The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene

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Abstract

Although the alternative oxidase (AOX) has been proposed to play a role in fruit development, the function of AOX in fruit ripening is unclear. To gain further insight into the role of AOX in tomato fruit ripening, transgenic tomato plants 3SS-AOX1a and 3SS-AOX-RNAi were generated. Tomato plants with reduced LeAOX levels exhibited retarded ripening; reduced carotenoids, respiration, and ethylene production; and the down-regulation of ripening-associated genes. Moreover, no apparent respiratory climacteric occurred in the AOX-reduced tomato fruit, indicating that AOX might play an important role in climacteric respiration. In contrast, the fruit that overexpressed LeAOX1a accumulated more lycopenone, though they displayed a similar pattern of ripening to wild-type fruit. Ethylene application promoted fruit ripening and anticipated ethylene production and respiration, including the alternative pathway respiration. Interestingly, the transgenic plants with reduced LeAOX levels failed to ripen after 1-methylcyclopene (1-MCP) treatment, while such inhibition was notably less effective in 3SS-AOX1a fruit. These findings indicate that AOX is involved in respiratory climacteric and ethylene-mediated fruit ripening of tomato.

Key words: Alternative oxidase, climacteric, ethylene, fruit ripening, tomato.

Introduction

Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes in colour, texture, flavour, and aroma (Alexander and Grierson, 2002; Klee, 2010). Fruit with different ripening mechanisms can be divided into two groups: climacteric fruit, in which ripening is accompanied by a peak in respiration and a concomitant burst of ethylene, and non-climacteric fruit, in which respiration shows no dramatic change and ethylene production remains at a very low level (White, 2002).

Ethylene has been identified as the major hormone that initiates and controls ripening in climacteric fruit, and its biosynthesis in plant tissues has been studied extensively (Srivastava and Handa, 2005; Argueso et al., 2007). Two systems of ethylene production have been defined in plants. System-1 represents basal ethylene in unripe fruit and vegetative tissues and is regulated in an auto-inhibitory manner, whereas system-2 operates during the ripening of climacteric fruit and flower senescence and is autocatalytic (Barry and Giovannoni, 2007; Yokotani et al., 2009). Studies have shown that the suppression of ethylene production by knocking down the expression of 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) and ACC synthase (ACS) resulted in a strong ripening inhibition (Hamilton et al., 1990; Oeller et al., 1991). Conversely, application of ethylene to climacteric fruit at the mature stage stimulates system-2 ethylene biosynthesis, accelerating ripening (Nakatsuaka et al., 1998).

It has been proposed that the alternative oxidase (AOX) pathway plays a role in fruit development (Kumar et al., 1990; Considine et al., 2001) but knowledge of its function is still
lacking. The AOX branches from the cytochrome c oxidase (COX) pathway at the level of ubiquinone (UQ) and couples the oxidation of ubiquinol to the four-electron reduction of oxygen to water in a manner insensitive to cyanide (CN; an inhibitor of COX) (Vanlerbergh and McIntosh, 1997; Affourtit et al., 2001). AOX is encoded by a small nuclear gene family. In tomato, four isoforms of two types of AOX have shown different expression patterns. LeAOX1a and LeAOX1b transcripts were found in most tomato tissues, including leaves, root, flowers, and fruit. The LeAOX2 transcript was detected in carpels and roots, and the LeAOX1c transcript was expressed preferentially in roots but not in fruit (Holtzapfel et al., 2003; Fung et al., 2006).

