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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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A B S T R A C T

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

adjusted to 25 mL with acetone (80% v/v). Absorbance was measured at 645 nm (chlorophyll *b* – Chl *a*) and 662 nm (chlorophyll *a* – Chl *b*) and concentrations were calculated according to the formulas described by Lichtenthaler (1987) (Chl $a = 12.25A_{662} - 2.79A_{645}$; Chl $b = 21.50A_{645} - 5.10A_{662}$), and results were expressed as chlorophyll per fresh weight mass, mg kg^{-1}.

2.2.7. Carotenoid content

Total carotenoid content was determined using 10 g of rind or flesh ground up in liquid nitrogen, following the same procedure described for the chlorophyll content evaluation. Absorbance was measured at 470 nm. Results were calculated using the equation described by Lichtenthaler (1987) ($C = 1000A_{470} - 1.82Ca - 85.02Cb/198$) and expressed on a fresh weight basis as mg kg^{-1}.

2.2.8. Ester volatiles

Ester volatile analysis followed protocol described by Bauchot et al. (1998) except that here SPME carboxen-PDMS (0.75 μm × 1 cm, Supelco, USA) was used as the adsorbent matrix. All analyses were performed on a Varian 3800 gas chromatograph interfaced with a Shimadzu QP-50000 mass spectrometer. Volatiles were identified by comparison to spectra of standards and to reference collections (NIST 98/EPA/NIH Mass Spectral database).

2.2.9. Cytokinin content

Zeatin (Z) and zeatin riboside (ZR) were separated using HPLC and detected using an immunoenzymatic assay according to Zieslin and Algom (2004) with some modifications. Rind, flesh, and root tissues from NT, pMEL1AS, and pAP4AS melon were ground in liquid nitrogen and cytokinins were extracted with ethanol during 30 min incubation. Nine volumes of ammonium acetate solution (40 mM, pH 6.5) were added to the extract, which was then filtered through a 0.22 μm filter membrane and purified through a polyvynilpolypyrrolidone column. Eluted cytokinins were separated by HPLC (Shimadzu HPLC system) using an EC 250/4 Nucleosil 100-5 C_{18} column and monitored by a UV detector set at 254 nm. Cytokinin containing fractions were submitted to immunochemical detection using monoclonal antibodies for zeatin and zeatin riboside. Cytokinin content was expressed as mass of zeatin and zeating riboside per fresh weight mass of fruit, μg kg^{-1}. Commercial standards were used for calibration and the recovery obtained was 89.87%.

2.2.10. Polyamine content

Extraction of free polyamines was performed as described by Hao et al. (2005) with minor changes: 1 g of rind or flesh was homogenized in perchloric acid (5%, 5 mL) and extracted on ice for 30 min. After centrifugation at 12,000 × *g*, 4 °C for 15 min, the supernatant was transferred to another tube and kept on ice. The pellet was extracted again with perchloric acid (5%, 1 mL) on ice for 30 min and then centrifuged at 12,000 × *g* and 4 °C for 15 min. The supernatants were combined and adjusted to pH 7.0 with saturated Na_2CO_3. Separation was performed in a μBondapak C18 column, 300 mm × 3.9 mm i.d., 10 μm (Waters, Milford, Massachusetts, EUA), and putrescine and spermidine were derivatized post-column with *o*-phthalaldehyde and monitored using a fluorescence detector (Ex: 340 nm; Em: 445 nm). Refer to Vieira et al. (2007) for a more detailed description of the analytical method. Results were expressed on a fresh weight basis as mg kg^{-1}.

