Role of ethylene in the biosynthetic pathway of aliphatic ester aroma volatiles in Charentais Cantaloupe melons

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Abstract

Compared to other melon types, Cantaloupe Charentais melons are highly aromatic with a major contribution to the aroma being made by aliphatic and branched esters. Using a transgenic line in which the synthesis of the plant hormone ethylene has been considerably lowered by antisense ACC oxidase mRNA (AS), the aliphatic ester pathway steps at which ethylene exerts its regulatory role were found. The data show that the production of aliphatic esters such as hexyl and butyl acetate was blocked in AS fruit and could be reversed by ethylene. Using fruit discs incubated in the presence of various precursors, the steps at which ester formation was inhibited in AS fruit was shown to be the reduction of fatty acids and aldehydes, the last step of acetyl transfer to alcohols being unaffected. However, treating AS fruit with the ethylene antagonist 1-methylcyclopropene resulted in about 50% inhibition of acetyl transfer activity, indicating that this portion of activity was ethylene-dependent and this was supported by the low residual ethylene concentration of AS fruit discs (around 2 nL L⁻¹). In conclusion, the reduction of fatty acids and aldehydes appears essentially to be ethylene-dependent, whilst the last step of alcohol acetylation has ethylene-dependent and ethylene-independent components, probably corresponding to differentially regulated alcohol acetyltransferases.

Key words: Aliphatic esters, Charentais melon, Cucumis melo, ethylene, aroma volatiles.

Introduction

Charentais cantaloupe melons (Cucumis melo L., var. cantalupensis Naud.) are orange-fleshed fruits that differ from the so-called American cantaloupes or Australian rockmelons (reticulatus variety) by a higher degree of fragrance and a faster ripening rate that is associated with a sharper peak of ethylene production and respiration climacteric. The aroma volatiles in Charentais type cantaloupe melons, as in other cantaloupes, are predominantly a complex mixture of esters and to some extent of saturated and unsaturated aldehydes and alcohols, and sulphur compounds among others (Homatidou et al., 1992). The volatile esters containing a branched alkyl chain originate from valine, isoleucine and other amino acids (Yabumoto et al., 1977; Willye et al., 1995), while the aliphatic esters and alcohols are produced from free fatty acids such as linoleic and linolenic acids (Baldwin et al., 2000). Fatty acids can generate compounds with shorter chains by β-oxidation (Sanz et al., 1997). Both pathways converge in the formation of aldehydes, which are reduced to alcohols in a reaction catalysed by alcohol dehydrogenase (Speirs et al., 1998). The last step in the production of esters is catalysed by an alcohol acetyltransferase, AAT (Fellman et al., 2000). AATs represent a large group of enzymes which include the alcohol acetyltransferase responsible for the acetylation of alcohols (Ueda et al., 1997). However, the hormonal factors regulating the biosynthetic pathways of aroma volatiles are still poorly understood.

The inhibition of ethylene production by antisense ACC oxidase mRNA resulted in a considerable loss of production of aroma volatiles, mainly esters (Bauchot et al., 1998). This indicated the regulatory role of ethylene in the biogenesis of aroma volatiles, but the steps on which ethylene acts remain unknown. In the present

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work, ethylene-inhibited antisense ACC oxidase (ACO) Charentais cantaloupe melons (Ayub et al., 1996) and the ethylene action inhibitor 1-methyl cyclopropene, 1-MCP (Sisler et al., 1996) were used to identify the control points of the action of ethylene. The study focused on the biosynthetic route leading to hexyl and butyl acetates, which are important volatile compounds identified in the headspace of Charentais cantaloupe melons (Bauchot et al., 1998). The pathway involves the following steps: hexanoate → hexanal → hexanol → hexyl acetate. On the other hand, the conversion of hexanoate into butyrate by β-oxidation leads to butyl acetate through the following pathway: butyrate → butanal → butanol → butyl acetate. By assessing the bioconversion of the various precursors by wild type (WT) and antisense ACO (AS) fruit discs, treated with 1-MCP or not, it was concluded that the reduction of fatty acids and aldehydes was essentially an ethylene-dependent process, while the last step of ester formation comprised both ethylene-dependent and -independent components.