In general, AOX is thought to play roles in heat production of thermogenic floral organs and cell adaptation under environmental stresses, such as inhibition of reactive oxidase species (ROS) formation and optimization of photosynthesis (Yoshida et al., 2008; Vanlerbergh et al., 2009; Zhang et al., 2010). To date, limited information exists on the contribution of the AOX pathway to fruit ripening. Some reports suggested that the expression of AOX and the CN-insensitive respiration of isolated mitochondria decreased during post-harvest ripening of tomato (Almeida et al., 1999; Costa et al., 1999; Sluse and Jarmuszkiewicz, 2000). Nevertheless, Holtzapfel et al. (2002) showed that AOX protein levels increased dramatically in tomato fruit ripened on the vine. Similar increases have been reported in mango and apple fruit in which climacteric bursts were associated with an enhanced CN-insensitive respiration (Duque and Arrabaca, 1999; Considine et al., 2001). In this regard, the role of AOX in climacteric fruit ripening requires further investigation.

In this study, a combination of chemical and transgenic approaches were undertaken to explore the role of AOX during tomato ripening. Here, evidence is provided that AOX plays an important role in the respiratory climacteric and may affect system-2 ethylene synthesis during tomato ripening. The relationships and possible mechanisms of these processes are discussed.

Materials and methods

Plant materials and growth conditions

Tomato (Solanum lycopersicum L. cv Hongyan) was used as the wild-type (WT) plant. Tomato seeds were surface-sterilized for 5 min in 70% (v/v) ethanol, then for 8 min in 10% (w/v) NaClO, followed by several washes in sterile distilled water. Tomato seeds were germinated and grown in artificial climate incubators (25 ºC day, 18 ºC night; 16 h light, 8 h dark) and transplanted into a greenhouse. The homozygous progeny of transgenic plants were selected and used for subsequent experiments.

Plasmid construction and tomato transformation

To generate the tomato overexpression LeAOX1a vector, the complete open reading frame (ORF) of AOX1a (accession no. AY034148) was amplified by reverse transcription-PCR (RT-PCR) using primers AOX1a-F (5′-TATTTGGACTCTTCTCTCAAGTTTC-3′) and AOX1a-R (5′-AAAAAGGAGAAAATAAGTGACGGCAG-3′), incorporating restriction sites for BamHI and EcoRI at the product ends. The amplified 1231 bp product was cloned into the pBl121 vector (Clontech, Palo Alto, CA, USA). The AOX1a insert was excised by BamHI and EcoRI, and cloned in the sense orientation driven by the Cauliflower mosaic virus (CaMV) 35S promoter (Fang et al., 1989), and was designated as 35S-AOX1a.

To construct AOX-RNAi vectors, the homologous sequence of the LeAOX1a (accession no. AY034148), LeAOX1b (accession no. AY034149), and LeAOX2 (accession no. AY324396) cDNA fragment was used for a double-stranded RNA interference (RNAi) trial. Sequences from AOX1a cDNA were isolated by RT-PCR using primers AOX1a-UF (5′-TGATTATTTCAGAGGAGATGTTG-3′), AOX1a-UR (5′-ATTAGTGCGTTATGATCCA-3′), AOX1a-D (5′-AGAGCAGATAATAGTAGAGACAGTGGA-3′), and AOX1a-DR (5′-GCTAGATGAACTCGTGATC-3′). An inverted repeat fragment of AOX1a was inserted downstream of the CaMV 35S promoter at the BamHI and EcoRI restriction sites of the modified PBl121. The construct AOX-RNAi was thus generated.

Transgenic plants were generated by Agrobacterium tumefaciens (strain EHA105)-mediated transformation according to the method described previously (Wang et al., 2009), and transformed lines were first selected for kanamycin (70 mg l⁻¹) resistance and then analysed by PCR to determine the presence of T-DNA. The primers designed to the NPTII (Kan resistance) marker of pBl121 for confirmation were 5′-GAGAGCTATTCCGCTATG-3′ and 5′-CTCAGAGAACACTCGTCAAGA-3′.

