

# Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit

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## Abstract

Cantaloupe melons have a typical climacteric behaviour with ethylene playing a major role in the regulation of the ripening process and affecting the ripening rate. Crossing of Cantaloupe Charentais melon with a non-climacteric melon indicated that the climacteric character is genetically dominant and conferred by two duplicated loci only. However, other experiments made by crossing two non-climacteric melons have generated climacteric fruit, indicating that different and complex genetic regulation exists for the climacteric character. Suppression of ethylene production by antisense ACC oxidase RNA in Charentais melon has shown that, while many ripening pathways were regulated by ethylene (synthesis of aroma volatiles, respiratory climacteric and degreening of the rind), some were ethylene-independent (initiation of climacteric, sugar accumulation, loss of acidity and coloration of the pulp). Softening of the flesh comprised both ethylene-dependent and independent components that were correlated with differential regulation of cell wall degrading genes. These results indicate that climacteric (ethylene-dependent) and non-climacteric (ethylene-independent) regulation coexist during climacteric fruit ripening. In addition, ethylene-suppressed melons allowed demonstrating that the various ethylene-dependent events exhibited differential sensitivity to ethylene and that ethylene was promoting sensitivity to chilling injury. Throughout this review, the data generated with melon are compared with those obtained with tomato and other fruit.

*Keywords:* Antisense ACC oxidase melons; Genetics of the climacteric; Cell wall-degrading genes; Ethylene sensitivity; Aroma volatiles; Chilling injury

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## 1. Introduction

The plant hormone ethylene regulates a wide range of plant developmental processes and environmental responses [1,2]. One of the most studied examples of ethylene regulation is the ripening of climacteric fruit in which, contrary to non-climacteric fruit, the ripening process is accompanied by a peak of respiration and a concomitant burst of ethylene production [3–5]. The ethylene burst results from autocatalytic stimulation of ethylene synthesis. The discovery of the ethylene biosynthesis pathway [6] has been a crucial step in the isolation of the two main regulatory enzymes, ACC synthase and ACC oxidase and the encoding genes. Suppression of ethylene production by knocking-down the expression of ACC oxidase and ACC synthase has resulted in a strong inhibition of the ripening process [7–9]. Ethylene affects the expression of many ripening-related genes [10,11] but, although it has been less studied, ethylene-independent regulation also exists in climacteric fruit. Most of the recent studies on climacteric fruit ripening have been carried out using tomato as a model fruit due to the relatively small genome, well characterized developmental mutants, availability of genomic tools, ease of genetic transformation and relatively short life cycle. The melon, although possessing less favourable characteristics, has some advantages over the tomato in comprising climacteric and non-climacteric genotypes. In addition, melons in which ethylene production has been almost totally abolished through down-regulation of ethylene biosynthesis genes have been generated [12]. In these conditions, melon has proved useful for undertaking generic studies devoted at understanding the inheritance of the climacteric character and at discriminating between ethylene-dependent and independent regulation. This review is aimed at bringing together recent advances made using melon fruit on these two aspects and at comparing with data obtained with tomato and other fruit.

## 2. Ethylene and ripening rate

As mentioned earlier, the melon comprises climacteric and non-climacteric genotypes. Typical climacteric phenotypes with high ethylene production, such as *Cucumis melo* var *cantalupensis*, have a fast ripening rate and short shelf-life. Non-climacteric melon, such as *C. melo* var *inodorus*, unable to produce autocatalytic ethylene, generally have a slow ripening rate associated with a long shelf-life. Hybrids of the Charentais *cantalupensis* type with an un-characterized non-ripening Charentais genotype has led to the generation of mid or long shelf-life melons. The characteristics of the non-ripening parent lines and hybrids have not been studied in terms of ethylene biosynthesis or sensitivity. Data are available only for the production of aroma volatiles which are shown to be strongly reduced in long shelf-life varieties [13]. Nevertheless, by screening different cantaloupe melons, Zheng and Wolff [14], have found a correlation between ethylene production and post-harvest decay. In addition, using

ACC oxidase (ACO) cDNAs probes, they were able to demonstrate that low ethylene production was associated with the presence of an RFLP ACO allele *Ao*, whereas high ethylene production was associated with the *Bo* allele in homozygous conditions [15].

In other fruit species, such as the apple, it has been established that low ethylene production was correlated with long storage life [16]. The amount of ethylene in ripening Fuji apples parallels the transcription level of the ripening-specific 1-aminocyclopropane-1-carboxylic acid synthase (ACS) gene, *MdACS1* [17]. An allele of this gene (*MdACS1-2*) contains an insertion of a retro-transposon-like sequence in the 5'-flanking region and is transcribed at a lower level than the wild type allele *MdACS1-1*. Cultivars that are homozygous for the *MdACS1-1* allele have a long storage life [18].