2.2.11. Transcript accumulation

Transcript accumulation was evaluated by quantitative PCR (q-PCR). RNA was extracted from 0.1 g of melon flesh according to manufacture instructions using PureLinK™ reagent (*Plant RNA Reagent* – Invitrogen™). Total RNA was treated with DNAse I (Invitrogen™), and RNA quality was confirmed in agarose gel (2%, w/v), by PCR, and spectroscopically quantified. cDNAs were obtained from 2 μg of RNA using SuperScript First-Strand System (Invitrogen™). Specific primers were designed for GenBank deposited sequences using Vector NTI Advance 10 (Invitrogen™). Criteria for primer selection were: size of amplified fragment between 100 and 230 bp; % CG bases between 40 and 60%; not more than two C or G bases among the last five nucleotides at the 3′ end; annealing temperature 60–65 °C; according to Applied Biosystems guidelines. Amplicons were evaluated in agarose gel (2%, w/v) and sequenced prior to RT-qPCR. Dissociation curves were evaluated and only primers yielding one peak, indicating specific amplification of the respective target gene, were utilized. A standard curve was prepared for each gene using six cDNA dilutions and only genes with amplification efficiency close to 100% were used. For each cDNA, *β-actin* and *18S* transcripts were used as normalizer for each cDNA, given their consistent transcription level in fruit samples in which the Ct varied less than 1.4. q-PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems) using fluorescent SYBR Green. Amplification reaction was performed in 25 μL total volume containing 2 μM of each primer, 12.5 μL of the PCR Master Mix SYBR Green, 4 ng of cDNA (1 μL), and water to make up the total volume. PCR conditions were: denaturing at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of three steps (95 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min), and final extension at 72 °C for 5 min. Since reaction efficiency was high (close to 100%), relative transcript accumulation was calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). For relative quantification of *ACCO* and *ACCS* transcripts, the expression level of NT fruit at 34 DAA (on the vine) was selected as the baseline. For all the other genes studied after harvest, NT fruit expression immediately after harvest served as baseline expression. The following genes were selected based on specificity and efficiency: ethylene synthesis (*ACCO* – F: 5′-AATCCGCACAAACCAAATCTTGTAC-3′/R: 5′AAGGATCCTAAGCTGAAAGTGAATA-3′; *ACCS1* – F: 5′-GAAAGCG TAC GATAACGATCCG-3′/R: 5′-CGGTATAAATAGAGGCTTTCGGAA-3′; *ACCS2* – F: 5′-GATGTCTCTC TAAATATTAA ACAG-3′/R: 5′-CATTAT CGTTGCTAGGAAACAAGTC-3′; *ACCS3* – F: 5′-GGTCTGGCAGA GAATCAGCTATCA-3′/R: 5′-GTAGCGCCAGCTGTAAGGACTAT-3′; *ACCS4* – F: 5′-TATGACATAATTAAGG TCACTAAT-3′/R: 5′-TGATTAGT GGAATATATAGGTTTTAT-3′; *ACCS5* – F: 5′-GACGCCTTTCTT CTGCCCACCCCCTAC3′/R: 5′CAATGTGAACTTGTTTACGGATTACGA-3′); cytokinin synthesis and response (*CYP735A2* – F: 5′-CTTCAACGT CTTTGTGTCCAAG-3′/R: 5′-CTACTCCGACCGATCTCTACAC-3′; *ARR1* – F: 5′-TTCATATGCCTGACATGGACGG-3′/R: 5′-AACCGCACCGTGCG TTACTCCC-3); flesh firmness (*PG1* – F: 5′-CACGCCTTGACT GCTGCTGCTG-3′/R: 5′-CGGCTTGGCTCCAAGATTGACG-3′); ester synthesis (*LOX* – F: 5′-AGAAGG CACTCCTGAGTATGAG3′/R: 5′-CTTCCAGCTTCTTTCTAAAATCCT-3′; *HPL* – F: 5′-GCATGGCGCCGCCG CGAGCCAACT-3′/R: 5′-CAGCGCGCGCCGCCGCTTGACACT-3′; *AAT1* – F: 5′-CCACAGGGGCCAGAATTACA-3′/R: 5′-TGGAGGAGGCAAGCA TAGACTT-3′; *AAT2* – F: 5′-CTATAATTGGAGGGTGTGGAATTATC-3′/R: 5′-AACATTTGCCCTAAATCTTTCCAT-3′; *AAT3* – F: 5′-CG CTTGATGACATGGCACAT-3′/R: 5′-GGCCTTACGGATAGCAGAGATC-3′; *AAT4* – F: 5′-CAGTTGTACCCCCGTCGAGTA-3′/R: 5′-AATAT CGCTTCTGATCGGAACAC-3′); and constitutive expression (*β-actin* – F: 5′-GTGATGGTGTGAGTCACACTGTTC-3′/R: 5′-ACGACC AGCAAGGTCCAAAC-3′; *18S* – F: 5′-AAAACGACTCTCGGCAACGG ATA-3′/R: 5′-ATGGTTCACGGGATTCTGCAATT-3′).

2.2.12. Experimental design and statistical analysis

All treatments had six biological replicates and two analytical duplicates, and were distributed in a completely randomized design. qPCR analysis were also carried out following a block design

Table 1
Cytokinin (zeatin and zeatin riboside) content ($\mu g\,kg^{-1}$) in root, rind and flesh of non-transformed (NT) and transformed Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), using *ACCO* pMEL1AS and pAP4AS antisense genes.

Melon	Plant part	Zeatin	Zeatin riboside
NT	Root	4.87 ± 2.01[a]	5.01 ± 1.25
	Rind	5.02 ± 1.25	6.23 ± 1.58
	Flesh	2.52 ± 1.07	5.25 ± 2.01
pMEL1AS	Root	5.68 ± 1.89	6.69 ± 0.87
	Rind	9.14 ± 2.31	8.75 ± 1.11
	Flesh	4.02 ± 1.24	7.98 ± 2.01
pAP4AS	Root	7.87 ± 2.34	9.78 ± 2.12
	Rind	21.01 ± 3.25	36.84 ± 3.69
	Flesh	8.25 ± 2.05	30.63 ± 4.25

[a] Mean of six biological replicates ± standard error.

(each plate as a block), with six biological replicates and three analytical duplicates. Data was subjected to an ANOVA, performed using the F test at the 5% significance level and means of treatment were compared using Tukey's test at the 5% significance level ($p \leq 0.05$), using SAS version 9.2 for Windows (SAS Institute, Cary, NC).

3. Results

3.1. Physicochemical and physiological changes in melon fruit during ripening on the vine

In order to establish an association between physiological responses to ethylene and hormones levels, cytokinins zeatin (Z) and zeatin riboside (ZR) were measured in roots, fruit flesh and rind (Table 1), and polyamines spermidine and putrescine were determined in the rind and flesh of fruit (Table 2). pAP4AS fruit that stayed green had higher Z and ZR content in the roots, fruit flesh and rind (Table 1). Spermidine and putrescine, although higher in the rind when compared to fruit flesh, showed no difference among genotypes (Table 2). Theses results indicate that genetic transformation of pAP4AS that suppressed ethylene production also affected cytokinin synthesis with no effect on polyamine content. pAP4AS (low ethylene production and high cytokinin content) was then compared to pMEL1AS (low ethylene production and normal cytokinin content) and NT melons (high ethylene production and normal cytokinin content) in order to study the interaction between ethylene and cytokinins on the physiological and molecular changes during ripening and under ethylene treatment.