Ethylene and 1-MCP treatments

Mature green (MG) fruit of uniform sizes were collected, washed with water, and air-dried. For ethylene treatment, fruit were incubated in 500 µl l⁻¹ ethephon (ET) solution in a closed container at room temperature for 12 h, then air-dried and placed at 25 ± 1 ºC. For 1-MCP treatment, fruit were placed in 20 litre containers and exposed to 0.5 µl l⁻¹ 1-MCP gas (SmartFresh™, 0.14% a.i., Rohm and Haas, Philadelphia, PA, USA) for 12 h at room temperature. Immediately following 1-MCP treatment, fruit were removed from the chambers and stored at 25 ± 1 ºC. Control fruit were treated with deionized water instead of ET or 1-MCP. Three replicates each of 20 fruit were used for each treatment.

Real-time quantitative RT-PCR

Total RNA extraction and qRT-PCR were performed as previously described (Xu et al., 2012). Three replicates were performed for each experiment. ACTIN1 and histone H4 genes were used as internal controls (German et al., 2002; Galpaz et al., 2008). The qRT-PCR primers are listed in Supplementary Table S1 available at JXB online. All the mRNA data were expressed as a percentage of the corresponding ACTIN1 transcript levels.

Respiration measurements

Oxygen consumption was measured using Clark-type electrodes (Hansatech, King’s Lynn, UK) based on the methods of Møller et al. (2002) with some modifications. Fruit pulp (0.05 g; adjacent to the peel) was weighed and cut into small pieces, then pre-treated with 5 ml of deionized water for 15 min in order to minimize the effect of wound-induced respiration. Measurements were performed at 25 ºC in a final volume of 2 ml of 2 mM phosphate buffer (pH 6.8), and the cuvette was tightly closed to prevent diffusion of oxygen from the air. KCN at a final concentration of 1 mM was used to inhibit the COX pathway, and 100 µM n-propyl gallate (nPG) was used to inhibit the AOX pathway. Total respiration (Vt) is defined as the O2 uptake rate without any inhibitor. AOX pathway respiration (Vox) is defined as the O2 uptake rate in the presence of KCN that was sensitive to nPG. Residual respiration (Vres) is defined as the O2 uptake in the presence of both 1 mM KCN and 100 µM nPG. COX pathway respiration (Vcyt) was calculated by the formula: \( V_{cyt} = V_t - V_{alp} - V_{res} \).

Ethylene production

A whole tomato fruit was placed in a 10 cm × 10 cm closed container at 25 ± 1 ºC, 85% relative humidity for 2 h. Then, a 1 ml gas sample from the headspace of each container was injected into a flame ionization
detection gas chromatograph (Agilent 6890 Series GC system, UK) equipped with an activated alumina stainless steel column. The carrier gas (helium) flow rate was 0.5 ml s⁻¹. The detector and injector were operated at 100 °C and the oven temperature was 50 °C.

**ATP and HCN measurements**

ATP was extracted from tomato tissues and quantified as previously described (Zheng et al., 2009). A 1 g aliquot of tomato tissue was finely sliced and put into 5 ml of acetone, and then kept in a boiling water bath for 5 min. A 3 ml aliquot of 20 mM TRIS-HCl buffer at pH 7.6 was added and samples were heated in a boiled water bath for 10 min, and then immediately cooled in an ice bath. The extract was centrifuged at 3000 g for 10 min, and the supernatant was collected. Bioluminescence was measured with the ATP Bioluminescent Assay Kit (Sigma, St Louis, MO, USA) using an SHG-D Bioluminescence Meter (Analytical and Testing Center, Chengdu, China).

Hydrogen cyanide (HCN) was measured by the method of Dicenta et al. (2002). A 1 g aliquot of pulp was processed with 0.2 g of β-glucosidase (Sigma) in 4 ml of acetate buffer (pH 5.5) in a cylindrical glass vessel for 2 h at 35 °C. The HCN released was collected by micro-diffusion in 1 ml of 0.2 M NaOH, located in a very small glass collector in the interior of the vessel. HCN content was estimated at 580 nm using a spectrophotometer (TU1800 spectrophotometer, P-general Limited Comp., Beijing, China).

