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Effect of low oxygen, temperature and 1-methylcyclopropene on the expression of genes regulating ethylene biosynthesis and perception during ripening in apple

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Abstract

Ethylene initiates and controls ripening in climacteric fruit which is developmentally regulated. During this process, ethylene production generates a strong signaling process inducing/suppressing various target genes that are associated with several attributes of fruit ripening. In apple, low temperature, low oxygen and 1-methylcyclopropene (1-MCP) treatments have been used to increase shelf life. In the present study, effort has been made to understand the molecular basis of the increase in shelf life under the influence of temperature, low oxygen and 1-MCP in Granny Smith apple. The apple fruit were exposed to these treatments either individually or in combination and levels of ethylene as well as transcript accumulation of the genes responsible for ethylene biosynthesis and ethylene receptors were measured. A tight regulation of the ethylene production was observed through differential expression of *MdACS1* and *MdERS1* genes. The ethylene levels were highly dependent on temperature, oxygen concentration and 1-MCP and effects of each were not only additive but associated with the expression of *MdACS1* and *MdERS1*.

Keywords: 1-methylcyclopropene; Ethylene biosynthesis; Ethylene receptor; Fruit ripening; Hypoxia; *Malus domestica*; Temperature

1. Introduction

Ethylene plays an important role in many plant processes including seed germination, organ senescence, stress responses and fruit ripening (Nath et al., 2006). Depending on the massive increase in respiration or ethylene production during ripening, fruit are classified as climacteric and non climacteric (Biale and Young, 1981). At the onset of ripening, climacteric fruit exhibit a peak in respiration followed by a burst in ethylene production. In non-climacteric fruit, no such burst in ethylene production and respiration is observed. Though a basal level of ethylene production is present even in non-climacteric fruit, ripening in these fruits is ethylene independent. Based on the nature and amount of ethylene produced during plant growth and development including fruit ripening, the concept of system-1 and system-2 ethylene was introduced (McMurchie et al., 1972). System-1 ethylene is produced at low level and contributes to the basal levels produced by the plant during various stages of growth and development and is auto-inhibitory (Oetiker and Yang, 1995). System-2 ethylene production is at a high level, and operates during ripening of climacteric fruits and senescence of some flowers and is auto-stimulatory (Oetiker and Yang, 1995). The transition from system-1 to system-2 ethylene production is an important step during fruit ripening and is developmentally regulated. It is now established that genes responsible for ethylene biosynthesis and signal transduction are stimulated during a climacteric burst of ethylene production and may be tightly associated during the transition from system-1 to system-2 (Nakatsuka et al., 1998).

The ethylene biosynthesis pathway is well characterised in higher plants with ACC synthase and ACC oxidase playing key

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roles in this pathway (Yang and Hoffman, 1984). Both enzymes are encoded by multi-gene families and differential expression of their members has been reported during fruit ripening (Zarembinski and Theologis, 1994; Barry et al., 2000). In tomato, eight ACS genes have been identified among which *LeACS2* and *LeACS4* are highly expressed during tomato fruit ripening (Barry et al., 2000). Of the three ACC oxidases in tomato, *LeACO1* expresses during fruit ripening while other two play role in other aspects of plant development (Alexander and Grierson, 2002). Ethylene produced by plants is perceived by a family of receptors, ethylene receptors, which have similarity to histidine kinases and are negative regulators of ethylene signal transduction (Guo and Ecker, 2004). These receptors also belong to multi-gene family and their differential expression during ripening has also been observed in various climacteric fruits (Nath et al., 2006). After ethylene receptors, signal passes to downstream components through MAP kinase cascade and induces expression of ethylene responsive factors (ERFs). These ERFs modulates expression of ethylene responsive genes through binding to ethylene responsive elements in promoters of the genes (Johnson and Ecker, 1998).

Apple is a climacteric fruit and use of controlled atmosphere such as low oxygen and low temperature storage as well as treatment with 1-methylcyclopropene (1-MCP), an ethylene perception inhibitor, are effective for delaying fruit ripening (Fan et al., 1999; Rupasinghe et al., 2000; Watkins et al., 2000; DeEll et al., 2002). Since controlled atmosphere storage and treatment with 1-MCP significantly delays ripening of apple fruit by prolonging the time for ethylene burst, we suspected that these treatments may be regulating ethylene biosynthesis and perception at transcriptional level. To confirm this, we stored Granny Smith (GS) apples, an export variety which is susceptible to superficial scald formation (Rudell et al., 2005), in either low oxygen or treated with 1-MCP before storing at different temperatures for various lengths of time. Ethylene production and transcript accumulation of different genes related to ethylene biosynthesis and perception were monitored in various treatments and at different time in order to elucidate regulatory role of these genes during fruit ripening.

