to 1-MCP was performed by Bobokalonov et al. [13]. They applied RNA-Seq analysis to tomatoes at MG, one day after the treatment. In the present research, tomato fruit were exposed to 1-MCP immediately after harvest at three ripening stages, MG, BR, and EO, and gene expression was monitored one day later, using tomato microarray chips. The objective of this experiment was to identify, in a more accurate approach than in previous studies, which genes are regulated by ethylene at different ripening stages, prior to the climacteric peak. We also analyzed metabolites in the 1-MCP treated tomatoes and found changes in the aspartate family, related to methionine biosynthesis, suggesting their involvement in the ripening process, possibly by promoting ethylene production. In addition, the AtSR1 (*Arabidopsis thaliana* signal responsive 1) binding motif that is recognized by an early ripening ethylene induced transcription factor was identified in a few methionine and ethylene biosynthetic pathway genes.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Tomatoes (*Solanum lycopersicum* cv. Ailsa Craig) were grown in an open field. Fruit at mature green (MG), breaker (BR), and early orange (EO) stages were harvested in the early morning hours and immediately transferred to the laboratory. Tomatoes of each ripening stage were equally divided into two groups: one group was treated with 1-MCP while the second group remained untreated and served as a control. Two biological replicates (each replicate was a pool of ten fruit) of 1-MCP treated tomatoes at each ripening stage and three biological replicates of untreated tomatoes were frozen in liquid nitrogen and stored at -80°C until analysis.

2.2. 1-MCP Treatment

1-MCP treatment was applied as described by Gamrasni et al. [14]. In brief, 1-MCP powder (Rohm and Haas, Philadelphia, PA, USA) containing 0.14% active ingredient, was dissolved in an airtight calibration bottle with warm water to a final concentration of 1000 $\mu\text{L L}^{-1}$ 1-MCP (stock solution). In addition, 30 mL of the stock solution were injected into a 30 L airtight barrel containing the tomatoes to obtain a final gaseous concentration of 1 $\mu\text{L L}^{-1}$ 1-MCP. Tomatoes at similar ripening stages were enclosed in airtight barrels, under identical conditions served as a control. After 20 h at 20 °C, the barrels were opened and the fruit were aerated for another 24 h at 20 °C, after which samples were frozen in liquid nitrogen for RNA extraction.

2.3. RNA Extraction, Gene Chip Analysis, and Data Analysis

Total RNA extraction, microarray analysis, and functional annotation were conducted as described by Itkin et al. [15]. In brief, total RNA was extracted using the hot phenol method [16], treated with DNase 1 (Sigma). cDNA was synthesized with Invitrogen SuperScript II reverse transcriptase and hybridized to the Affymetrix GeneChip[®] Tomato Genome Array (10,209 genes) as described in the Affymetrix technical manual (available at <http://www.affymetrix.com>). Statistical analysis of the microarray data was performed using the Partek[®] Genomics Suite (Partek, www.partek.com) and the Robust Microarray Averaging (RMA) algorithm [17]. Changes in expression level were determined by ANOVA analysis. False discovery rate (FDR) was applied to correct for multiple comparisons [18]. Differentially expressed genes were chosen according to $\text{FDR} < 0.05$ and there was at least a two-fold difference between 1-MCP treated or untreated samples at the same ripening stage, with a signal above background in at least one of the microarray chips. Functional annotation analysis was performed manually, using publicly available databases.

2.4. Real-Time PCR Validation of Microarray Data

To confirm the reliability of the microarray results, real time PCR was applied to selected genes from each ripening stage and treatment. The following genes were chosen: chalcone isomerase (Les.1968.1.A1_at), ACC synthase 4 (Les.3661.1.S1_at), ACC oxidase homologue (Les.3225.3.S1_at), SAM synthase1 (Les.226.1.S1_at), lipoxygenase C (Les.3980.1.S1_at), and pectate lyase (Les.5579.1.S1_at). A high and significant correlation indicated consistency between the two methods (Figure S1).

2.5. Extraction and Analysis of Free Amino Acids and Derivatives

Samples of amino acid content in fruit pericarp were prepared according to Golan et al. [19], and the extractions were analyzed by GC-MS according to Katz et al. [20] for the detection of free amino acids and their derivatives.

2.6. Detection of the AtSR1 Binding Motif Sequence

A search of the tomato genome sequence revealed that the (A/C/G)CGCG(G/T/C) element was present in the promoter regions of the methionine and ethylene biosynthesis pathway genes [21]. These genes were retrieved from the NCBI genome database and SOL Genomics (<https://solgenomics.net>). The promoter regions are assumed to be within 3 kb upstream the first ATG sequence.

2.7. Gas Chromatography Analysis of Ethylene Production

Fruit were held at 20 °C, 70% RH for 18 days. For monitoring ethylene production, three replicates of individual fruit were enclosed every other day for 2 h in 600 mL airtight containers. Two 2.5 mL samples were withdrawn with a syringe from the headspace of each container and injected into a Varian 300 gas chromatograph (GC), equipped with an alumina column and a flame ionization detector (FID). Temperatures of the injector, column, and detector were 85 °C, 75 °C, and 65 °C, respectively. Ethylene production was expressed as $\text{pmole kg}^{-1} \text{ s}^{-1}$.

3. Results

3.1. Physiological Effects of 1-MCP Treatment on Tomatoes at Pre-Climacteric Stages

Tomatoes at three ripening stages MG, EO, and BR, all before the climacteric peak, were treated with 1-MCP. In addition, 1-MCP delayed peel color alteration noticeably in EO tomatoes and slightly in BR tomatoes, while no visual alteration was observed in MG tomatoes (Figure 1). The ethylene peak was delayed following 1-MCP treatment of MG, Br, and EO tomatoes by five, seven, and two days, respectively (Figure 2). These results confirmed the efficacy of the treatment.