NT melons showed a typical climacteric behavior, with a rise in ethylene production after 34 DAA and a peak at 42 DAA accompanied by significant flesh firmness reduction (Fig. 1B) Fig. 1. In contrast, transgenic melon did not present a peak of ethylene production (Fig. 1A) and showed smaller changes in flesh firmness. NT flesh firmness reduced drastically during ripening, varying from 57 N at 34 DAA to 5 N at 46 DAA (Fig. 1B). At 48 DAA NT fruit were completely soft. Although pMEL1AS and pAP4AS fruit also showed a reduction in flesh firmness, it occurred to a lesser extent going from 65 N at 34 DAA to 40 N at 52 DAA (Fig. 1B). pMEL1AS and pAP4AS stayed greener and less yellow than NT during ripening on the vine (Fig. 1C and D). NT fruit color variation during 34 DAA through 42 DAA (Fig. 1D) was associated with changes in the major compounds responsible for rind color: chlorophyll content declined (Fig. 1E) and total carotene content increased (Fig. 1F). In pMEL1AS and pAP4AS fruit, these compounds did not vary as much. Flesh carotenoid content was not affected by treatments (data not shown), reaching 453.5 $mg\,kg^{-1}$ of total carotenoid at harvest.

Table 2
Polyamine content (spermidine and putrescine $mg\,kg^{-1}$), at harvest, of the rind and flesh of non-transformed (NT) and transformed Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), using *ACCO* pMEL1AS and pAP4AS antisense genes.

Melon	Plant part	Spermidine	Putrescine
UNT	Rind	2.35 ± 0.31[a]	7.53 ± 0.72
	Flesh	1.25 ± 0.29	4.56 ± 0.29
pMEL1AS	Rind	2.06 ± 0.45	8.01 ± 0.94
	Flesh	1.15 ± 0.45	4.98 ± 0.45
pAP4AS	Rind	2.23 ± 0.36	7.85 ± 0.89
	Flesh	0.96 ± 0.37	5.01 ± 0.67

[a] Mean of six biological replicates ± standard error.

3.2. Physicochemical and physiological changes in melon fruit during ripening after harvest

NT melons were harvested at 36 DAA (beginning of climacteric) and pMEL1AS and pAP4AS at 44 DAA (when an abscission zone was observed and soluble solids content was approximately 16%). After harvest, ethylene production of NT melon increased and reached a maximum (7.65 $nmol\,kg^{-1}\,s^{-1}$) at 72 h after harvest (Fig. 2A). pMEL1AS and pAP4AS fruit did not show a peak in ethylene production (Fig. 2A), and levels were 0.04 $nmol\,kg^{-1}\,s^{-1}$, 99.5% lower than NT fruit.

pMEL1AS and pAP4AS kept at room temperature without ethylene treatment had a reduction in flesh firmness from 50 N to 32 N, while fruit treated with ethylene showed a variation going from 50 N to 9 N (Fig. 2B). NT fruit without ethylene application showed a reduction in flesh firmness going from 47 N to 5 N. Thus, confirming that melon flesh softening is a physiological change accelerated by ethylene.

Following similar ripening behavior observed on the vine (Fig. 1C), NT melon showed degreening (color parameter "a" changing from −33 to −19) after harvest (Fig. 2C). pMEL1AS and pAP4AS fruit showed significantly less rind degreening ("a" from −38 to −33). Responses were varied upon ethylene application; pMEL1AS showed degreening similar to NT fruit, while pAP4AS fruit had only a slight change in green color with "a" values (green/red scale) close to −30 (Fig. 2C). In addition, fruit that had a higher decrease in green color after harvest (NT and pMEL1AS + C_2H_4) also showed higher yellowing (Fig. 2D). Rind color variation was similar between pMEL1AS and pAP4AS during ripening on the vine (Fig. 1C and D), but differed from fruit exposed to ethylene after harvest (Fig. 2C and D). Harvested pMEL1AS fruit treated with ethylene showed degreening (Fig. 2C) and yellowing (Fig. 2D), associated with higher reduction of chlorophyll content (Fig. 2E) and increase in rind carotenoid content (Fig. 2F). In pAP4AS these changes occurred only partially, indicating a lower sensitivity to ethylene. Flesh carotenoid content did not change significantly after harvest (data not shown), staying at approximately 449 $mg\,kg^{-1}$, despite ethylene supply. Inhibition of ethylene production in pMEL1AS and pAP4AS fruit resulted in 93% reduction of ester volatile production from pMEL1AS and 87% from pAP4AS (Fig. 3). Reduction of volatile production occurred for all compounds evaluated, including compounds with low odor values (for example, methyl propyl acetate) and potent odorants (methyl propanoate) (Fig. 3). Five days after ethylene treatment pMEL1AS fruit restored normal ester production. However, pAP4AS fruit only partially restored ester volatile production reaching about 26% of the production observed from NT fruit.

3.3. Gene transcript accumulation during ripening on the vine

During NT fruit ripening on the vine *ACCO* transcripts accumulated beyond 32 DAA, with maximum accumulation at 38 DAA (Fig. 4A), followed by subsequent increase in ethylene production (Fig. 1A). Transformed fruit had little to no *ACCO* transcripts detected, in agreement with a reduction of more than 99.5% in ethylene production from these fruit.