In the tomato, the *rin* mutation [19] that encodes a MADS box type transcription factor and regulates the ripening process confers ethylene insensitivity to the fruit [20] and has been used for generating long shelf-life commercial varieties. However, the *rin* gene has been shown to control ripening of both climacteric and non-climacteric fruit [21] indicating that it probably acts upstream of the climacteric switch.

## 3. Inheritance of the climacteric character

As emphasized above, the climacteric character represents an important determinant of the ripening rate and storability. Because genetically compatible climacteric and non-climacteric types of melon are available, it has been possible to study the inheritance of the climacteric character. Périn et al. [22] have generated a segregating population resulting from a cross between a typical climacteric type Charentais melon (*C. melo* var *cantalupensis* cv *Védrantais*) and a non-climacteric melon, Songwhan Charmi PI 161375 (*C. melo* var *chinensis*). By studying the segregation of the formation of the abscission layer (Al) of the peduncle and ethylene production, it was found that the climacteric character was controlled by two duplicated independent loci (*Al-3* and *Al-4*) and that the intensity of ethylene production was controlled by at least four quantitative trait loci (QTLs) localized in other genomic regions. None of the QTLs matched with known genes of the ethylene biosynthetic or transduction pathways. It was recently reported that some introgression lines generated from two non-climacteric melons, Piel de Sapo (var *inodorus*) and Songwhan Charmi PI 161375 (var *chinensis*) presented a climacteric character [23]. The QTLs associated with ethylene production and respiration rate in this work were not located at the same position with the *Al* loci described by Périn et al. [22]. Taking together, these data suggest that different and complex genetic regulation exists for the climacteric character.

Whatever the genetic control involved, crossing climacteric with non-climacteric melons generates climacteric melons. Crossing of long shelf-life honeydew melon (*C. melo* var *inodorus*) with cantaloupe Charentais type melon (*C. melo* var *cantalupensis*) gives hybrids of the climacteric type [24].

#### **4. Control of ethylene production in melon through biotechnology**

Although genetic transformation of melon is not as easy as tomato, several papers report on the successful control of ethylene production in melon through biotechnology. Cantaloupe Charentais melons (cv Védraçais) have been transformed with an antisense construct of an ACC oxidase cDNA driven by the 35S promoter [12]. A line of the antisense lines generated showed a reduction of ethylene production by more than 99.5% which resulted in strong effects on the ripening process [25]. Similar work on the same variety of Cantaloupe Charentais melon, Védraçais, has been carried out using an antisense construct of an apple ACC oxidase gene. It resulted in a severe reduction of ethylene production and strong effect on fruit ripening [26].

Reducing ethylene production has also been achieved by expressing the T3 bacteriophage S-adenosylmethionine hydrolase (SAMase) under the control of a chimeric fruit-specific promoter in American-type cantaloupes [27]. By catalyzing the degradation of SAM, a precursor to ethylene synthesis, melons expressing SAMase produced 75% less ethylene than control fruit. However, the inhibition of ethylene production was not enough to change significantly the ripening and post-harvest behaviour. One of the very few effects of the transgene was a delay in the formation of the abscission zone which frequently allowed greater sugar accumulation on the vine.

Transformation of the Krimka parent line of Galia type melon with an antisense gene of ACO has been achieved. It resulted in strong reduction of ACC oxidase activity and ethylene production that caused a delay in softening of the flesh and yellowing of the rind [28].

#### **5. Evidence for ethylene-dependent and independent ripening pathways and gene expression**

The availability of ethylene-suppressed melons allowed discriminating between ethylene-dependent and independent ripening pathways. It has been observed that pulp coloration, accumulation of sugars and loss of acidity were ethylene-independent processes, whereas yellowing of the rind, softening of the flesh, development of the peduncular abscission zone, aroma formation and climacteric respiration were totally or partially ethylene-dependent [25,29,30]. Similar observations were made in Charentais melons transformed with an antisense ACO from apple Silva et al. [26] and in apples silenced for either ACC synthase or ACC oxidase [31].

In tomato fruit in which ethylene production and sensitivity was reduced by low oxygen and high carbon dioxide, some evidence has early been given of ethylene-independent events, such as sugar and organic acid metabolism [32]. Later, transgenic tomatoes with 97% inhibition of ethylene production were shown to present after harvest a delay in colour development, in the loss of acidity and sugar accumulation, but not in the softening rate [33]. The 3% residual ethylene was altering only partly the ripening process and fruit were capable of ripening normally on the vine.

In banana fruit, MCP treatments led to the observation that peel degreening and aroma volatiles production were two ethylene-dependent processes whereas sugar accumulation was ethylene-independent [34]. Regulation of the softening process and of the biosynthesis of aromas will be studied in details below.

