Ripening-associated Biochemical Traits of Cantaloupe Charentais Melons Expressing an Antisense ACC Oxidase Transgene

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ABSTRACT. Transgenic Cantaloupe Charentais melons (*Cucumis melo* var. *cantalupensis* Naud. 'Védrantais') exhibiting strong inhibition of ethylene production were used as a model to discriminate between ethylene-regulated and ethylene-independent ripening pathways. Compared to wild-type fruit, transgenic fruit did not undergo significant yellowing of the rind and softening of the flesh. However, these effects were completely reversed by treating transgenic fruit with 50 $\mu\text{L}\cdot\text{L}^{-1}$ exogenous ethylene. Pigmentation of the flesh occurred early before the onset of the climacteric and was thus unaffected by ethylene inhibition in transgenic fruit. Total soluble solids accumulated at the same rate in both types of fruit until 38 days after pollination when wild-type fruit abscised. However, as ethylene-inhibited fruit failed to develop a peduncular abscission zone, they remained attached to the plant and accumulated higher amounts of sugars, mainly sucrose. Harvesting transgenic fruit resulted in a small but significant increase of internal ethylene associated with softening of the flesh.

The plant hormone ethylene regulates the ripening of climacteric fruit and therefore represents an obvious target for controlling climacteric fruit ripening by genetic manipulation. Strong inhibition of the ripening process has been achieved in transgenic tomato (*Lycopersicon esculentum* L.) by incorporating silencing genes coding for two enzymes of the ethylene biosynthetic pathway—ACC synthase (Oeller et al., 1991) and ACC oxidase (Hamilton et al., 1990)—or by expressing genes coding for enzymes involved in the degradation of the ethylene precursor ACC—ACC deaminase (Klee et al., 1991) and S-adenosyl hydrolase (Good et al., 1994). Until recently, these genetic engineering methodologies have been restricted to tomato as a unique plant model. Melons of the Cantaloupe Charentais type (*Cucumis melo* var. *cantalupensis* Naud.) exhibit good sensory attributes, but their poor keeping quality is limiting their wide commercial acceptance. The cultivar Védrantais is an orange-fleshed and smooth-skinned French Cantaloupe Charentais melon. These melons are highly aromatic but usually have a sharp climacteric phase (Hadfield et al., 1995) associated with poor keeping qualities. They have been classified by Naudin (1859) as the *cantalupensis* group within the *Cucumis melo* L. species. The American cantaloupes have either orange or green flesh, are usually netted or have warts on the skin, and generally display better keeping qualities (Dunlap et al., 1990). They have been classified in the *reticulatus* group (Naudin, 1859). We have recently shown that the expression of an ACC oxidase antisense gene strongly reduces ethylene production and inhibits

the ripening process of Cantaloupe Charentais melons (Ayub et al., 1996). In this paper, we report on the characterization of the ripening behavior of these transgenic melons by analyzing for certain biochemical traits (sugar content, acidity, firmness, and color of the flesh and rind) and demonstrate that the ripening process includes ethylene-independent and ethylene-regulated pathways.

Materials and Methods

PLANT MATERIAL. Plants of a T₂ progeny of a B17 line of transgenic Cantaloupe Charentais melon (*C. melo* var. *cantalupensis* Naud. 'Védrantais') harboring an ACC oxidase gene (Balagué et al., 1993) in an antisense orientation (Ayub et al., 1996) were used. Untransformed wild-type plants were used as controls.

Plants were cultivated in a greenhouse in 1995 and 1996 and trained to a trellis using standard cultural practices for fertilization and pesticide treatments. Flowers were self-pollinated by hand and tagged for date of fruit set. Only two fruit were kept on each plant. Abscission of wild-type fruit was observed at ≈ 38 d after pollination (DAP) in 1995 and 42 DAP in 1996.

COLOR AND PIGMENT MEASUREMENTS. The b parameter corresponding to the chromacity coordinate along the yellow-blue axis in the *L,*a,*b color system was measured with a Minolta CR-300 chromameter. Measurements in quadruplets of the rind of the entire fruit and the flesh of a transversely cut fruit were recorded. Chlorophyll from the rind was extracted using 80% acetone and measured according to Hill et al. (1985). The carotenoid content of the flesh and rind tissues was determined by spectrophotometry (Romojaro et al., 1979).