ACCS transcript accumulation varied among *ACCS1* (Fig. 4B), *ACCS2* (Fig. 4C), *ACCS3* (Fig. 4D) and *ACCS5* (Fig. 4E). *ACCS4* transcripts were not detected. *ACCS1* (Fig. 4B) and *ACCS3* (Fig. 4D) had higher transcript accumulation in NT than in pMEL1AS and pAP4AS fruit, and the observed variations had high positive correlation with *ACCO* transcript accumulation (Fig. 4A) and ethylene production (Fig. 1A). These results indicate that these genes have strong association with ripening evolution. On the other hand, high *ACCS5* transcript accumulation in pMEL1AS and pAP4AS fruit (Fig. 4E), demonstrated that this gene is negatively regulated by ethylene. *ACCS2* mRNA accumulation seems to be independent of ethylene since there was an up-regulation of *ACCS2* in all three genotypes (Fig. 4C).

3.4. Gene transcript accumulation during ripening after harvest

ACCO mRNA accumulation decreased over time when NT fruit harvested 36 DAA were kept at room temperature to complete ripening (Fig. 5). This is in agreement with the maximum transcript accumulation observed at 36 and 38 DAA (Fig. 4A). pMEL1AS and pAP4AS melon even after ethylene application did not show increase in *ACCO* transcript levels. As expected, *PG1* transcripts of NT fruit accumulated during ripening and were up-regulated by ethylene in pMEL1AS earlier and more intensely when compared to pAP4AS.

LOX, *HPL*, *AAT1*, *AAT3* and *AAT4*, all ester biosynthesis associated genes, had elevated transcript accumulation between 24 and 72 h in NT fruit (Fig. 5). pMEL1AS and pAP4AS fruit, however, showed considerably low transcript accumulation of the same genes. Only *AAT2* had an increased mRNA content in transformed fruit when compared to NT. pMEL1AS treated with ethylene showed a general induction of transcript accumulation. In general, these results indicate that those fruit producing more ester volatiles (NT and pMEL1AS + C_2H_4) (Fig. 3) also had in common an increased mRNA content of *HPL*, *LOX*, *AAT1*, *AAT3* and *AAT4*.

CYP735A2, a cytokinin hydroxylase gene, showed higher transcript accumulation in the root, and fruit of pAP4AS when compared to pMEL1AS and NT (Fig. 6). The highest relative expression of this gene was observed in the roots of pAP4AS. Similarly, *ARR1* known to be a transcription factor induced by cytokinins, also showed higher transcript accumulation in the pAP4AS genotype, specially in the fruit (Fig. 6).