**Statistical analysis**

Data were analysed by one-way analysis of variance (ANOVA) and means were compared by a Dunnett’s test at *P* < 0.05.

**Results**

**LeAOX gene expression and CN-insensitive respiration during fruit ripening**

As shown in Fig. 1A, the total respiration (Vₜ) changed little from the mature green (MG) to the turning (T) stage. It peaked at the pink (P) stage and subsequently decreased. CN-insensitive respiration (Vₐₜ) followed a pattern similar to that of Vₜ. Vₐₜ increased 3-fold from the MG to the P stage when it accounted for 40% of the Vₜ at the P stage (Fig. 1A). The results suggest that the AOX pathway respiration may contribute to the respiration climacteric during fruit ripening.

To identify the possible involvement of the tomato AOX genes, their expression at different ripening stages was measured. LeAOX1a expression increased significantly at the T stage and peaked at the P stage, whereas LeAOX1b and LeAOX2 were expressed at a relatively low level during fruit ripening, although their expression pattern was similar to that of LeAOX1a (Fig. 1B). These data suggest that LeAOX1a might play a dominant role in the AOX pathway respiration.

**LeAOX1a expression is stimulated by ethylene and inhibited by 1-MCP treatment**

To investigate whether AOX expression is ethylene dependent, MG fruit were treated with ET or 1-MCP. As shown in Supplementary Fig. S1A at *JXB* online, the respiratory climacteric of the ET-treated fruit occurred 2 d earlier than in the control fruit. Also, CN-insensitive respiration was up-regulated and peaked at the same time as the respiratory climacteric following ET treatment (Supplementary Fig. S1B). 1-MCP treatment had the opposite effect on fruit respiration, in that both Vₜ and Vₐₜ were markedly suppressed and their peaks occurred later (Supplementary Fig. S1B).

Notably, LeAOX1a was significantly anticipated when ET was applied to MG fruit compared with the control fruit (Supplementary Fig. S1C). In contrast, 1-MCP treatment reduced the transcript levels of LeAOX1a, suggesting that its expression is ethylene regulated. No alterations in LeAOX1b or LeAOX2 transcript levels were observed upon the exposure to ET, although they were repressed by the 1-MCP treatment (Supplementary Fig. S1D, E at *JXB* online).

**Effect of AOX overexpression and suppression on fruit ripening**

To gain further insight into LeAOX function, transgenic tomato plants overexpressing LeAOX1a (35S-AOX1a) or suppressing LeAOX (AOX-RNAi) were generated. As shown in Supplementary Fig. S2A at *JXB* online, all eight 35S-AOX1a transgenic lines that were tested showed increased levels of LeAOX1a, and the
35S-AOX1a-8 and 35S-AOX1a-20 lines showed the greatest expression levels. In contrast, there were no significant differences in the expression of LeAOX1b and LeAOX2 between the transgenic and WT plants. Among the six AOX-RNAi transgenic lines, lines 1 and 6 exhibited the most severe LeAOX reduction (90% for the LeAOX1a transcript and ~50% for the LeAOX1b and LeAOX2 transcripts) (Supplementary Fig. S2B). Therefore, the 35S-AOX1a-8, 35S-AOX1a-20, aox-1, and aox-6 transgenic lines were selected for further experiments.

AOX overexpression or down-regulation did not result in noticeable changes in flowering time but affected ripening (Supplementary Fig. S3 at JXB online). The AOX-RNAi fruit had a longer ripening time [from the MG to the red (R) stage], and, on average, the fruit was larger than control and AOX-overexpressing tomatoes (Fig. 2). Reduction of AOX increased fresh weight and reduced soluble solids and lycopene upon ripening (Fig. 2C). AOX silencing also led to prolonged post-harvest ripening (Fig. 3A). These fruit had reduced peel colour (hue angle), loss of firmness, and water loss in comparison with WT and 35S-AOX1a tomatoes (Supplementary Fig. S4). In contrast, the 35S-AOX1a fruit reached maturity first during on-vine or off-vine ripening and accumulated more lycopene content at the R stage when compared with WT fruit (Fig. 2).