FIRMNESS. Four measurements of flesh firmness were recorded from cut fruit using an 8-mm cylinder pressure Penefel tester (Copa Technologie, St-Etienne du Gres, France).

SUGARS AND ACIDITY. Total soluble solids ($^{\circ}\text{Brix}$) of mesocarp tissue were assessed by refractometric reading of four juice samples collected from each fruit. Sucrose, glucose, and fructose were measured enzymatically using a commercial kit (Boehringer Mannheim GmbH, Germany). Titratable acidity was measured by

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Fig. 1. Changes in the chlorophyll content of the rind (A) and the carotenoid content (fresh mass basis) of the flesh (B, continuous lines) and the rind (B, dotted lines) of antisense ACC oxidase (□) and wild-type (■) melons attached to the vine. Each point represents a mean of three replications $\pm$ SE (1995 experiment).

titration against 0.1 N NaOH according to Alavoine et al. (1988) and reported as citric acid equivalents.

ETHYLENE TREATMENTS. Transgenic fruit were harvested at 33, 42, and 47 DAP and treated with 50 μ L.L⁻¹ ethylene at 25 °C in 32-L vessels in the presence of KOH to eliminate respiratory CO₂. Each vessel containing three fruit was ventilated every 12 h to prevent oxygen deprivation and adjusted for ethylene concentration.

SAMPLING AND STATISTICAL ANALYSIS. To minimize variability, fruit of uniform size and of the same age (DAP) were used for physical and biochemical analysis. In addition, internal ethylene concentration of each fruit was monitored on the vine, as described previously (Ayub et al., 1996), to determine the ethylene climacteric. Values were expressed as means $\pm$ SE of four independent measurements of three different fruit.

Results and Discussion

COLOR OF THE RIND AND FLESH. The ripening behavior of transgenic 'Védrantais' exhibiting almost complete inhibition (>99%) of ethylene production was analyzed for various biochemical parameters associated with fruit ripening. In wild-type melon, the onset of ripening resulted in a rapid loss of chlorophyll of the rind starting 34 DAP (Fig. 1A), while transgenic melon failed to display loss of rind chlorophylls throughout its development on the vine. This observation agrees with the demonstration that chlorophyll degradation was ethylene regulated (Trebitsh et al., 1993). Carotenoid content of the rind increased in wild-type fruit but remained unchanged in transgenic fruit (Fig. 1B). However, no differences were observed in the carotenoid content of the flesh between control and ethylene-inhibited fruit (Fig. 1B). The flesh undergoes color changes early on before the onset of the climacteric, indicating that synthesis of carotenoids is ethylene independent. This agrees with previous data demonstrating that, in muskmelons of the *reticulatus* group, the pigmentation of the flesh develops well before the onset of ethylene production (Reid et al., 1970).

Fig. 2. Changes in flesh firmness of antisense ACC oxidase (□) and wild-type (■) melons attached to the vine. Each point represents a mean of three replications $\pm$ SE (1995 experiment).

More recently, it has been shown that, in melons of the *cantalupensis* group, expression of the phytoene synthase-encoding gene (an enzyme involved in the early steps of carotenoid biosynthesis) is detected at an early stage of fruit development (Karvouni et al., 1995). In contrast with melon, inhibition of ethylene production by antisense ACC oxidase in tomato results in severe inhibition of carotenoid synthesis in the flesh and skin (Murray et al., 1993; Picton et al., 1993).