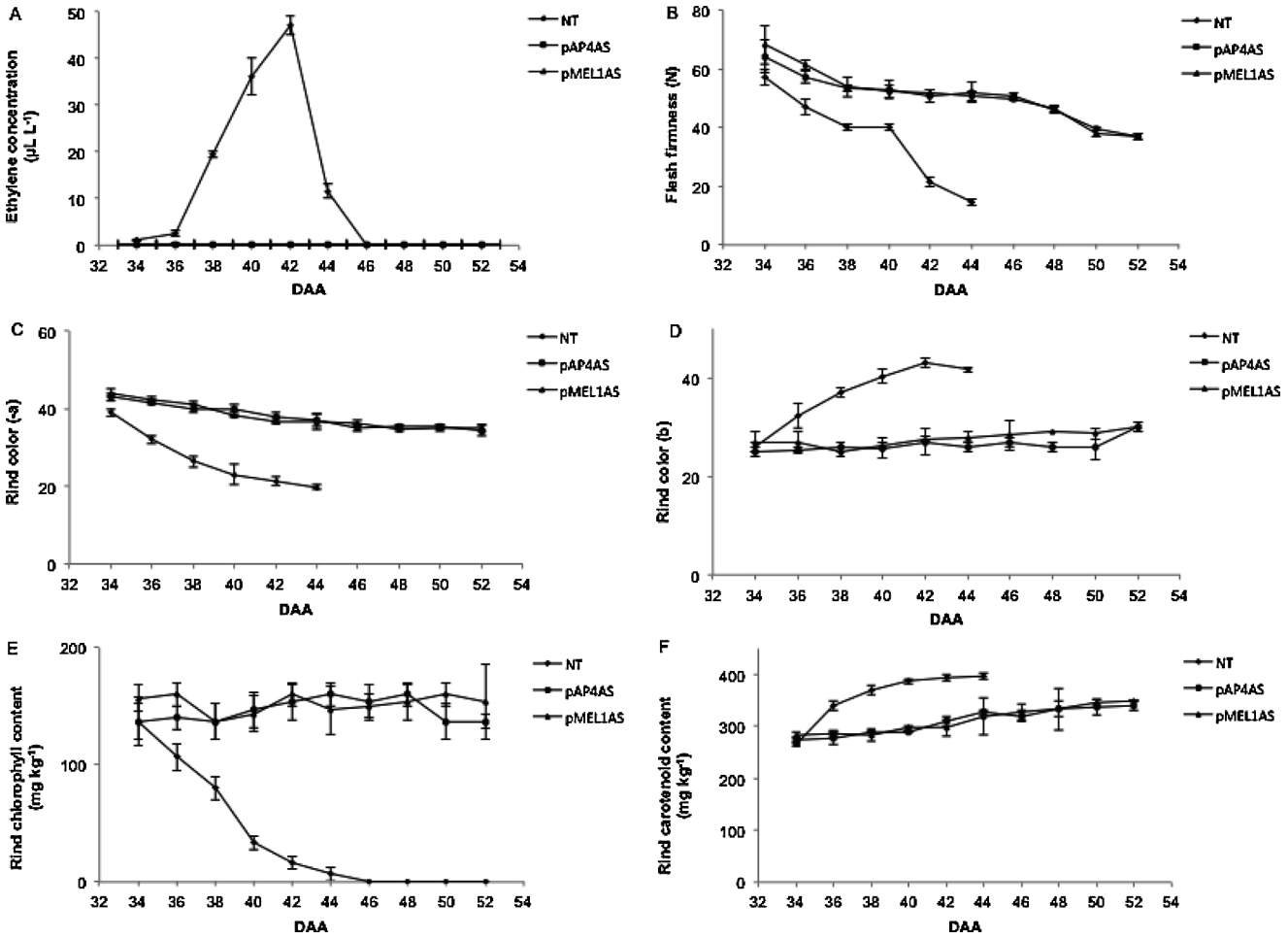

Fig. 1. Physicochemical and physiological changes in non-transformed (NT) and transformed Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais) using *ACCO* pMEL1AS and pAP4AS antisense, during ripening on the vine. (A) Ethylene concentration ($\mu L\,L^{-1}$); (B) flesh firmness (N); (C) rind color (-a); (D) rind color (b); (E) rind chlorophyll content ($mg\,kg^{-1}$); (F) rind carotenoid content ($mg\,kg^{-1}$). Vertical bars represent standard error of the mean ($n = 6$). DAA (days after anthesis).

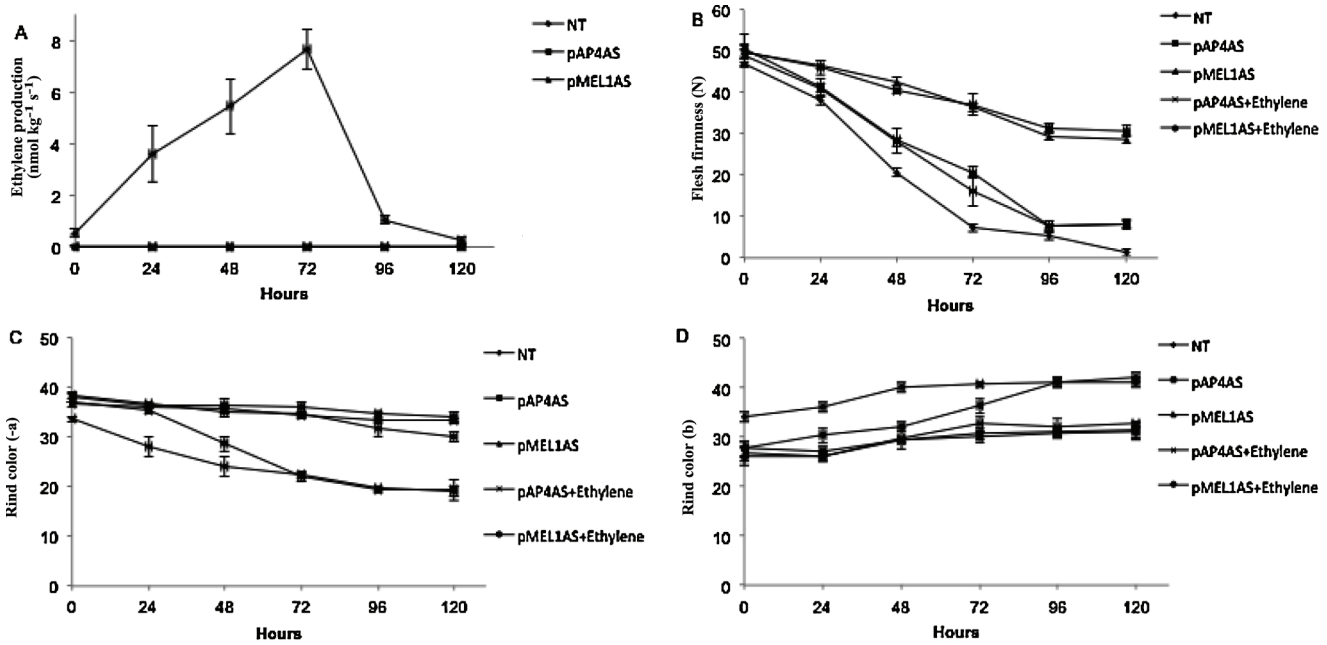

Fig. 2. Physicochemical and physiological changes in non-transformed (NT) and transformed Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais) using *ACCO* pMEL1AS and pAP4AS antisense, during postharvest. Ethylene production ($nmol\,kg^{-1}\,s^{-1}$) (A), Flesh firmness (N) (B), Rind color (-a) (C); Rind color (b) (D); Rind chlorophyll content ($mg\,kg^{-1}$)(E); Rind carotenoid content ($mg\,kg^{-1}$) (F); Vertical bars represent standard error of the mean ($n = 6$).

4. Discussion

Inhibiting ethylene production by the transgenic *ACCO* antisense approach prolongs ripening and shelf-life in fruit (Ayub et al., 1996; Defilippi et al., 2005; Silva et al., 2004). *ACCO* antisense pMEL1AS and pAP4AS melon studied here also had an extended ripening period (10 d). pMEL1AS and pAP4AS did not differ regarding maturation, ethylene and ester production, flesh firmness, rind color, rind chlorophyll and carotenoid content, and polyamine content during ripening on the vine. However, after harvest, upon ethylene application only pMEL1AS recovered ester volatile production and color changes and developed a peduncular abscission zone, confirming previous findings (Bauchot et al., 1998; Guis et al., 1997).

Exposure of pAP4AS fruit to ethylene for 120 h did not result in complete green color reduction or in rind yellowing, and ester volatile production was only 26% of NT. The high cytokinin content observed in these fruit may be responsible for reducing their sensitivity to ethylene. This explanation is supported by studies using Arabidopsis mutants with high cytokinin content that have slowed senescence (Catterou et al., 2002). In addition, plants expressing low levels of pheophorbide oxidase gene or cultivated under moderate water stress have shown induced cytokinin synthesis resulting in less sensitivity to ethylene and better preservation of green color (Buchanan-Wollaston et al., 2005; Cogo et al., 2011; Pruzinska et al., 2003).