**Changing AOX levels affects ethylene production and respiration**

No respiratory climacteric was found during the ripening of AOX-RNAi fruit (Fig. 4). The respiration pattern of 35S-AOX1a fruit was similar to that of WT fruit, although the climacteric peak of 35S-AOX1a fruit was higher than that of WT fruit (Fig. 4). Consistent with the changes in CN-insensitive respiration during ripening, the AOX protein levels were altered in the transgenic fruit. The expression of AOX in WT and 35S-AOX1a fruit peaked at the P stage, and then decreased slightly, whereas it was barely detectable in AOX-RNAi fruit throughout ripening (Fig. 2B). Interestingly, ethylene production was higher in 35S-AOX1a fruit than in WT fruit, whereas it was suppressed

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**Fig. 2.** Physiological, morphological, and metabolic characterization of the wild-type (WT) and transgenic fruit. (A) Representative red fruit of WT and transgenic plants in the T2 generation. (B) Changes in the AOX protein (~33 kDa) levels during fruit ripening in WT and transgenic fruit. (C) Average fruit size and fresh weight of the WT and transgenic fruit at the MG (mature green) stage were measured, respectively. Soluble sugar and lycopene content of the WT and transgenic fruit at the R (red) stage were measured, respectively. Data are the means ±SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruit (P < 0.05).
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in AOX-RNAi fruit (Fig. 4), especially at the P stage, suggesting that the down-regulation of AOX influences ethylene synthesis.

The characterization of the expression of ethylene biosynthesis genes indicated that the ACC synthase4 (LeACS4) mRNA in AOX-RNAi fruit was substantially repressed at climacteric (P stage), as compared with WT fruit, and the LeACS2 transcript was repressed by 20% (Fig. 4D). Moreover, AOX-repressed fruit showed dramatic reductions in the transcript level of ACC oxidase1 (LeACO1), which was repressed by >40%, whereas LeACO4 expression was repressed to an extent similar to that of the expression of LeACS2 (Fig. 4D). In contrast, the mRNA levels of these genes were slightly higher in AOX1a-overexpressing fruit than in WT fruit at the P stage (Fig. 4D). Therefore, the ethylene reduction in AOX-RNAi fruit may be attributed to the down-regulation of the key genes in ethylene biosynthesis, especially via the down-regulation of LeACS4 and LeACO1 expression. In addition, there was a reduced accumulation in the transcript levels of a number of ethylene-regulated genes, including polygalacturonase (LePG) and the carotenoid synthesis enzyme, phytoene synthase1 (LePSY1); these results are consistent with the reduced ethylene levels in AOX-silenced fruit (Supplementary Fig. S5 at JXB online).

The results also showed that several genes that are involved in ethylene signal transduction were altered in the transgenic fruit. Transcripts of NR (LeETR3), LeETR4, LeEIL3, and LeERF1 were slightly up-regulated in the 35S-AOX1a fruit compared with WT fruit, but were suppressed in AOX-RNAi fruit (Fig. 4E), suggesting that AOX might affect the flux through the ethylene signalling pathway during ripening.

AOX-silenced fruit fail to ripen after 1-MCP treatment

The interactions between ethylene and AOX were further studied following the application of ET or 1-MCP. ET application at the MG stage promoted fruit ripening, as WT and 35S-AOX1a fruit reached ripening at 5 d after the treatment (Fig. 3B). This

