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Putative role of cytokinin in differential ethylene response of two lines of antisense ACC oxidase cantaloupe melons

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ABSTRACT

Two transgenic lines of 'Cantaloupe' melon derived from the same wild type genotype were previously generated using ACC oxidase antisense constructs from melon (pMEL1AS) and apple (pAP4AS). Both lines yielded fruit with reduced ethylene production and low ACC oxidase (ACCO) expression. ACCO antisense fruit also exhibited lower expression of ACC synthase genes, ACCS1 and ACCS3, indicating that these genes are positively regulated by ethylene and participate in the autocatalytic ethylene production process. In contrast, a higher expression of ACCS5 was observed in antisense lines when compared to the wild type indicating a negative feedback regulation of ACCS5 by ethylene. Fruit of both transformed lines exhibited delayed ripening and reduction in ester volatile production but differed in their response to exogenous ethylene supply. While postharvest ethylene application fully restored the ripening process in pMEL1AS melon, it only restored flesh softening of pAP4AS melon but not rind color change or aroma volatile production. Up-regulation of lipoxygenase pathway associated genes (hydroxyperoxide lyase, lipoxygenase, and alcohol acyl transferases 1, 3 and 4) occurred in ethylene-treated pMEL1AS fruit but not in pAP4AS melons. Polygalacturonase1 gene transcript accumulation increased in pMEL1AS and pAP4AS fruit upon ethylene supply. Zeatin and zeatin riboside content of roots and fruit (rind and flesh) of pAP4AS plants were 5-fold higher than the wild type and pMEL1AS counterparts. Higher relative transcript accumulation of a gene involved in the cytokinin synthesis and a gene involved in cytokinin response were also found in the roots and fruit of pAP4AS. In addition, polyamines, which are known to reduce sensitivity to ethylene, remained unchanged in all fruit. Collectively the results suggest a putative role for the increased endogenous cytokinin content in counteracting ethylene action in some aspects of the fruit ripening process.

1. Introduction

Melon (*Cucumis melo* L.) var. cantalupensis Naud cv. Vedrantais is a typical climacteric fruit with a negative relationship between ethylene production and shelf life, and a positive relationship between ethylene and aroma production. Such physiological behavior has been confirmed by transformation of melon using 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) antisense genes (Ayub et al., 1996; Silva et al., 2004); Ayub et al. (1996) utilized an ACCO antisense gene from melon (pMEL1AS), previously isolated and characterized by Balagué et al. (1993), and Silva et al. (2004) utilized an ACCO antisense gene from 'Royal Gala' apple (pAPAS). In both cases, the wild type genotype was the same and the transformation resulted in *ACCO* transcription suppression and low ethylene production.

Among pAPAS transgenic events (Silva et al., 2004), pAP4AS exhibited phenotypic changes characterized by high axillary bud growth, greenish color, and reduced leaf senescence. These features are commonly observed in plants expressing high cytokinin content; for example, *HOC* Arabidopsis mutant (Catterou et al., 2002) and transgenic plants over expressing *IPT* gene (Merewitz et al., 2011; Zhang et al., 2010), or plants submitted to moderate drought stress (Cogo et al., 2011). Because of these characteristics, it was hypothesized that cytokinin content could have been affected in pAP4AS melon.

Yellowing of fruit rind, flesh firmness reduction, formation of a peduncular abscission zone, and reestablishment of ester production, resulting from postharvest ethylene application to pMEL1AS

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melon, indicate that these are ethylene-dependent or partially ethylene-dependent events (Flores et al., 2002; Nishiyama et al., 2007). On the other hand, flesh color and sugar content are not affected by ethylene (Bauchot et al., 1998; Bower et al., 2002; Guis et al., 1997; Pech et al., 2008). In the case of pAP4AS melon, ripening was not completely recovered with ethylene application (0.1–400 $\mu L\,L^{-1}$). Although the cause for such behavior is not clear, it is known that high cytokinin (Akhtar et al., 1999; Chen et al., 2001; Cogo et al., 2011; Martineau et al., 1995) or polyamine-containing organs (Neily et al., 2011) are less sensitive to ethylene.

Transgenic melon (pMEL1AS) (Ayub et al., 1996) and transgenic apples (pAE12AS) expressing an *ACCO* antisense (Dandekar et al., 2004), with suppressed ethylene and ester volatile production (Bauchot et al., 1998; Silva et al., 2004; Yahyaoui et al., 2002), were able to restore volatile production when ethylene was exogenously supplied. Ester volatile synthesis is dependent on fatty acid metabolism involving lipoxygenase (LOX), α- and β-oxidations, followed by reduction into aldehydes and alcohols assisted by alcohol dehydrogenase (ADH), and alcohol acyltransferase (AAT) that catalyzes the last esterification step (Beekwilder et al., 2004). Flores et al. (2002) and Yahyaoui et al. (2002) noticed that ester volatile synthesis is promoted by *AAT* activation. In melon, four *AAT* (*AAT1*, *AAT2*, *AAT3* and *AAT4*) clones were isolated and characterized; with *AAT1* and *AAT4* being up-regulated during ripening under ethylene control (El-Sharkawy et al., 2005; Yahyaoui et al., 2002).