Although carotenoid synthesis in melon is thought to be ethylene-independent (Guis et al., 1997), in pMEL1AS rind carotenoid content was affected by ethylene leading to yellowing

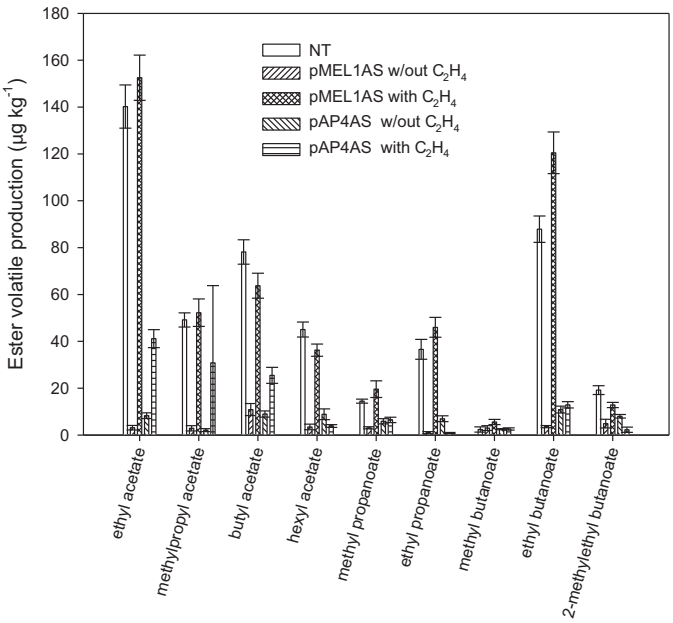

Fig. 3. Ester volatile production ($\mu g\,kg^{-1}$) by non-transformed (NT) and transformed (using ACCO pMEL1AS and pAP4AS antisense) Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), treated with ethylene (with C_2H_4) and measured 120 h after treatment or not treated with ethylene (w/out C_2H_4) measured immediately after harvest.

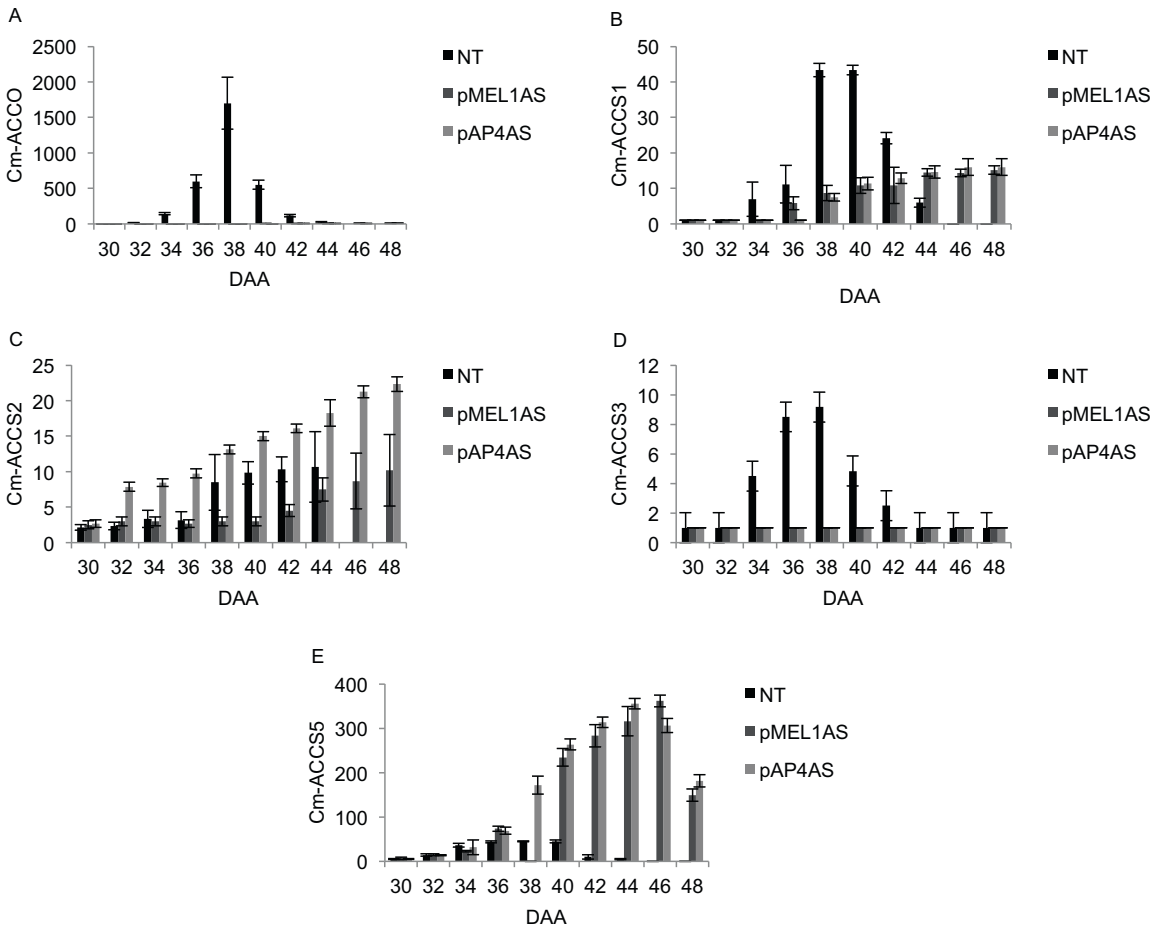

Fig. 4. Relative transcript accumulation of genes associated with ethylene biosynthesis during ripening on the vine of non-transformed (NT) and transformed melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), using *ACCO* pMEL1AS and pAP4AS antisense gene. Relative transcript accumulation calculated according to the formula $2^{-\Delta\Delta ct}$. *Cm-ACCO* (A), *Cm-ACCS1* (B), *Cm-ACSS2* (C), *Cm-ACCS3* (D) and *Cm-ACCS5* (E) vertical bars represent standard error of the mean ($n = 6$). DAA (days after anthesis).