Fig. 3. Post-harvest ripening of wild-type (WT) and transgenic fruit with or without 500 μl l⁻¹ ET or 0.5 μl l⁻¹ 1-MCP treatment. (A) Representative ripening of WT, 35S-AOX1a, and AOX-RNAi tomatoes. (B) Representative ripening of WT, 35S-AOX1a, and AOX-RNAi tomatoes in response to ET or 1-MCP treatment. (C and D) Representative phenotypes of the 1-MCP-treated WT, 35S-AOX1a, and AOX-RNAi tomatoes after 30 d of storage.
treatment also promoted ripening in AOX-RNAi fruit, which were fully ripe 5–7 d earlier than the water-treated control fruit. It is important to note that ET treatment recovered the impaired ripening phenotype but did not recover the ripening rate of AOX-silenced fruit to an extent similar to that of the ET-treated WT fruit. In contrast, fruit ripening was significantly delayed by the 1-MCP treatment. However, ripening of 35S-AOX1a was less affected by the 1-MCP treatment compared with WT fruit, which were fully ripe after 11 d of storage (Fig. 3B). When AOX-RNAi fruit was treated with 1-MCP, ripening was nearly blocked (Fig. 3C, 3D).

Consistent with the observed fruit ripening, the ET treatment significantly promoted total respiration, CN-insensitive respiration, and ethylene emission in WT and 35S-AOX1a fruit, while only a slight increase occurred in AOX-RNAi fruit (Fig. 5), supporting the idea that AOX plays an important role in ethylene autocatalysis. 1-MCP treatment delayed respiration and ethylene peaks in WT and AOX1a-overexpressing fruit (Fig. 5). Indeed, ethylene production was further suppressed in 1-MCP-treated AOX-RNAi fruit. In agreement with the observed lower ethylene production, several key genes involved in ethylene biosynthesis in AOX-RNAi fruit were significantly down-regulated after the 1-MCP treatment (Fig. 6). In addition, characterization of the ripening metabolism indicated that the lycopene accumulation and the soluble sugar content were substantially repressed in 1-MCP-treated AOX-RNAi fruit, which still maintained higher fruit firmness after 30 d of storage than the 1-MCP-treated WT and AOX1a-overexpressing fruit (Fig. 7A).

To confirm these results further, the expression profiles of regulatory genes previously described as involved in the ripening of tomato fruit, such as RIPENING INHIBITOR (RIN), COLORLESS NON RIPENING (CNR), NON-RIPENING (NOR), and NEVER RIPE (NR) were analysed (Tigchelaar et al., 1978; Wilkinson et al., 1995; Vrebalov et al., 2002; Manning et al., 2006). NR and CNR were down-regulated in 1-MCP-treated AOX-RNAi fruit, and their transcript levels were lower in AOX-RNAi fruit than in WT and 35S-AOX1a fruit, especially for the expression of NR (Fig. 7B). These results are consistent with the notion that NR expression in tomato fruit is positively regulated by ethylene (Wilkinson et al., 1995; Nakatsuka et al., 1998). No difference in the expression of RIN and NOR was observed between WT and transgenic fruit in response to 1-MCP (Fig. 7B).
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ATP and HCN contents correlate with ethylene production and AOX levels

As shown in Fig. 8A, the ATP content increased at the T stage and peaked at the P stage during the ripening of WT and 35S-AOX1a fruit; this result was consistent with the changes in total respiration (Fig. 4A). AOX-RNAi fruit had a normal basal level of ATP, which however did not show a similar changing pattern to WT and 35S-AOX1a fruit. In contrast, at the P stage, the ATP levels in AOX-RNAi fruit were significantly lower than in WT and 35S-AOX1a fruit (Fig. 8A). Notably, the HCN content was very low before the climacteric, abundant at the climacteric, and then rapidly diminished (Fig. 8B). Compared with WT fruit, the peak HCN content was higher in 35S-AOX1a fruit but lower in AOX-RNAi fruit.

Discussion

The data presented here demonstrate that AOX plays an important role in controlling tomato ripening. The overexpression of LeAOX1a did not change the pattern of fruit ripening, but it contributed to offsetting the inhibitory effect of the ethylene perception inhibitor, 1-MCP. In contrast, the reduction of AOX expression affected ethylene action and delayed ripening. Thus, AOX might be a newly identified component of the currently known regulatory network that controls fleshy fruit ripening.