Concerning gene expression, Hadfield et al. [35] have isolated sixteen cDNAs whose abundance is regulated during ripening of Charentais melons, the majority of them being fruit specific. When examined in antisense ACO fruit, three distinct patterns of mRNA accumulation were observed. One group of cDNA corresponded to mRNAs whose abundance was reduced in transgenic fruit but inducible by ethylene treatment. A second group was not significantly altered in the transgenic fruit and was unaffected by treatment with ethylene, indicating that these genes are regulated by ethylene-independent developmental cues. The third and largest group showed an unexpected pattern of expression, with mRNAs reduced in transgenic fruit and remaining low after exposure to ethylene. Regulation of this third group of genes thus appears to be ethylene-independent but may be regulated by other developmental factors that require ethylene at a certain stage of development. These data demonstrate that both ethylene-dependent and independent pathways exist for the regulation of ripening-related genes in climacteric fruit.

#### **6. Role of ethylene in the initiation of the climacteric**

Ethylene-suppressed melons exhibit no climacteric rise in respiration [30]. However, despite the absence of ethylene, ACC accumulation and ACC synthase activity start to increase at the same time as in wild type fruit, indicating that the initiation of the climacteric rise in ethylene and respiration are not controlled by ethylene (Fig. 1A). The ethylene precursor ACC strongly accumulates in antisense ACO (AS) fruit due to the absence of ACC oxidase activity. ACC synthase activity is steadily increasing to reach much higher levels in AS than in WT fruit probably as a result of the absence of retro-inhibition of ACC synthase gene(s) (Fig. 1B). Auto-inhibition of ethylene production has long been demonstrated in the pre-climacteric period [36].

#### **7. Ethylene-dependent and independent softening**

Fruit softening is a major factor that determines fruit quality and post-harvest life. Charentais type cantaloupes show a very rapid decrease in flesh firmness during ripening and represent a good model fruit for understanding the molecular events involved in the softening process [37]. Since ethylene is playing an important role in softening, transgenic suppression of ethylene production has been a powerful tool to discriminate between ethylene-dependent and independent softening events. Antisense ACO melons exhibit strong reduction of softening, but significant residual softening still persists indicating the presence of an ethylene-independent component in flesh softening [25]. In the apple, fruit obtained from plants silenced for either ACC synthase or ACC oxidase, showed reduced fruit softening [31]. In

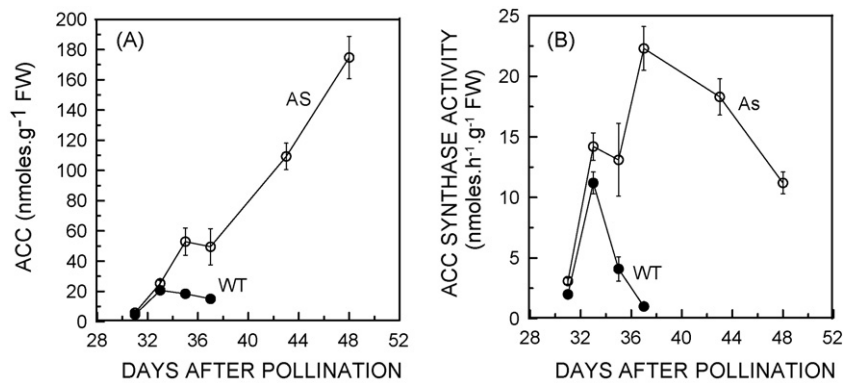


Fig. 1. ACC accumulation (A) and ACC synthase activity (B) in antisense ACO (AS) and wild type (WT) Cantaloupe Charentais melons. Values represent the means of three replicates  $\pm$ S.E.

antisense ACO tomatoes, a lack of inhibition of the softening has been observed [33], indicating that the 3% residual ethylene was sufficient to activate the cell wall degradation events.

The inhibition of the ethylene-dependent component of softening can be achieved by treating fruit with the ethylene antagonist 1-MCP, but, similarly to ethylene-suppressed transgenic melon residual softening still occurs [38]. The ethylene-independent component of softening is variable. It can be important in some fruit such as kiwis where 1-MCP has a moderate effect on softening [39]; it can be much more marked in other fruit such as pears where MCP causes total inhibition of softening [40].