Materials and methods

Plant material

A homozygous line (T6 progeny) of Charentais cantaloupe melons (Cucumis melo L., var. cantalupensis Naud. cv. ‘Védrantais’) harbouring melon ACC oxidase cDNA in antisense orientation (AS) and exhibiting 99.5% inhibition of ethylene production (Ayub et al., 1996) was used for comparison with the corresponding untransformed wild type (WT). Plants were cultivated in a greenhouse as previously described (Guis et al., 1997). AS and WT fruit were harvested at the same age (i.e. 43–47 d after pollination) when the internal ethylene concentration had started to increase (> 3 μl l⁻¹) in WT fruits.

Ethylene analysis and treatment with 1-MCP and ethylene

The internal ethylene concentration of the fruit was monitored using an external gas collection reservoir as described previously (Ayub et al., 1996). AS and WT fruits were treated on the vine using an external gas collection reservoir as described previously (Ayub et al., 1996). The internal ethylene concentration of the fruit was monitored using an external gas collection reservoir as described previously (Ayub et al., 1996). AS and WT fruits were treated after harvest with a flow of 50 μl l⁻¹ of 1-MCP for 3 d in sealed 3.0 l jars with periodic ventilation and adjustment of 1-MCP concentration every 12 h. The 1-MCP used was prepared chemically (Sisler et al., 1996). AS fruit were treated after harvest with a flow of 50 μl l⁻¹ ethylene for 3 d.

In vivo bioconversion of precursors of aliphatic esters

A modified version of the method described by Paillard was used (Paillard, 1979). Fruits were surface-sterilized and fruit discs (7 mm diameter and 2 mm thick) were withdrawn from the middle part of the pulp using a cork borer. Each sample consisted of 15 discs taken from one fruit. The discs were briefly rinsed with sterile water to remove the intercellular material and incubated in 250 ml sterilized Erlenmeyer flasks containing 50 ml of sodium phosphate (0.3 M, pH 5.8) buffer. An aqueous solution of the substrates was introduced at a concentration of 10⁻³ M for hexanal and sodium hexanoate and 10⁻⁴ M for hexanol. Flasks were sealed, shaken (120 rpm) at 25 °C and the medium was withdrawn at the desired periods of time for freezing at –20 °C before analysis of volatiles. The whole experiment was performed under sterile conditions.

Analysis of volatiles

Butyl acetate, butanol, hexyl acetate, and hexanol present in whole fruit were analysed by grinding 250 g of melon flesh in liquid nitrogen. The powder was homogenized in 750 ml phosphate buffer (0.3 M, pH 7) containing α-pinene as an internal standard. After thawing, the volatile components were extracted three times with 50 ml pentane. The organic phases were pooled, dried on sodium sulphate and concentrated to 0.1 ml under nitrogen flow.

For the analysis of volatiles produced by bioconversion and released in the medium, 25 ml of the incubated medium were thawed and extracted with 1 ml of pentane containing 5 μl l⁻¹ of α-pinene. After vigorous stirring for 1 min with a Vortex, the organic phase was transferred to a 2 ml Eppendorf tube and centrifuged 3 min at 13 000 rpm.

For analysis, 1 μl of the pentane phase was injected into a Hewlett-Packard gas chromatograph (model 5890) equipped with a flame ionization detector and a HP-Innowax cross-linked polyethylene glycol column (30 m × 0.25 mm × 0.25 μm). Injector and detector temperatures were 250 °C. The oven temperature was programmed from 40 °C (1 min) to 190 °C at a rate of 2 °C min⁻¹. Nitrogen was used as carrier gas at 100 kPa. Compounds were identified by comparison of retention times with those of authentic standards.

Results

Production of hexanol, butanol and hexyl and butyl acetate by untransformed (WT) and antisense ACO (AS) melons treated or not with ethylene

This work focused on the biosynthetic route leading to two important aliphatic esters present in the aroma volatiles of melon: hexyl acetate and butyl acetate. Table 1 shows that the two esters and hexanol are produced at high levels in WT fruit while they are undetectable in AS fruit. Butanol was not present at detectable levels in any type of fruit. However, the presence of butyl acetate in WT and ethylene-treated AS fruit indicates that it must play the role of a short-lived intermediate. Treatment of AS fruit with ethylene was

| Table 1. Levels of butyl acetate, hexyl acetate, butanol and hexanol in tissues of WT and AS melons treated or not with ethylene |
|---------------|---------------|---------------|---------------|
| Volatiles     | WT (μmol kg⁻¹) | AS (μmol kg⁻¹) | AS + 50 μl l⁻¹ ethylene for 3 d (μmol kg⁻¹) |
| Hexanol       | 1.5 ± 0.6     | tr            | 4.9 ± 1.8     |
| Hexyl acetate | 1.5 ± 0.6     | tr            | 2.7 ± 0.6     |
| Butanol       | 4.4 ± 1.6     | tr            | 3.3 ± 1.1     |
| Butyl acetate | nd            | nd            | nd            |

*tr = Levels below 0.3 μmol kg⁻¹.