Although several studies have revealed that AOX may play a role in the respiration climacteric (Duque and Arrabaca, 1999) or post-climacteric senescent processes during fruit ripening (Considine et al., 2001), no detailed study on tomato fruit has used transgenic plants or mutants; therefore, the precise role of AOX in respiratory climacteric is still to be determined. It was shown that in mango the activity of AOX peaks after climacteric respiration, which contributed to fruit senescence, rather than the respiratory climacteric (Considine et al., 2001). However, studies in apple fruit demonstrated that climacteric increases in respiration during fruit ripening were linked to increased AOX capacity and that AOX was induced at the climacteric during post-harvest storage (Duque and Arrabaca, 1999). The present data are consistent with the latter observation. In WT fruit, the respiratory climacteric occurred at the P stage (or after 5 d post-harvest storage), concomitant with a burst of CN-insensitive respiration (Fig. 1; Supplementary Fig. S1 at JXB online). Furthermore, ET treatment anticipated total respiration and the capacity of
the AOX pathway; while in the AOX-silenced fruit, no apparent respiratory climacteric occurred during on-vine or post-harvest ripening (Figs 4, 45). Even after the ET treatment, the respiratory pattern of the AOX-silenced fruit was not restored to that of the WT fruit (Fig. 5). These results indicate that the AOX pathway is an important component in achieving the respiration peak and that the role of AOX in the tomato respiratory climacteric cannot be substituted by ET treatment. Indeed, previous studies demonstrated that AOX activity in vivo may be regulated in a feed-forward fashion by upstream respiratory carbon metabolism; for example, intramitochondrial pyruvate, a potent activator of the AOX, was proved to stimulate the AOX capacity (Millar et al., 1993; Day and Wiskich, 1995; Pastore et al., 2001). Moreover, it was found that glycolysis is stimulated at the climacteric peak: rapid flux through the glycolytic pathway will lead to increased pyruvate production and accumulation of intramitochondrial reducing power, and these metabolic conditions are likely to result in an enhanced activity of the AOX (Duque et al., 1999). These findings reveal the importance of AOX in climacteric burst and fruit ripening.

Despite no direct connection between AOX and ethylene being found in previous studies, this work suggests that AOX plays an important role in climacteric ripening, probably through interactions with the ethylene pathway. The present data showed that AOX-RNAi fruit had a long shelf life during post-harvest storage and delayed on-vine fruit ripening (Fig. 3; Supplementary Fig. S3 at JXB online). Furthermore, no apparent ethylene climacteric was observed during the AOX-RNAi fruit ripening, and ET treatment did not fully restore the ripening rate of the AOX-silenced fruit (Fig. 3B). These observations suggest that AOX plays a partial role in ethylene signal transduction and might be necessary for ethylene autocatalysis. In fact, the overexpression of LeAOX1a led to slightly higher ethylene production during ripening, especially at the peak time (Fig. 4).

It should be noted that AOX-silenced fruit do not show ethylene or respiration bursts but can reach the R stage, indicating that the climacteric is dispensable for ripening to occur in tomato. More interestingly, the 1-MCP-treated AOX-RNAi fruit failed to ripen. It thus seems likely that both AOX and ethylene contribute to fruit ripening and that the inhibition of both AOX and ethylene is required to halt tomato ripening completely. Expression analyses of ripening genes such as NR, CNR, RIN, and NOR revealed that NR and CNR mRNA accumulation was reduced in the 1-MCP-treated AOX-RNAi fruit (Fig. 7). Considering that the