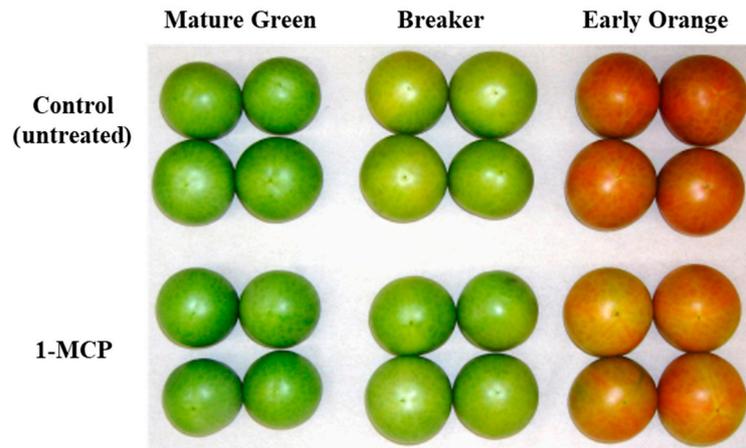


Figure 1. Tomatoes harvested at different ripening stages and treated with 1-MCP (untreated fruit served as control), after 24 h at 20 °C, the time of sampling for molecular analysis.

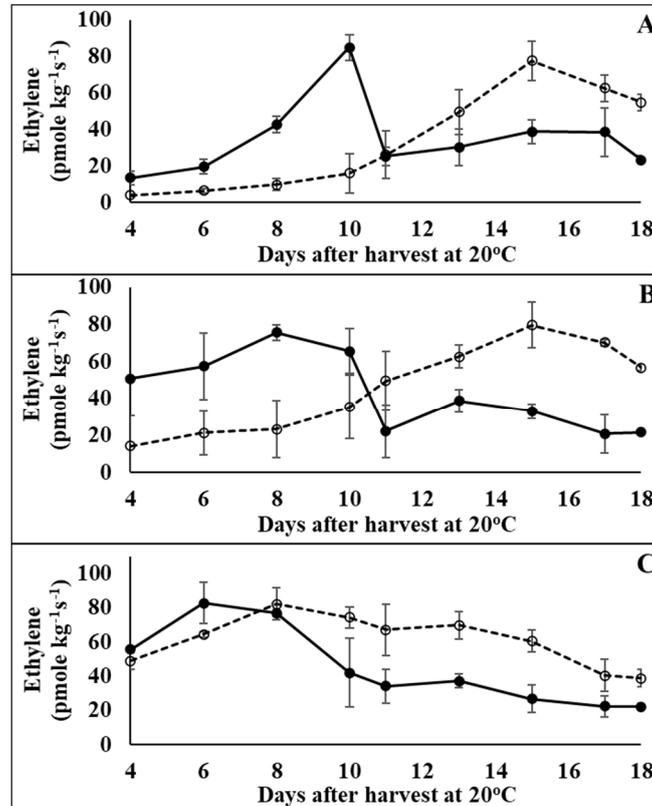


Figure 2. Ethylene production rate of 1-MCP treated and untreated tomatoes (control) after harvest at different maturity stages. (A) Mature green, (B) Breaker, (C) Early orange. Full lines—Untreated tomatoes, broken lines- 1-MCP treated tomatoes. Values are mean ± SE bars (*n* = 3).

3.2. The Effect of 1-MCP Treatment on the Expression Pattern of Tomatoes at Pre- Climacteric Stages

The differentially expressed genes data of 1-MCP treated tomatoes at the three pre- climacteric stages were analyzed by two-way hierarchical clustering. Two major branches were apparent for the samples: one that included 1-MCP treated MG and BR tomatoes and untreated MG tomatoes and the other that contained 1-MCP treated EO and untreated BR and EO tomatoes (Figure 3). The presence of 1-MCP treated BR tomatoes in one group and untreated BR in the other suggested the most distinct effect of the treatment was at BR (Figure 3).