2. Materials and methods

2.1. Plant material and treatments

Mature apple (*Malus domestica*, Var. Granny Smith) fruit, which were not producing detectable ethylene, were harvested from the orchard of the University of Maryland in Western Maryland, MD, USA. Fruits were transported to the University of Maryland, College Park and kept at 1 °C in air with 80% humidity. For the air and low oxygen treatments, fifteen to twenty fruits were placed in 20 L desiccators and connected to air cylinder (Air) or low oxygen cylinder (1.5% O_2) at a flow rate of 30–40 ml/min. For 1-MCP treatment, fifteen to twenty fruits were placed in 20 L desiccators and exposed to 10 μL/L 1-methylcyclopropene (Ethyl Bloc from Biotechnologies for Horticulture Inc, Walterboro, SC, USA) for 18–20 h. 1-MCP was generated in the desiccators by placing required inert powder in the Eppendroff tube and adding water through rubber septum using a syringe. After 1-MCP treatment the desiccators were connected to air cylinder with the above flow rate. Depending on the experiments done the above desiccators sets were kept at 18 °C, 7 °C or 1 °C as mentioned in the figure legends. Immature green GS apple fruits kept in air at 18 °C were also subjected to ethylene (10 ppm) for 24 h and 48 h to study influence of exogenous ethylene. Fruits were removed from the desiccators at different time points, peeled and the cortex tissue was cut in small pieces and frozen in liquid N_2. The frozen tissue was ground to a fine powder and stored at −70 °C until used.

2.2. Measurement of ethylene production

Two ml gas sample was withdrawn from continuous airflow from 20 L desiccators containing fifteen to twenty apples and injected into the gas chromatograph (HP, 6890 series) to measure ethylene every alternate day. The average of three readings from each desiccators was taken as measure of ethylene production for each set. Data are expressed as means ± SD (standard deviation) of at least 3–4 replicates.

2.3. Total RNA isolation, cloning and sequencing of cDNA

Total RNA from apple fruit was isolated as described by Asif et al. (2006). RTPCR was performed using RTPCR kit (Invitrogen Inc., USA) as per manufacturer's recommendations. Briefly, 2 μg total RNA was reverse transcribed by MuMLV Reverse Transcriptase at 42 °C using oligo(dT) primer. One tenth of the reaction was used for PCR product using degenerate or gene specific primers. The PCR products were cloned using TA Topo cloning kit (Invitrogen Inc., USA). To isolate full-length cDNA of the genes 5′- and 3′-RACE was performed using gene specific primers and respective kits (Invitrogen Inc., USA). The primers used for isolation of partial cDNA and 5′- and 3′-RACE are listed in Supplementary Table 1. Nucleotide sequence of each cDNA was established using M13 universal and reverse primers. Sequencing was carried out on an automated DNA sequencing system (ABI 377A, Applied Biosystems Inc., USA) using the dye terminator cycle sequencing kit. Sequence comparisons against databases were performed using BLAST and BLASTX algorithms (Altschul et al., 1990) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

2.4. Northern blot analysis

Total RNA was resolved on 1.2% formaldehyde denaturing agarose gel and transferred to Hybond XL membrane (Amersham Biosciences, Buckinghamshire, NA, USA) according to the manufacturer's protocol. The blot was prehybridized overnight at 42 °C in a mixture containing 50% formaldehyde, 1% SDS, 5X SSC and 5X Denhardts solution. Denatured ^{32}P dCTP radiolabelled probes were added to the same but fresh hybridization solution. The probes were prepared to a specific activity of approximately 10^8 cpm μg^{-1} using Invitrogen RadPrime labeling kit, and at least 1×10^6 cpm were added per ml hybridization solution. The hybridization was carried out at 42 °C for 14–16 h. The washing of the blot was carried out in SSC plus 0.1% SDS. The final wash of the blots was done at