FIRMNESS. In contrast to netted muskmelons (Lester and Bruton, 1986), Cantaloupe Charentais melons exhibit fast softening of the flesh during ripening and after harvest (Dumas de Vault and Aubert, 1976). A similar trend has been observed in control melons during ripening on the vine (Fig. 2). In the transgenic line, suppression of ethylene production has resulted in almost complete inhibition of softening, indicating that cell wall disassembly is triggered by ethylene. In transgenic tomatoes producing low levels of ethylene, the rate of softening is similar to wild-type fruit during ripening, but it is retarded during the over-ripening phase (Klee, 1993; Murray et al., 1993; Picton et al., 1993). Differences between melon and tomato may be explained by the relatively high level of residual ethylene (3% to 10%) in transgenic tomato compared to our melons (<0.5%). It has been reported that endo-polygalacturonase (PG) transcript accumulation is unaffected in ACS (Oeller et al., 1991) and ACO antisense (Picton et al., 1993) tomatoes although, in the latter case, PG transcript accumulation is delayed but eventually reaches a level similar to that attained in the wild-type fruit. Interestingly, while PG transcript accumulation is ethylene independent, ethylene has been reported to be required for the translation of PG transcripts in tomato (Theologis et al., 1993). No other ripening-related cell wall-degrading enzymes have been characterized in low-ethylene transgenic fruit.

Fig. 3. Changes in sugar content (fresh mass basis) of antisense ACC oxidase (open symbols) and wild-type (close symbols) melons attached to the vine; (A) total soluble solids, (B) glucose (triangle) and fructose (square), (C) sucrose, and (D) sweet taste. The sweet taste is calculated by using the relative sweetness values of 100 for sucrose, 180 for sucrose, and 67 for glucose as in Cohen and Hicks (1986). Each point represents a mean of three replications $\pm$ SE (1995 experiment).

Fig. 4. Changes in titratable acidity (citric acid equivalent) of antisense ACC oxidase (□) and wild-type (■) melons attached to the vine. Each point represents a mean of three replications $\pm$ SE (1995 experiment).

SUGARS AND ACIDITY. Sugar content is an important sensory attribute of Cantaloupe Charentais melon. In this study, total soluble solids accumulated at the same rate in both types of fruit; however, control fruit abscised

from the plant (38 DAP), while ethylene-inhibited fruit continued to accumulate more sugars. In wild-type plants, the climacteric rise of ethylene activates the peduncular abscission zone, causing fruit drop at 38 DAP; whereas, in transgenic plants, fruit remain attached to the vine for >48 DAP. To avoid over-ripening and postharvest deterioration, wild-type fruit are commercially harvested at the onset of the climacteric (34 DAP) when they reach 10° Brix (Fig. 3A), which represents a threshold for acceptable eating quality (Alavoine et al., 1988). As in other melon cultivars (Bianco and Pratt, 1977; Wang et al., 1996), sucrose is present at low levels in immature fruit and increases rapidly during ripening (Fig. 3C). Glucose and fructose concentrations reach 20 g·kg⁻¹ of fresh mass in immature fruit and do not undergo major changes during ripening of wild-type fruit and slightly decrease in transgenic fruit (Fig. 3B). Therefore, the rapid increase in total sugars during ripening is entirely due to sucrose, and premature harvesting of fruit is detrimental to quality. The contribution of soluble sugars to sweetness is not uniform. Using the relative sweetness values of 100 for sucrose, 180 for fructose, and 67 for glucose as in Cohen and Hicks (1986), we calculated changes in sweetness of control and transgenic melons (Fig. 3D). A continuous increase in sweetness in transgenic melons maintained on the vine up to 48 DAP is observed, while this is interrupted earlier in control fruit due to abscission. Because antisense fruit can be harvested later than 34 DAP without risk of over-ripening and fruit abscission, they can accumulate higher amounts of sucrose (Fig. 3C) and are sweeter (Fig. 3D).

A sharp decrease of titratable acidity was observed starting at 34 DAP, which corresponded to the early climacteric stage (Fig. 4). In transgenic fruit, the decrease in titratable acidity was severely delayed and reached low levels at the end of the experiment. This suggested that the organic acid metabolism was ethylene independent and probably related to the respiratory metabolism of the fruit. For optimum fruit quality, acidity should be low, but the threshold value should be determined. A delay in the loss of acidity was also observed in antisense ACC oxidase transgenic tomatoes throughout ripening (Murray et al., 1993).