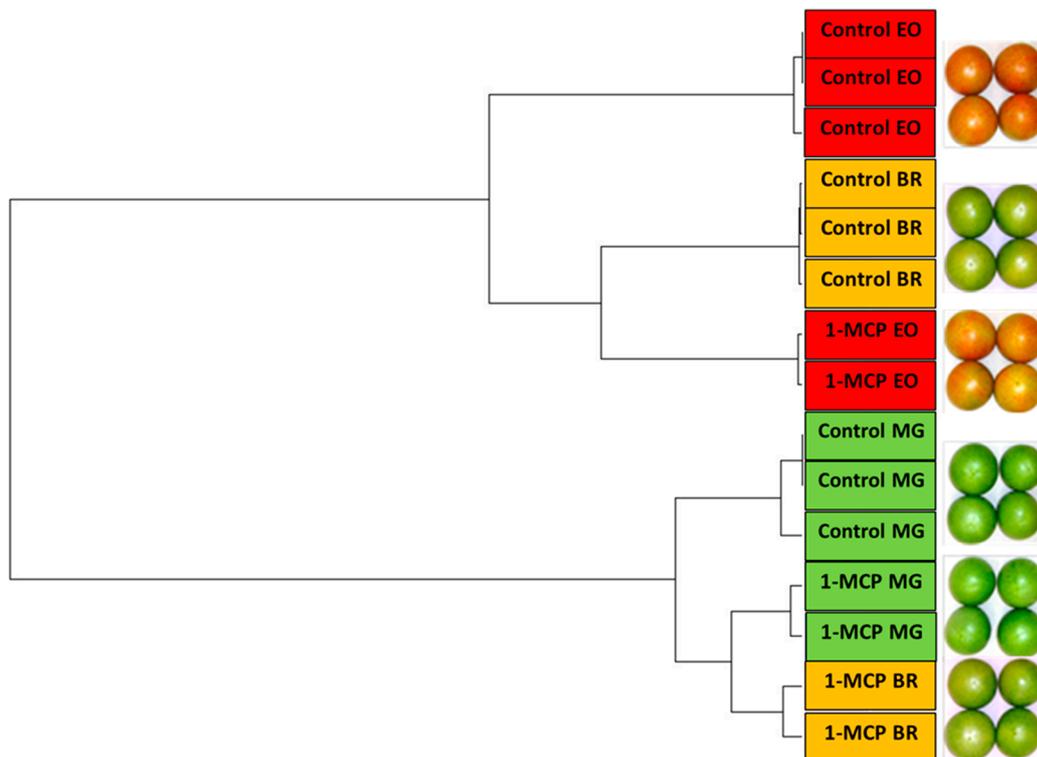


Figure 3. Clustering analysis of samples. The distance in the dendrogram was calculated using pairwise Pearson's dissimilarities between differentially expressed genes (fold change of ± 2 , FDR < 0.05). The clustering was performed using the made4 package of R statistical software. For each maturity stage, two replicates of 1-MCP treated and three replicates of untreated tomatoes were used. MG-mature green, BR-breaker, EO-early orange.

Another outcome of the analysis was that 1-MCP treated tomatoes had the highest similarity to untreated tomatoes of an earlier ripening stage, namely: 1-MCP treated BR—untreated MG and 1-MCP treated EO—untreated BR. This was as expected, since 1-MCP is known to slow down the ripening process.

3.3. 1-MCP Responsive Transcripts Common to Tomatoes at Pre- Climacteric Stages

Genes that displayed a two-fold expression difference between 1-MCP treated and untreated fruit at each ripening stage were subjected to Venn diagram analysis (Figure 4a,b, respectively). The overall 1-MCP upregulated and down regulated genes were 589 and 993, respectively. In addition, 51 upregulated and 85 downregulated genes were common to MG, BR, and EO (Figure 4a,b, respectively).

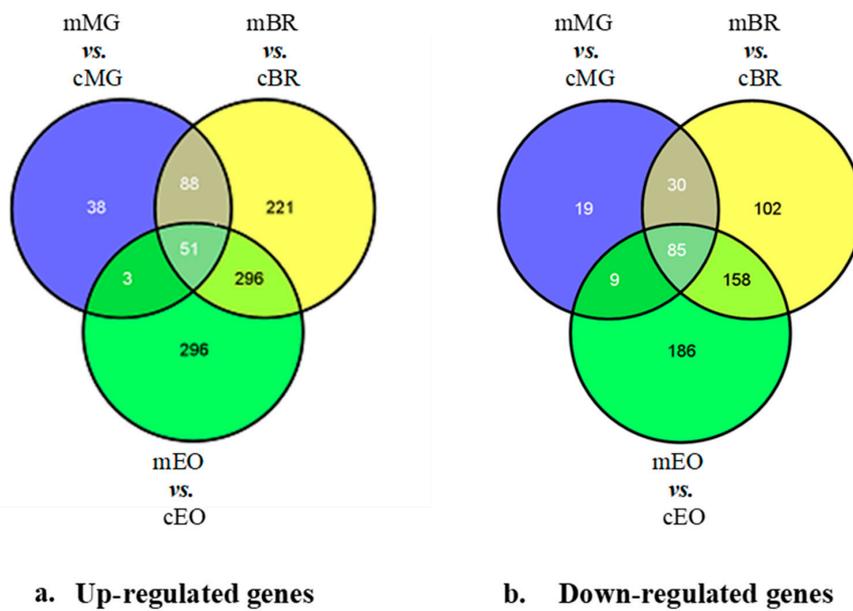


Figure 4. Venn diagram for 1-MCP treated vs. untreated tomato genes of the same ripening stage. (a) upregulated genes; (b) downregulated genes. The diagrams show the numbers of overlapping and non-overlapping genes differentially expressed. Each panel describes significantly up or down regulated transcripts as indicated (panel a ≥ 2 fold change, panel b ≤ 2 fold change), (adjusted $p < 0.05$). c—Control (untreated), m-1—MCP treated, MG—mature green, BR—breaker, EO—early orange.

The number of 1-MCP responsive transcripts increased dramatically as ripening progressed: in MG, 38 and 19, whereas in BR, 221 and 102 and in EO, 296 and 186 genes had higher or lower expression, respectively. The moderation in the magnitude of gene expression changes due to 1-MCP was greater at the transition from MG to BR than from BR to EO (Figure 5). This indicates a high responsiveness of the fruit to 1-MCP from MG to BR, which became much less from BR to EO.

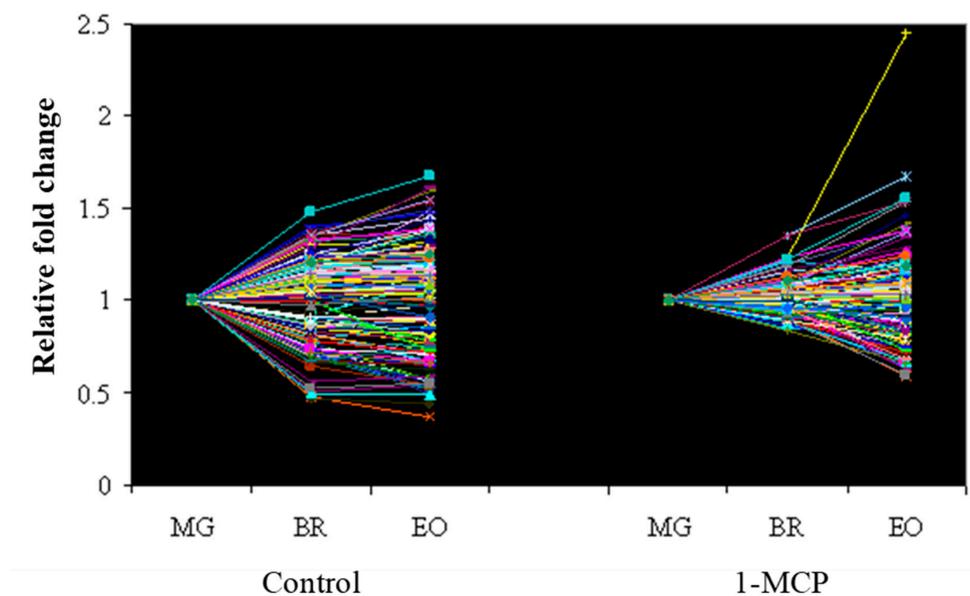


Figure 5. The difference in expression of 1-MCP responsive transcripts common to MG, BR, and EO tomatoes, between sequential ripening stages of 1-MCP treated or untreated tomatoes ($n = 136$). The expression of MG ripening stage served as a reference for the other stages in each comparison. MG—mature green, BR—breaker, EO—early orange.