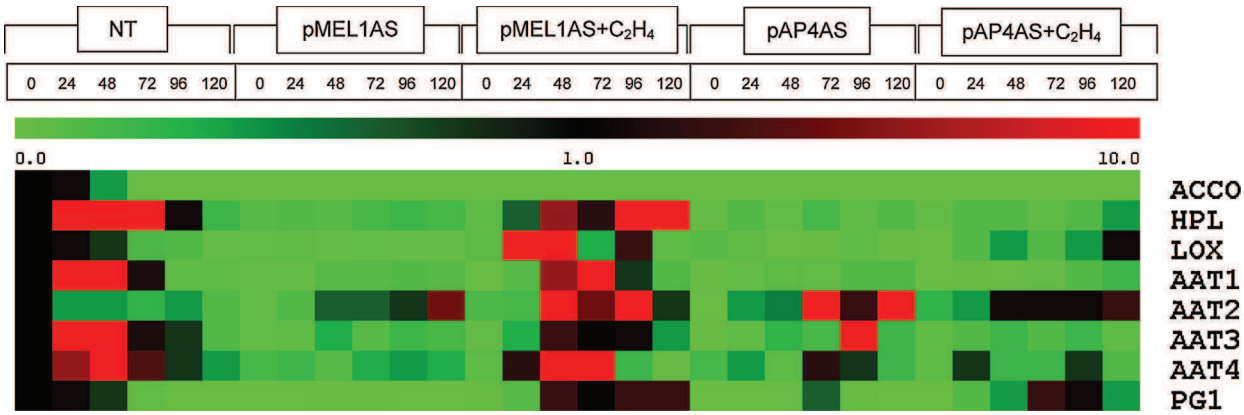

Fig. 5. Relative transcript accumulation of 1-aminocyclopropane-1-carboxylic oxidase (*ACCO*), hydroperoxide lyase (*HPL*), lipoxygenase (*LOX*), alcohol acyltransferase (*AAT1, AAT2, AAT3, AAT4*), and (*PG1*) genes of Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), non-transformed (NT) and transformed using *ACCO* pMEL1AS and pAP4AS antisense treated or not with ethylene. Samples were collected at 0, 24, 48, 72, 96 and 120 h. Transcript level is described in a 0 to 10 scale. Green color on the left indicates minimal accumulation, black color in the middle represents 5 times the mRNA content compared to green and red color on the right hand corner represents a 10 fold increase in mRNA content compared to the green end of the scale.

of fruit rind. This finding indicates that carotenoid synthesis and accumulation may be regulated differently in the rind and flesh of melon fruit. In addition, high cytokinin containing fruit (pAP4AS) did not show the same behavior, i.e., ethylene did not stimulate chlorophyll degradation or carotenoid accumulation in the rind.

Defilippi et al. (2005) showed that a reduction in ethylene production negatively affected ester volatile production. In addition, Pech et al. (2008) indicated that selection of genotypes for increased shelf-life either by classical breeding or a transgenic approach led to fruit with lower aromatic potential. As previously observed by Flores et al. (2002) and Guis et al. (1997), ethylene application to pMEL1AS restored volatile production (Fig. 3). In apple, the same aroma re-establishment occurred in addition to the restoration of rind color and the formation of a peduncular abscission zone (Defilippi et al., 2005). However, in pAP4AS fruit not all the classical maturation events were restored with ethylene application. This was not expected since both pMEL1AS and pAP4AS were transformed with *ACCO* antisense clones from melon and apple, respectively, and had 94.3% homology.

In order to correlate physiological and molecular responses, ethylene and ester biosynthesis and flesh firmness associated genes were monitored. First, transformation was efficient in suppressing mRNAs from *ACCO*, which also interfered with *ACCS* transcript accumulation. mRNA accumulation of ester biosynthesis genes *LOX*, *AAT1*, *AAT2*, *AAT3* and *AAT4* was higher in NT melon than in pMEL1AS and pAP4AS. Ethylene application induced a rapid increase of *LOX*, *AAT1*, *AAT3* and *AAT4* transcript accumulation in pMEL1AS melons but not in pAP4AS melons. pMEL1AS and NT showed similar physiological responses to ethylene. It is a general consensus that ester volatile biosynthesis in climacteric fruit is an ethylene-dependent event and that induction of *AAT* expression is necessary and sufficient for volatile production (El-Sharkawy et al., 2005). For pAP4AS melon however, ethylene supply was unable to restore ester production for both strong and low odorant intensity compounds.