EFFECT OF DETACHMENT ON INTERNAL ETHYLENE CONCENTRATION. As reported earlier (Ayub et al., 1996), transgenic

Fig. 5. Effect of detachment from the plant on internal ethylene concentration of antisense ACC oxidase melons (1995 experiment). The arrow points to a detached fruit (▲) and to a fruit that has been left on the vine (■).

fruit kept on the vine displayed low internal ethylene levels (<0.5 $\mu\text{L}\cdot\text{L}^{-1}$). However, upon harvesting at 38 DAP, internal ethylene increased immediately to reach 4 $\mu\text{L}\cdot\text{L}^{-1}$ and then remained constant (Fig. 5). In spite of this increase, the concentration of internal ethylene in detached transgenic fruit remained low compared to that of wild-type fruit, which reached 180 $\mu\text{L}\cdot\text{L}^{-1}$ (Ayub et al., 1996). An accelerated increase in internal ethylene has been already described upon detachment of apples (*Malus domestica* Borkh.) from the tree (Sfakiotakis and Dilley, 1973). It has been suggested that detachment of fruit from the vine resulted in inhibition of a translocation factor within the tree or a ripening inhibitor in the fruit, although the nature of these putative factors remains unknown. This subject has been reviewed by McGlasson et al. (1978).

RESTORATION OF RIPENING BY EXOGENOUS ETHYLENE. Transgenic melons harvested at 37, 42, and 47 DAP and placed at 25 °C in the presence of 50 $\mu\text{L}\cdot\text{L}^{-1}$ of ethylene showed rapid softening of the flesh (Fig. 6A), yellowing of the rind (Fig. 6B), and development of a peduncular abscission zone (Fig. 7). This indicated that ethylene was capable of restoring the ripening process and abscission of the peduncle with the same efficiency at 42 DAP (normal harvest date of control fruit) and at prior or later stages of development on the plant. This demonstrated that sensitivity to ethylene was unaffected following transformation with antisense ACC oxidase. Only partial restoration of the normal ripening process (coloration) has been achieved in ACC oxidase antisense tomatoes (Pictou et al., 1993), while complete restoration has been reported in ACC synthase antisense tomatoes (Oeller et al., 1991).

While transgenic fruit did not undergo any softening when left on the vine, they exhibited significant softening upon detachment from the plant without any yellowing of the rind. Detachment of ACC oxidase antisense tomatoes also resulted in enhanced softening (Murray et al., 1993). The increase in internal ethylene observed upon detachment (Fig. 5) might be sufficient for stimulating cell wall degradation in the flesh, while it was still below the threshold value needed for inducing yellowing of the rind. In 'Anjou' pears (*Pyrus communis* L.), softening was more sensitive than other ripening pathways to exogenous ethylene treatment (Wang and Hansen, 1970).

These data indicate that antisense ACC oxidase melons provide useful data for discriminating between ethylene-regulated and ethylene-independent ripening pathways. Obviously, yellowing of the rind, softening of the flesh, and development of the peduncular abscission zone are completely blocked in ethylene-inhibited

Fig. 6. Effect of exogenous ethylene on softening of the flesh (A) and pigmentation of the rind (B). Antisense ACC oxidase melons were either harvested after 37, 42, and 47 d after pollination (DAP) and stored in air (●) or in the presence of 50 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene (■) or kept on the vine (---). Arrow indicates the date of harvest and ethylene treatment. Each point represents a mean of three replications $\pm$ SE (1996 experiment).

Fig. 7. Ethylene-dependent activation of the peduncular abscission zone. Antisense ACC oxidase melons (1996 experiment) were stored for 4 d at 25 °C in air (left) or in the presence of 50 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene (right). Arrow points to the abscission zone of ethylene-treated fruit.

fruit, while coloration of the flesh and accumulation of sugars are not regulated by ethylene.

From a commercial stand point, these data indicate that controlled melon ripening is possible. The new transgenic line offers flexibility in selecting for a harvest date. Since transgenic melons do not ripen on the vine, fruit can be allowed to accumulate more sugars on the vine without risk of over-ripening. Furthermore, storage and transportation can be handled at room temperature or moderate temperatures. Ripening can then be induced at will by treating fruit with exogenous ethylene to recover the original fruit quality attributes.

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