3.4. Gene Ontology (GO) Analysis for 1-MCP Responsive Transcripts Shared at Different Ripening Stages

GO enrichment analysis of the differentially expressed genes (136) provides results in three term categories: Molecular Function (MF), Biological Process (BP), and Cellular Component (CC). The 'Tomato array GO term analysis' platform (TFGD: http://ted.bti.cornell.edu/cgi-bin/TFGD/array/GO_analysis.cgi) was applied for 1-MCP responsive transcripts that were common to MG, BR, and EO (Figure 4a,b). The enriched MF were a two-component sensor activity and a histidine kinase activity (Table 1). The BP of these transcripts was peptidyl-histidine phosphorylation and their CC was located on the endoplasmatic reticulum membrane. These transcripts included, among others, the ethylene receptors ETR2, ERS1, and the ethylene receptor homologs ETR4 and ETR5. Ethylene receptors are similar to the bacterial two-component histidine kinases, formed of a sensory histidine kinase and a response regulator domain [22]. The ethylene receptors are located at the endoplasmic reticulum membrane and, once ethylene is bound by the receptor, a signal transduction pathway is initiated [23].

Table 1. The gene ontology of 1-MCP responsive transcripts common to MG, BR, and EO tomatoes (n = 136). Corrected *p*-value according to Bonferroni with cutoff *p*-value < 0.05. MG—mature green, BR—breaker, EO—early orange.

Gene Ontology Terms Classification	Corrected <i>p</i> -Value
Molecular function	
two-component response regulator activity	<0.001
protein histidine kinase activity	<0.001
isocitrate dehydrogenase (NADP+) activity	<0.001
ion binding	0.041
oxidoreductase activity	0.009
monooxygenase activity	0.009
Biological process	
peptidyl-histidine phosphorylation	0.001
isocitrate metabolic process	0.002
glyoxylate cycle	0.028
response to hormone stimulus	0.034
response to endogenous stimulus	0.008
anatomical structure development	0.024
Cellular component	
endoplasmic reticulum membrane	0.02
extracellular region	<0.001

Another enriched term was related to the MF of isocitrate dehydrogenase (NADP+) and its metabolic processes in the glyoxylate cycle and ion binding, related to oxidoreductase activity and monooxygenase activity (Table 1). 1-MCP treatment reduced the expression level of the isocitrate dehydrogenase (NADP+) gene (*SIICDH1*) leading to moderation of the respiration rate [24,25].

Additional enriched functions were those belonging to the BP hormone stimulus response that included the terms endogenous stimulus and anatomical structure development. 1-MCP treatment enriched transcripts of hormones that regulate ripening related processes: ethylene, auxins, jasmonic acid, and gibberellin. The specific ethylene inhibition by 1-MCP was reported to influence the crosstalk between plant hormones affecting fruit metabolism and ripening [26].

The CC category was enriched with the term extracellular region, including the apoplast and the extracellular space. These included genes related to cell wall degradation (such as beta-galactosidase) and lipid metabolism (GDSL-esterase/lipase). The anatomical structure of the fruit could be affected by alteration of these biological processes.

3.5. Ripening Related Genes Common to Tomatoes at Pre-Climacteric Stages Affected by 1-MCP Treatment

As expected, the expression of ethylene biosynthesis and signal transduction related genes were affected by 1-MCP treatment (Table 2). ACS2 that executes the first step of ethylene biosynthesis was significantly reduced as were the ACC (1-aminocyclopropane-1-carboxylate) oxidation genes (ACO4, ACO5, ACO homolog transcript with a putative similar activity). The expression of ethylene receptors ETR2, ETR4, ETR5, and ERS1 was reduced. In addition, the expression of ethylene response factors ERF1 and ERF3 increased following 1-MCP treatment.

Table 2. The average fold change of genes related to ethylene biosynthesis and signal transduction, respiration, and amino acid metabolism that were affected by 1-MCP treatment at different ripening stages (for control samples $n = 3$, for 1-MCP samples $n = 2$).

Affymetrix Gene Code	MG	BR	EO	Annotation
les.3662.1.s1_at	−3.7	−5.1	−2.8	ACS2, 1-aminocyclopropane-1-carboxylate synthase 2
les.5917.1.s1_at	−7.1	−2.9	−6.0	ACO5, 1-aminocyclopropane-1-carboxylate oxidase 1
les.132.1.s1_at	−4.1	−12.8	−11.2	ACO4, 1-aminocyclopropane-1-carboxylate oxidase 4
les.244.2.s1_at	−5.6	−3.1	−2.3	1-aminocyclopropane-1-carboxylate oxidase homolog
lesaffx.18025.1.s1_at	−2.8	−7.8	−67.1	oxidoreductase, 2OG-Fe(II) oxygenase family protein
les.3465.1.s1_at	−24.8	−19.8	−8.2	ETR2, ethylene response 2 receptor
les.36.1.s1_at	−2.5	−3.0	−3.5	ETR4, ethylene response 4 receptor
les.35.1.s1_at	−3.7	−2.2	−2.3	ETR5, ethylene response 5 receptor
les.85.1.s1_at	−4.9	−7.2	−3.4	ERS1, ethylene-responsive sensor 1
les.876.1.a1_at	3.7	21.4	2.6	ERF1, Ethylene response factor 1
les.4101.2.s1_a_at	2.6	4.2	3.9	ERF2, Ethylene response factor 2
les.3311.3.s1_at	−2.6	−4.8	−5.6	ICDH1, Isocitrate dehydrogenase (NADP+)
les.2518.1.a1_at	−2.3	−5.7	−4.8	SAT-1, serine O-acetyltransferase
les.2518.2.a1_at	−2.4	−5.9	−8.6	SAT-1, serine O-acetyltransferase
les.2518.3.a1_at	−2.3	−5.0	−13.0	SAT-1, serine O-acetyltransferase
les.3323.1.s1_at	−3.3	−3.5	−2.9	CGS, cystathionine gamma synthase
LesAffx.67643.1.S1_at	−3.4	−19.5	−10.4	L-asparaginase
Les.290.1.S1_at	−69.9	−307.0	−17.3	HDC, histidine decarboxylase