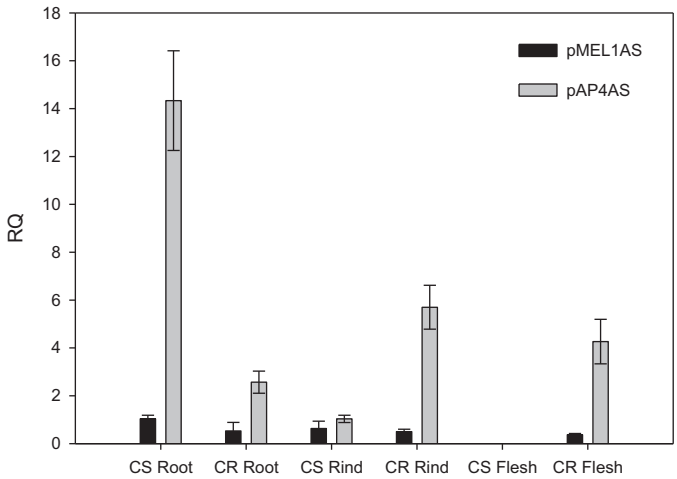

Fig. 6. Transcript accumulation in the root, rind and flesh of genes *CYP735A2* (CS) and *ARR1* (CR) of Cantaloupe melon (*Cucumis melo* var. Cantalupensis, Naud cv. Vedrantais), transformed using ACCO pMEL1AS and pAP4AS antisense Vertical bars represent standard error of the mean. RQ – relative quantitation.

It is known that cytokinins, mostly synthesized in the roots and translocated to other plant parts, are affected by ethylene and retard leaf and flower senescence (Buchanan-Wollaston et al., 2005; Martineau et al., 1995). Broccoli (Chen et al., 2001) and tomato (Martineau et al., 1995) overexpressing cytokinins are less sensitive to ethylene. Exogenous application of 6-benzylaminopurine to broccoli can also lead to these same effects (Downs et al., 1997). Application of cytokinins (6-benzylaminopurine, 6-BAP and N-(2-chloro-pyridin-4-yl)-N′-phenylurea, CPPU) to vegetative plant parts or fruit of NT, pMEL1AS, and pAP4AS did not show differences in ethylene production, firmness, soluble solids, titrable acidity, carotenoids, ester volatiles, or mRNA content except that NT and pMEL1AS plants had fruit that were 18% larger than pAP4AS. Although the physiological mechanism was not described, an association between cytokinin synthesis and increased shelf-life (Zaicovski et al., 2008) or the stay-green symptom (Akhtar et al., 1999) has been observed. In this study, higher cytokinin content was found in roots, fruit rind and flesh of pAP4AS melon. These results are in agreement with findings by Martineau et al. (1995), suggesting that a reduction in ethylene production beyond extending shelf-life, prolonged the vegetative cycle of tomato promoting root emission and consequently higher cytokinin synthesis and accumulation, reducing responsiveness to ethylene. The examination of transcript accumulation of two genes involved in the cytokinin synthesis and response (*CYP735A2* and *ARR1*) support this hypothesis. *CYP735A2* involved in cytokinin synthesis showed high transcript accumulation in pAP4AS roots. On the other hand, *ARR1* mRNA content, a transcription factor affected by endogenous cytokinins, was higher in pAP4AS fruit. The up-regulation of *CYP735A2* in pAP4AS roots may have led to the observed increased hormone level, which may consequently have affected the expression of cytokinin *ARR1* in the fruit.

It was possible for pAP4AS transgenic melon to have higher polyamine content due to a higher ACC availability, but this hypothesis was not confirmed. Spermidine and putrescine content, as expected, were higher in fruit rind than in fruit flesh. Melon transformation with highly homologous clones led to lines with distinct physiology. pMEL1AS was made to ripen with ethylene application, allowing for the characterization of ethylene-dependent, independent and partially dependent events. Unresponsiveness to ethylene in pAP4AS melon is likely due to the high cytokinin content, suggesting an involvement of plant hormones other than ethylene in ripening control.

In summary, the high cytokinin content found in pAP4AS melons (two fold higher than pMEL1AS) affected the fruit responsiveness to ethylene. The typical molecular and physiological changes known as ethylene-dependent, including transcript accumulation of genes involved in the ester synthesis, especially *AAT1* and *AAT4* (Lucchetta et al., 2007; Pech et al., 2008), degreening (Golding et al., 1998), and peduncular abscission zone (Guis et al., 1997) appear to be affected by higher cytokinin content. On the other hand, flesh firmness, a partially ethylene dependent event (Nishiyama et al., 2007) was not affected by high cytokinin content. Based upon these results it is proposed that fruit responses to ethylene are partially affected by cytokinin content.

5. Conclusions

Melons expressing an antisense ACC oxidase (pMEL1AS and pAP4AS) gene did not develop normal ripening. In addition, the silencing of *ACCO* revealed regulation of members of the ACC synthase gene family by ethylene. *ACCS1* and *ACCS3* were positively regulated by ethylene and participate in the autocatalytic ethylene production process, and the *ACCS5* gene was negatively regulated by ethylene. *ACCS2* mRNA accumulated continuously during ripening of NT and ethylene-suppressed fruit suggesting that this gene is ethylene-independent. The pMEL1AS and pAP4AS melons respond differentially to post-harvest ethylene treatment. *Polygalacturonase1* responded to ethylene in both pMEL1AS and pAP4AS fruit, while genes of the LOX pathway were stimulated by ethylene only in pMEL1AS fruit. As a consequence, the pMEL1AS restored ester volatile production. pAP4AS roots and fruit showed high accumulation of cytokinin but not polyamines. The elevated transcript accumulation of genes involved in the cytokinin synthesis (*CYP735A2*) and response (*ARR1*) supports that this hormone may be responsible for the differential physiological responses between pMEL1AS and pAP4AS.

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