Thus, the aforementioned transgenic lines pMEL1AS and pAP4AS serve as model for studying the interaction between ethylene and cytokinin in climacteric melon ripening. It is possible that hormone accumulation such as cytokinin (Cogo et al., 2011; Merewitz et al., 2011; Zhang et al., 2010) and/or polyamines (Neily et al., 2011) impact postharvest metabolism. In order to test this hypothesis, melons with normal ripening on the vine and after harvest (NT), were compared to others that either develop (pMEL1AS) or lack (pAP4AS) the classical responses to ethylene treatment. Ripening associated chemical and physiological variables as well as transcript accumulation of ethylene biosynthesis, cell wall disassembling, chlorophyll breakdown and ester biosynthesis genes were monitored.

2. Materials and methods

2.1. Plant material and experiments

Non-transformed Cantaloupe melon (*C. melo* var. Cantalupensis, Naud cv. Vedrantais) (NT) and melon transformed with *ACCO* antisense pMEL1AS (Ayub et al., 1996) and pAP4AS (Silva et al., 2004) clones were cultivated according to standard practices, leaving no more than four fruit per plant, following CTNBio (Brazilian regulatory council) biosafety regulation for greenhouse cultivation.

2.1.1. Ripening on the vine

In order to follow ripening evolution, ethylene, flesh firmness, rind color, rind chlorophyll content, rind and flesh carotene content, and soluble solids content, were evaluated during ripening on the vine at every two days starting 34 d after anthesis (DAA) until 44 DAA for NT melon, and 52 DAA for pMEL1AS and pAP4AS. Six fruit were evaluated in each analysis, totaling 48 NT fruit and 60 pMEL1AS and pAP4AS fruit each per treatment. Transcript accumulation of ethylene biosynthesis genes (*ACCO* and *ACCS*) was quantified starting at 30 DAA until 48 DAA for NT, pMEL1AS, and pAP4AS fruit.

2.1.2. Ripening after harvest

Postharvest physiological and molecular changes were also evaluated. NT fruit were harvested at 36 DAA, when abscission zone started to form and were kept at 23 ± 2 °C and 80 ± 5% relative humidity. pMEL1AS and pAP4AS were harvested at 44 DAA since they had a longer maturation cycle. Thirty-six fruit per treatment were kept at 23 ± 2 °C and 36 more were exposed to ethylene (100 $\mu L\,L^{-1}$) for 120 h, in 7.2 L flasks containing a KOH solution (150 mL, 1 N). At every 12 h the flasks were opened to replace the KOH solution and ethylene concentration was adjusted to 100 $\mu L\,L^{-1}$. Postharvest ethylene production, rind color, rind chlorophyll and carotene content, and soluble solids analyses were performed after 1, 24, 48, 72, 96 and 120 h of harvest in fruit kept at room temperature (NT, pMEL1AS and pAP4AS) and fruit kept under ethylene (pMEL1AS and pAP4AS). Volatile compounds were evaluated immediately after harvest and for transformed fruit the measurement was repeated 120 h after ethylene treatment. Individual fruit were considered biological replicates and each analysis was performed in duplicate.

2.1.3. Cytokinin (root and fruit) and polyamine (fruit) accumulation

Cytokinin content and relative accumulation of genes associated to cytokinin synthesis and response were quantified in root tips (sampled after the harvest of the second fruit), in addition to fruit rind and flesh of NT, pMEL1AS and pAP4AS plants. Upon collection samples were washed with water containing diethylpyrocarbonate (DEPC), frozen in liquid nitrogen and stored at −80 °C. Sample collection for polyamine analysis followed the same protocol except at this time only rind and flesh were collected.

2.2. Analyses

2.2.1. Ethylene

Ethylene concentration of individual fruit was monitored on the vine as described by Ayub et al. (1996), and results expressed as $\mu L\,L^{-1}$. After harvest, melons were enclosed in 7.2 L flasks at room temperature (23 ± 2 °C). After 30 min, 1 mL of the headspace was sampled and injected into a GC (Varian 3300), as described by Silva et al. (2004). Ethylene production after harvest was expressed in $nmol\,kg^{-1}\,s^{-1}$.

2.2.2. Firmness

Flesh firmness was determined using a texturometer (TA.XT plus) with a 2 mm probe, with 50% penetration at $1\,mm\,s^{-1}$ in cut opened fruit. Results were expressed in Newtons (N) (Silva et al., 2004).

2.2.3. Soluble solids

Soluble solids content was determined using an Abbe refractometer (ATAGO-N1) and data was expressed as percentage (Silva et al., 2004).

2.2.4. Color

Color was determined using a colorimeter (Minolta Chromometer CR 300, D65, Osaka, Japan), with 8 mm aperture and standard CIE-L*a^*b^*. a^* and b^* values were utilized. Measurements were performed on opposite sides of the fruit at the equatorial region (Silva et al., 2004).

2.2.5. Titratable acidity

Determined by titration using NaOH 0.1 N. Results were expressed in % of citric acid (Silva et al., 2004).

2.2.6. Chlorophyll content

1 g of fruit rind was ground in 5 mL acetone (80% v/v) and left stirring for 15 min. The mixture was centrifuged (10,000 × *g*; 10 min; 4 °C) and the supernatant transferred to a 25 mL volumetric flask (this step was repeated three times), and the final volume was