Genes involved in amino acid metabolism were significantly reduced by 1-MCP treatment. This included the serine O-acetyltransferases (SATs) genes that catalyze the conversion of serine to cysteine, which later donates the sulfur moiety to methionine; the cystathionine gamma synthase gene (CGS)—the first enzyme unique to methionine biosynthesis; the asparaginase gene—the enzyme that hydrolyzes the amino acid asparagine to aspartate. These genes are involved in the biosynthesis of methionine, the precursor of ethylene, and can hence regulate its production. It should be noted that histidine decarboxylase was dramatically inhibited at all stages examined, but most extensively in BR fruit (300 fold).

3.6. Methionine Related Metabolites in 1-MCP Treated Tomatoes at Pre- Climacteric Stages

Climacteric ethylene production is an autocatalytic process fed by the essential amino acid methionine, a member of the aspartate family (Figure 6). We analyzed the metabolites in 1-MCP treated tomatoes to identify changes in methionine precursors during the pre-climacteric stages of ripening (Figure 7). Homoserine is the precursor for O-phosphohomoserine, the first committed metabolite for methionine synthesis. This was the only metabolite that was significantly higher in 1-MCP treated MG tomatoes (Figure 7b).

Homocysteine and methionine, metabolites downstream to homoserine, were significantly affected at BR by 1-MCP treatment (Figure 7c,d, respectively): the natural decline in homocysteine was noticeably prevented, probably due to an increase in its demand for ethylene synthesis at BR (Figure 7c), while methionine was less affected, probably due to strong regulation (Figure 7d).

Aspartate, the carbon skeleton for methionine biosynthesis, was somewhat higher (not significantly) in 1-MCP tomatoes in BR and EO (Figure 7a) and may reflect the increased demand for ethylene during climacteric ripening.