![Fig. 6. Changes in the transcript levels of ethylene synthesis-related genes in wild-type (WT) and transgenic fruit during post-harvest ripening in response to 0.5 μl l⁻¹ 1-MCP treatment. Relative expression profiles of LeACS2 (A), LeACS4 (B), LeACO1 (C), and LeACO4 (D) in 1-MCP-treated WT and transgenic fruit obtained by quantitative RT-PCR. Data are the means ±SD of three independent experiments. The asterisks indicate statistically significant differences from the water-treated WT fruit (P < 0.05).](image-url)
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NR gene acts downstream of the ethylene pathway but CNR acts upstream (Adams-Phillips et al., 2004; Barry and Giovannoni, 2007), the reduction of these transcripts implicates that AOX has some unexpected roles in fruit ripening. Further work will be necessary to understand the cross-regulation between the AOX pathway and ripening-associated transcription factors, since both ethylene-dependent and ethylene-independent regulatory pathways co-exist to coordinate the ripening process in climacteric fruit (Alba et al., 2000; Pech et al., 2008).

Relationships between AOX and ethylene probably exist. ATP, which is generated during respiration and is required for ethylene biosynthesis (Yang and Hoffman, 1984; Genard and Gouble, 2005) and fruit metabolism (Barry and Giovannoni, 2007), is a possible candidate, although AOX respiration is uncoupled from ATP generation. It is known that AOX can allow carbon flow through glycolysis and the citric acid cycle by removing excess carbohydrates and avoiding the over-reduction of the electron transport chain as well (Borecky and Vercesi, 2005). Therefore, it seems that the AOX pathway respiration increases rapidly and is accompanied by the respiratory climacteric to enable high turnover rates of carbon, thus allowing the large amount of ATP that is needed for system-2 ethylene synthesis and a series of ethylene-regulated ripening processes to be produced. Increased ethylene, in turn, further induces the CN-insensitive respiration by itself or by its co-product CN (Yip and Yang, 1988). CN has been reported to activate the AOX genes transcriptionally in tobacco and maize (Ederli et al., 2006). Moreover, CN treatment causes a rise in respiration and the ripening response in many fruit very similar to that evoked by ethylene (Solomos and Latties, 1974, 1976; Tucker and Latties, 1984). Therefore, it is possible that increased ethylene biosynthesis during fruit ripening results in increased HCN levels, which in turn induces AOX expression and promotes CN-insensitive respiration. Here, an interesting observation was that the HCN content accumulated extensively at the climacteric during wild-type fruit ripening (Fig. 8), which was consistent with the burst of ethylene production (Fig. 4). In the AOX-silenced fruit, the detectable HCN content was lower than that in the wild-type fruit. It should be pointed out that, although β-cyanoalanine synthase (β-CAS), which detoxifies HCN, is localized mainly in the mitochondria (Millenaar and Lambers, 2003; Ebbs et al., 2010), the HCN content that is produced during ethylene synthesis might be high enough to induce AOX expression and promote CN-insensitive respiration at the climacteric. In fact, whether CN acts as a signal

Fig. 7. Fruit quality and ripening-related gene comparison of the 1-MCP-treated wild-type (WT) and transgenic fruit. (A) Comparison of the lycopene content, soluble sugar content, and fruit firmness in WT and transgenic fruit 30 d after 1-MCP treatment. (B) Comparison of the transcript levels of NR, CNR, RIN, and NOR in WT and transgenic fruit 30 d after 1-MCP treatment. Data are the means ±SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruit (P < 0.05).
molecule, or only as a toxic byproduct of ethylene metabolism, needs to be determined in further studies.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Response of respiration and LeAOX genes to ET or 1-MCP treatment during post-harvest ripening in tomato.

Figure S2. Transcript levels of LeAOX in the transgenic plants.

Figure S3. Representative phenotypes of wild-type and transgenic plants in the T1 generation.

Figure S4. Changes in the hue angle, fruit firmness, and water loss during ripening in wild-type and transgenic tomatoes.

Figure S5. Changes in the expression of LePG and LePSY1 during ripening in wild-type and transgenic fruit.

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