*nd = Non detectable.
able to restore the biosynthesis of hexyl and butyl acetate and hexanol to a level similar to WT fruit.

Effects of suppression of ethylene synthesis on the capacity of melon discs to metabolize various precursors of the biosynthetic pathway of aliphatic esters

In order to determine which step of aliphatic ester biosynthesis was inhibited/attenuated in ethylene-inhibited AS melons, the capacity of AS and WT fruit discs to convert various precursors of the pathway was assessed. When both WT and AS disc tissues were fed with hexanol ($10^{-4}$ M), the immediate precursor of hexyl acetate, there was a fast decline in the concentration of hexanol associated with an increase in hexyl acetate (Fig. 1A, B). Interestingly, the rate of conversion was identical in AS tissues, indicating that the strong inhibition of ethylene production had no effect on the esterification step. The ability to reduce aldehydes into alcohols was tested using hexanal as a precursor and measuring the

![Image](image.png)

Fig. 1. Bioconversion of various precursors of the aliphatic esters biosynthetic pathway by melon discs incubated for 24 h. Inserts of the left column correspond to wild-type control melon fruit (A, C, E). Inserts of the right column correspond to antisense ACC oxidase fruit (B, D, F). Data of inserts A and B represent the bioconversion of hexanol (●) into hexyl acetate (♦), C and D of hexanal (○) into hexyl acetate (♦) and E and F of Na-hexanoate into hexanal (○), hexyl acetate (♦) and butyl acetate (▲).
formation of hexyl acetate. Incubation of WT tissues with 10⁻³ M hexanal (Fig. 1C) resulted in a sharp decline in the concentration of hexanal for the first 3 h, followed by a plateau and then a steady accumulation of hexyl acetate over 24 h. In AS discs (Fig. 1D), the changes of hexanal concentration followed the same trend as in WT discs. However, the production of hexyl acetate was considerably reduced in AS with a plateau at 0.4 μmol kg⁻¹ FW. After 24 h there was about 10 times less hexyl acetate in AS discs than in WT (3.4 μmol kg⁻¹ FW). These data, therefore, indicate that ethylene-suppressed AS tissues had lost most of their capacity to reduce aldehydes into alcohols.

Tissues were also incubated in the presence of Na-hexanoate (10⁻³ M), a precursor upstream in the biosynthetic pathway. Figure 1E shows that WT melon discs generated high amounts of hexyl acetate and butyl acetate and low amounts of hexanal. By contrast, AS melon discs were totally unable to produce esters (Fig. 1F). Hexanal remained at a very low level. In both cases hexanol and butanol were present at trace levels. These data indicate that AS tissues had lost the capacity to convert fatty acids into the corresponding aldehydes.

**Effects of inhibiting ethylene action by 1-MCP**

The role of ethylene was further assessed by treating WT and AS melon fruit with the potent inhibitor of ethylene action, 1-MCP. The capacity of fruit discs of WT 1-MCP-treated melons to produce esters (hexyl and butyl acetates) was inhibited almost completely when Na-hexanoate was given as a precursor, but only by 50% in the presence of hexanol (Fig. 2). As previously observed, untreated AS fruit discs were capable of producing as many esters as the WT when hexanol was provided. However, AS discs from 1-MCP-treated fruit, underwent a 50% reduction of the conversion of hexanol into esters. The low level of ethylene production in AS fruit was sufficient to support a portion of the activity of ester formation. Conversely, a significant part of this activity was ethylene-independent.