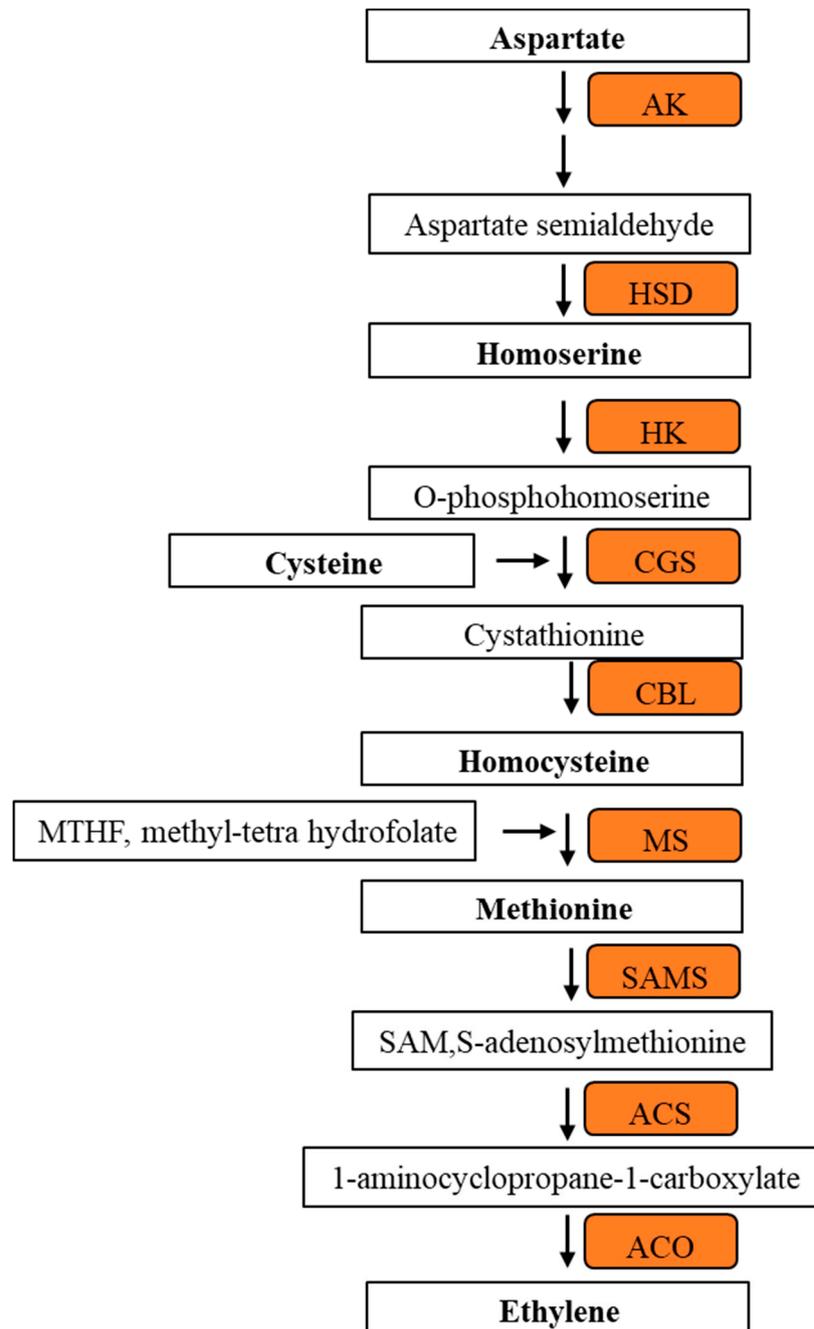


Figure 6. Metabolites of aspartate and additional precursors related to ethylene biosynthesis. The levels of metabolites in bold letters were measured during ripening. Abbreviations: AK, Aspartate kinase; HSD, homoserine dehydrogenase; HK, homoserine kinase; CGS, cystathionine-gamma-synthase; CBL, cystathionine-beta-lyase; MS, Methionine synthase; SAMS, S-adenosyl-methionine synthase; ACS, ACC synthase; ACO, ACC oxidase.

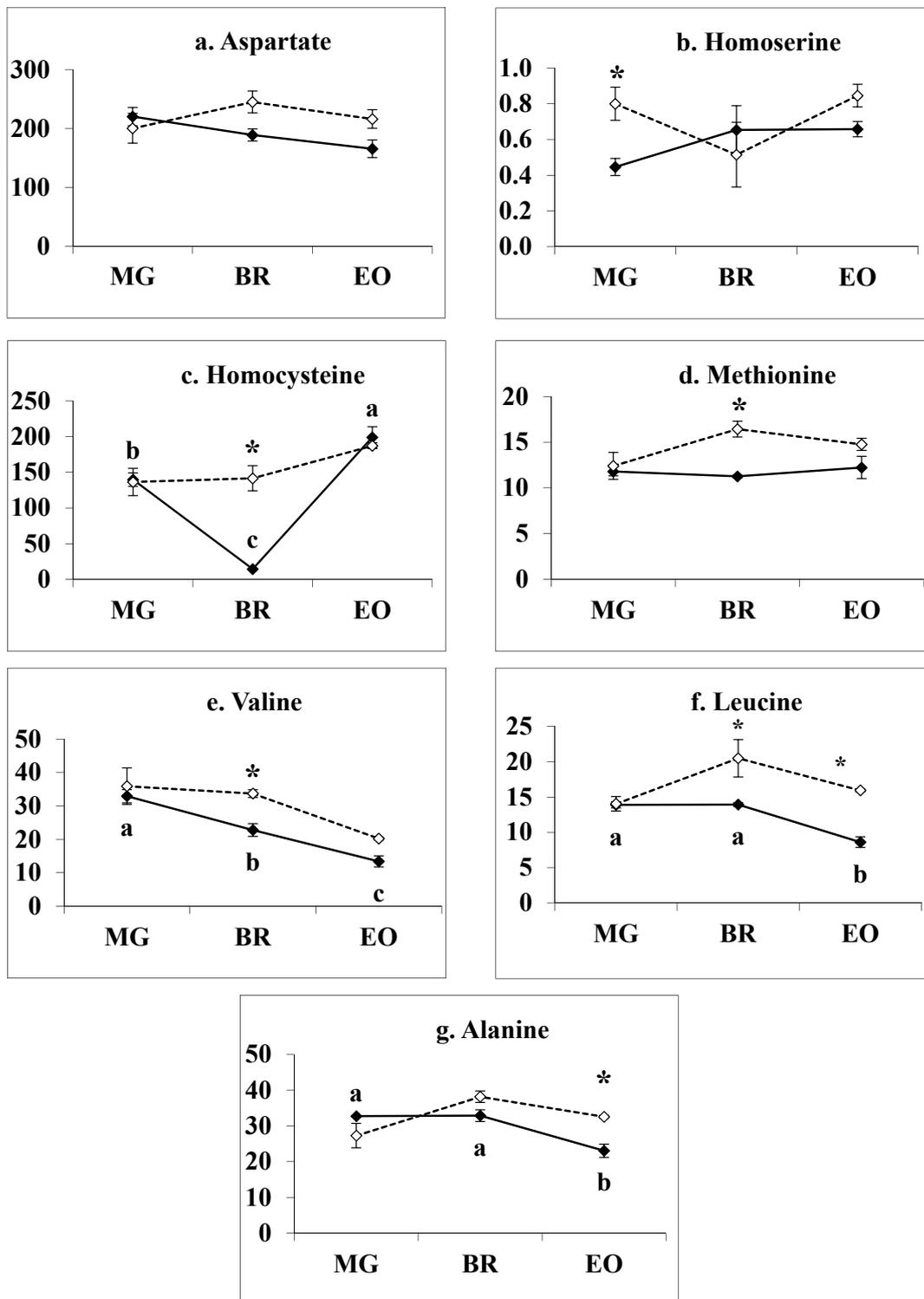


Figure 7. Intermediate metabolites and free amino acids (nmol gFW⁻¹) in ripening tomatoes, treated or untreated with 1-MCP. (a) aspartate, (b) homoserine, (c) homocysteine, (d) methionine, (e) valine, (f) leucine, and (g) alanine. The broken line represents the 1-MCP treated fruit and the full line represents the control fruit. MG—mature green, BR—breaker, EO—early orange. Values are mean ± SE bars (for control samples *n* = 3, for 1-MCP samples *n* = 2). (a–c) different letters represent significant differences between the ripening stages of the same treatment at *p* < 0.05 (Tukey HSD). * Asterisk represents significant difference between treatments at the same ripening stage at *p* < 0.05 (Student *t*-test). Letters/Asterisk were not added if there were no statistical differences.

3.7. Branch-Chain Amino Acids in 1-MCP Treated Tomatoes during Pre-Climacteric Ripening

The reduction in respiration is one of the major effects of 1-MCP treatment [27]. Pyruvate, the final metabolite of glycolysis and a precursor for the TCA cycle (tricarboxylic acid cycle), is the starting compound for the branched-chain amino acids valine, leucine, and alanine. In 1-MCP treated tomatoes, higher levels of the branched chain amino acids (Figure 7e–g, respectively) might result from reduced pyruvate demand.

3.8. AtSR1 Binding Motif in the Promoters of Methionine and Ethylene Biosynthesis Pathway Genes

Yang and Poovaiah [21] showed by gel retardation in *Arabidopsis thaliana* that AtSR1 targets the nucleus and specifically recognizes a novel 6-bp CGCG box (A/C/G)CGCG(G/T/C). Analysis of promoter regions upstream of genes of the methionine to ethylene biosynthesis pathway was applied to identify the AtSR1 binding motif. The motif was identified upstream of two aspartokinase/homoserine dehydrogenases and two cystathionine beta lyase genes that sensitize homoserine and homocysteine, respectively. In addition, the motif was identified in the promoter regions of two ACO and three ACS genes along the ethylene biosynthesis pathway (Table 3). The microarray chip that was used in our screen contains two of these genes: 1-aminocyclopropane-1-carboxylate synthase 1a (ACS1a) and 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1). The exposure to 1-MCP affected the mRNA level of ACS1a by -1.2, -1.4 and 1.9 fold changes, respectively for MG, BR, and EO tomatoes, and the ACO1 mRNA level by -2.1, -1.7 and -2.1 fold changes, respectively.

Table 3. The position of methionine and ethylene biosynthetic genes with an AtSR1-binding motif in the promoter region.

Description	Locus Name	Upstream to ATG
Aspartokinase/homoserine dehydrogenase	Solyc11g040390	−1004
Aspartokinase/homoserine dehydrogenase	Solyc11g072010	−1361
Cystathionine beta-lyase	Solyc04g055230	−691
Cystathionine beta-lyase	Solyc08g066620	−1598
* 1-aminocyclopropane-1-carboxylate synthase 1a	Solyc08g081550	−2191
1-aminocyclopropane-1-carboxylate synthase 3	Solyc02g091990	−352
1-aminocyclopropane-1-carboxylate synthase 8	Solyc03g043890	−309
* 1-aminocyclopropane-1-carboxylate oxidase 1	Solyc07g049530	−653
1-aminocyclopropane-1-carboxylate oxidase 2	Solyc12g005940	−630

* represents the genes that have AtSR1 binding motif in their regulation region and their expression was represented in the microarray chip.

4. Discussion

4.1. The Genetic Tomato Pre-Climacteric Response to 1-MCP

We aimed to deepen our understanding of ethylene regulated gene expression at the early stages of the ripening process and the beginning of the climacteric ethylene burst. Our research focused on gene expression, 24 h after 1-MCP treatment, of freshly harvested tomatoes at three pre-climacteric stages of ripening: MG, BR, and EO. 1-MCP treatment did not block ethylene production; however, it did postpone the ‘ethylene burst’ in the three pre-climacteric stages. The expression analysis provided a snapshot of the immediate changes of genes that are ethylene responsive.

In general, as expected, the gene expression pattern for each 1-MCP treated ripening stage resembled that of the previous untreated one, namely: 1-MCP BR tomatoes and MG untreated tomatoes had a similar expression profile and likewise 1-MCP EO tomatoes and BR untreated tomatoes had a comparable expression profile (Figure 3).

In addition, BR 1-MCP treated and non-treated tomatoes were divided, by hierarchical clustering, into different branches, indicating high receptiveness of this ripening stage to 1-MCP and consequently to ethylene. The largest impact of 1-MCP was at the transition from MG to BR (Figure 4). These two

observations support the perception that ethylene is needed to enhance gene expression at early stages of ripening while, at later stages, it is required to maintain it.

Venn diagrams enabled the detection of genes at different ripening stages that were significantly affected by 1-MCP (Figure 4). These genes probably play an important role in the ripening process and are ethylene regulated. GO analysis of these genes revealed two major significant enrichments that were detected at all examined ripening stages. The terms related to ethylene receptors (Table 1) and *SIICDH1*, the cytosolic isocitrate dehydrogenase, an NADP⁺ dependent gene that catalyzes the oxidative decarboxylation of isocitrate to 2-oxaloglutarate (Table 2), suggesting that these constitute the core impact of 1-MCP treatment.

Ethylene receptors have been described previously to play an important role in the early ripening process. We reported the reduction in the expression of ethylene receptor genes in 1-MCP treated fruit. Similar results were found by Mata et al. [28]. Hence, it could have been expected that this would lead to enhanced ripening as ethylene receptors negatively regulate ripening responses and their degradation controls the timing of ripening [29]. However, in contrast, the ripening of most 1-MCP treated fruit was delayed (Figure 1). This could be explained by a later work of Mata et al. suggesting that the receptor proteins are maintained due to post translation modifications [30].

The enrichment of cytosolic isocitrate dehydrogenase terms has not been previously indicated. We have suggested, based on virus induced gene silencing of *SIICDH*, that low *SIICDH1* levels lead to an accumulation of citrate, due to a decline in the conversion of citrate to 2-oxaloglutarate in the cytosol (the GABA shunt). Thereafter, citrate reduces phosphofruktokinase activity, leading to an increase of pyruvate, the initial metabolite of the TCA cycle and hence we concluded that *SIICDH1* is a key step in the initiation of climacteric respiration [24].

4.2. Methionine Biosynthesis Metabolites Involved in Climacteric Ethylene Induction

The amino acid methionine belongs to the aspartate family and donates the carbon skeleton for ethylene biosynthesis [31]. In an analysis of the metabolites of the methionine biosynthetic pathway, homoserine was significantly higher in MG tomatoes treated with 1-MCP than in untreated fruit. In addition, AtSR1 binding motif, which binds early responsive ethylene induced transcription factors, was found in the promoters of two aspartokinase/homoserine dehydrogenase genes that catalyze the biosynthesis of homoserine, indicating their regulation by early ethylene response factors. The availability of homoserine was reported to be the limiting factor for methionine biosynthesis [32].