**Discussion**

Aroma volatiles represent a major quality attribute of cantaloupe melon fruit, particularly of the Charentais type which is renowned for its fragrance. In recent years, breeders have generated long or mid-shelf-life varieties that exhibit a reduction of aroma volatile production. Considering that extension of the shelf-life is generally coupled with a reduction or delay in the production of the plant hormone ethylene (Lacan and Bacou, 1996), it was tempting to correlate low aroma volatile production with low ethylene synthesis or action. The direct demonstration of such a correlation has come from the generation of transgenic fruit in which ethylene production was strongly reduced by an antisense RNA strategy. In tomato, inhibition of ethylene production by suppression of ACC synthase (Oeller et al., 1991) or ACC oxidase (Hamilton et al., 1990) activity has resulted in a significant reduction of aroma volatiles (Baldwin et al., 2000). Also, non-ripening natural mutants of tomato that are affected for ethylene response are deficient in the most potent odorants (McGlasson et al., 1987). In melon, F₁ hybrids generated using the AS parent line of Ayub et al. (Ayub et al., 1996) produce low amounts of aroma volatiles, especially esters (Bauchot et al., 1998). In the present work, where the original homoyzgous AS line has been used, the inhibition of hexanol, hexyl acetate and butyl acetate was even more marked than in the F₁ hybrids anlaysed by Bauchot et al. (Bauchot et al., 1998).

In addition, it is shown that ethylene is capable of restoring the production of these compounds. The presence of very low amounts of hexanol in AS fruit indicates an inhibition of the biosynthetic pathway upstream of the acetyl transfer. Restoration of fragrance by ethylene was also observable by sniffing.

In order to elucidate the critical steps at which the production of aroma volatiles could be regulated in melon, the capability of fruit discs to convert precursors of the aliphatic esters pathway was evaluated. These data are in agreement with previous work showing that incubation of apple fruit discs with short chain fatty acids and/or alcohols resulted in the production of ester volatiles (Paillard, 1979). Similar observations were made by applying vapours of the precursors directly onto intact apples (De Pooter et al., 1983) and strawberries (Yamashita et al., 1977). The generation of esters with reduced chain length (butyl acetate) as compared to the initial substrate (Na-hexanoate) is a result of β-oxidation of fatty acids. This has also been observed in apple fruit...
(De Pooter et al., 1983). The possible role of lipoxygenases (LOX) that are involved in the early steps of the biosynthesis of some C6 aldehydes and alcohols and of phospholipase D which is assumed to yield fatty acid precursors (Marangoni et al., 1996) has not been evaluated here. However, down-regulation of LOX activity in tomatoes did not induce significant changes in aroma volatiles of tomatoes (Griffiths et al., 1999).

Concerning the limiting steps of the ester biosynthetic pathway, little information is available. Ueda et al. found that a wide range of genotypes of the reticulatus, momordica, makuwa, conomon, agrestis, and F1 hybrids from these groups have the capacity to convert isobutyl alcohol into isobutyrlacetate (Ueda et al., 1997). However, members of the inodorus, flexuosus and dudain genotypes either lacked the capacity or exhibited very low capacity. Shalit et al. demonstrated that acetyl-CoA: alcohol acyltransferase activity (AAT) was high in climacteric melons of the reticulatus group, but was negligible in non-climacteric melons of the inodorus group (Shalit et al., 2001). The comparison of ethylene-inhibited transgenic material producing low levels of aroma volatiles with the wild-type control, associated with the use of the ethylene antagonist 1-MCP, have now allowed the role of ethylene in the regulation of aroma volatiles production to be addressed using the same genetic background. Both ethylene-suppressed transgenic fruit (tomatoes, Baldwin et al., 2000; melons, Bauchot et al., 1998) and 1-MCP-treated fruit (plums, Abdi et al., 1998; bananas, Golding et al., 1998) produced lower levels of volatiles. In this paper it has been shown that ethylene regulates the steps of reduction of fatty acids and aldehydes and, partly, the step of esterification, in the aliphatic ester pathway. This indicates that the alcohol dehydrogenases (ADH) involved in the pathway are predominantly ethylene-dependent. A strong increase in ADH activity has been observed during the ripening of climacteric fruit (tomato; Chen and Chase, 1993). However an increase in ADH activity was also found in non-climacteric fruit (grape; Sarni-Manchado, 1997) that can be considered as ethylene-independent. The presence of ethylene-independent ADHs in climacteric fruit cannot be excluded.

The esterification of alcohols through the AAT gene family. The possible role of lipoxygenases (LOX) that are involved in the early steps of the biosynthesis of some C6 aldehydes and alcohols and of phospholipase D which is assumed to yield fatty acid precursors (Marangoni et al., 1996) has not been evaluated here. However, down-regulation of LOX activity in tomatoes did not induce significant changes in aroma volatiles of tomatoes (Griffiths et al., 1999).

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The esterification of alcohols through the AAT activity has been observed during the ripening of melon (Cucumis melo L.) fruits. (Aharoni et al., 1997). Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. The Plant Cell 12, 647–661.