Possibly, the tomato AtSR1 homologue induced by ethylene binds to the promoter regions of genes involved in methionine and ethylene biosynthesis that contain AtSR1 binding motif in the promoter (Table 3)—hence resulting in increased homoserine and subsequently a rise in ethylene production. To the best of our knowledge, this is the first report that proposes this pivotal role for homoserine in climacteric ethylene induction.

Homocysteine is the direct precursor for methionine. BR tomatoes exhibited a sharp decrease in homocysteine levels, compared to MG tomatoes, which was prevented by 1-MCP, probably due to reduced consumption for ethylene production. The AtSR1 binding motif was identified in the promoter regions of genes of the methionine and ethylene biosynthesis pathways (Table 3). Among them, *ACS1a* and *ACO1* were present in the microarray chip, and their expression in tomatoes that were exposed to 1-MCP was reduced at MG and BR. Similarly, in our previous report, we noted that *SIICDH1* contains AtSR1 binding motif and its expression was reduced at MG, and EO, and significantly at BR [24]. Thus, suggesting a coordinated regulation for the autocatalytic ethylene biosynthesis and respiration in which this motif, probably together with additional elements, takes part.

Katz et al. [20] showed a positive correlation between elevated ethylene production and increased CGS mRNA, the first enzyme unique to methionine biosynthesis, during the ethylene burst of tomato climacteric ripening. They suggested that de novo synthesis of methionine might be required in addition to the recycling of the methionine moieties, when ethylene is auto-catalytically produced. Here, we identified a significant reduced expression of CGS in 1-MCP treated tomatoes at different

ripening stages, but methionine levels were significantly affected only at BR (Figure 7c). This indicates a strong regulation of methionine levels, probably because it serves as an important precursor for many metabolites and biological processes [33,34].

4.3. Possible Shared Regulation of Respiration and Ethylene Production

An additional assumption for the effect of 1-MCP on the ripening process is related to the reduced expression of *SIICDH1*. An inhibition of respiration would reduce the consumption of pyruvate, the initial metabolite of the TCA cycle, and therefore its alternative products, such as alanine, would be elevated. Alanine is able to regulate the levels of pyruvate and affect the respiration rate [35]. The increase in the level of alanine detected in 1-MCP treated tomatoes (significantly in EO), is probably involved in the reduction of the respiration rate. Higher levels of valine and leucine, additional pyruvate products, were also detected in 1-MCP treated tomatoes. The effect of these metabolites on respiration during fruit ripening has not been reported; however, it was described that the oxidation of some amino acids, including valine and leucine, in other plant organs can directly feed electrons into the mitochondrial electron transport chain [36]. Hence, the high levels of valine and leucine could have accumulated due to reduced respiration in 1-MCP treated tomatoes.

In summary, we were able to widen our understanding of the climacteric ripening onset, most notably regarding the molecular association between autocatalytic ethylene production and climacteric respiration in MG.

In addition, the AtSR1 binding motif which was identified previously in *SIICDH1* [24] was also identified in methionine and ethylene biosynthesis genes (*HSD*, *CBL*, *ACS1a*, *ACS3*, *ACS8*, *ACO1* and *ACO2*), suggesting a possible coordinated regulation of ethylene production and respiration involving the tomato AtSR1 homologue (Figure 8).

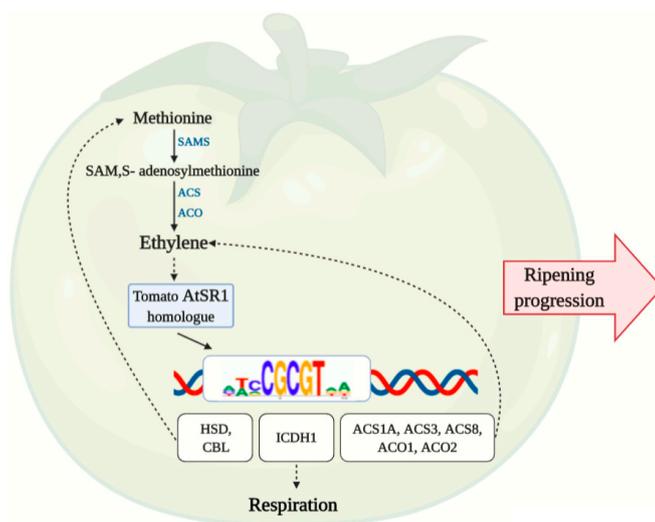


Figure 8. A proposed scheme for the synchronized regulation of fruit respiration and ethylene production during MG pre-climacteric stage by the tomato AtSR1 homologue. SAMS, S-adenosylmethionine synthase; ACS, ACC synthase; ACO, ACC oxidase; HSD, homoserine dehydrogenase; CBL, cystathionine-beta-lyase; ICDH1, Isocitrate dehydrogenase 1.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/11/1669/s1>. Figure S1: microarray gene expression validation with real time PCR. Selected transcripts of 1-MCP treated and untreated tomatoes at MG, BR, and EO ripening stages were referred to the untreated MG tomato gene expression that served as reference. The regression equation, correlation coefficient (r) and correlation significance (p) values between the methods were: $Y = 1.0494 X + 0.3854$, $r = 0.9132$, $p < 0.0001$, respectively.

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methodology, software and analysis, D.G., M.G. (Martin Goldway), E.F., A.R., M.G. (Michal Glikman) and A.T.A., writing—first draft preparation D.G. and M.G. (Martin Goldway); review and editing, D.G., M.G. (Martin Goldway), R.B.-A. and E.F.; supervision, M.G. (Martin Goldway) and A.A.; All authors have read and agreed to the published version of the manuscript.

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