Size exclusion chromatography of cell wall polysaccharides of ethylene-suppressed fruit, with or without exogenous ethylene, indicated that depolymerisation of both pectins and xyloglucans are strongly ethylene-dependent [41]. Northern analysis of a diverse range of cell wall-related genes, including those encoding polygalacturonases, xyloglucan endotransglycosylase/hydrolases, expansin and  $\beta$ -galactosidases demonstrated that specific genes of each families could be categorized as totally ethylene-dependent, totally ethylene-independent or partially ethylene-dependent [41]. These data suggest that cell wall-modifying proteins from each family contribute to the disassembly of different elements of the cell wall, with ethylene-regulated genes contributing to the ethylene-dependent softening and genes that are not regulated by ethylene contributing to the ethylene-independent component of softening. The discrimination between the ethylene-dependent and independent expression of cell wall-degrading genes has been possible in the transgenic melons of Ayub et al. [12] used in these studies thank to an inhibition of ethylene production close to 100%. In antisense ACS tomatoes where inhibition of ethylene production was not so high, polygalacturonase gene expression has been considered as ethylene-independent [8]. But it was later demonstrated that sufficient residual ethylene was present in the fruit to stimulate PG gene expression due its very high sensitivity to ethylene [42].

## 8. Role of ethylene in aroma volatiles production

The aroma volatiles evolved by Cantaloupe melons are mainly made of a complex mixture of esters, including sulfur-

containing esters and of saturated and unsaturated aldehydes and alcohols. Traditional Charentais melons are highly aromatic, but new types of Charentais melons with mid or long shelf-life have been generated by the breeders in which some of the original sensory qualities have been affected, especially aromas [13], giving a good example of antagonism between extension of storage life and aroma volatile production. The genetic background of the parents used for generating these new phenotypes is not well defined, but it is predictable that they are impaired for either ethylene synthesis or perception. Using hybrids of Charentais melons obtained by crossing with the antisense ACO line of Ayub et al. [12], Bauchot et al. [29] demonstrated that the transgenic hybrids evolved 60–85% less total volatiles than the non-transgenic hybrids. In addition, volatiles with low odour values, such as ethyl, 2-methylpropyl and 2-methylbutyl acetates were half to fifth lower than in controls while potent odorants, such as ethyle-2-methylpropanoate and ethyl-2-methylbutanoate were reduced by 97%. These data indicate that ethylene is controlling preferentially the synthesis of the most potent odorants.

Using fruit disks incubated in the presence of various precursors, it was shown that the steps at which the formation of esters was inhibited in the ethylene-suppressed fruit was the reduction of fatty acids and aldehydes. The acyl transfer to alcohols to form esters was strongly but not totally inhibited, indicating that this step which is controlled by alcohol acyltransferases had both ethylene-dependent and independent components [43]. Three genes encoding alcohol acyltransferases [44,45] and two genes encoding alcohol dehydrogenases [46] have been isolated and functionally characterized. All of them are strongly regulated by ethylene.

Ethylene-suppressed apples exhibited strong reduction of ester and alcohol production but were unaffected for aldehydes and alcohols [31,47]. In line with the synthesis of aromas, the activity and gene expression of alcohol acyl transferase were strongly reduced but not of alcohol dehydrogenase [48]. These data show that some differences exists between the melon and the apple with the synthesis of aldehydes and alcohols being ethylene-dependent and independent, respectively. The last step of esters biosynthesis is ethylene-dependent in both fruit.

## 9. Sensitivity to ethylene of some of the ripening pathways

Treating antisense ACO melons with ethylene allowed complete recovery of ripening [25]. It also enabled the determination of the gradual sensitivity of the various pathways to the hormone. The threshold level of ethylene capable of physiological activity varied from  $1 \mu\text{L L}^{-1}$  for degreening of the rind to  $2.5 \mu\text{L L}^{-1}$  for softening of the flesh, for membrane deterioration and for cell separation in the peduncle abscission zone [49]. The saturation levels for total accomplishment of the pathway were  $2.5\text{--}5.0 \mu\text{L L}^{-1}$ , which is by far much lower than the internal ethylene found at the climacteric peak (over  $100 \mu\text{L L}^{-1}$ ), indicating that the fruit evolves more ethylene than necessary, at least for the pathways under study. Cessation of the ethylene treatment resulted in a complete arrest of degreening of the rind and of peduncle abscission, demonstrating that both ripening pathways are completely dependent on ethylene. On the contrary, softening of the flesh and membrane deterioration, though significantly slowed-down upon removal of ethylene, continued to proceed suggesting that an ethylene-independent component exist in these two processes.

The sensitivity to ethylene of the climacteric respiration is affected by detachment of the fruit [30]. The application of ethylene to antisense ACO melons stimulated  $\text{O}_2$  consumption only if they were detached from the vine, showing that attachment to the plant inhibits the effect of ethylene on respiration. This effect of detachment on sensitivity to ethylene is known as tree factor by post-harvest physiologists and implies that an inhibitor of fruit ripening or ethylene action could be translocated by the phloem. The nature of this

inhibitor remains unknown. Auxin has been suggested to be a candidate but experimental evidence is still lacking.

## 10. Chilling injury

Non-freezing low temperature storage causes injury to melon and most other fruit and vegetables of tropical and subtropical origin. Ethylene suppression in antisense ACO melons considerably reduced the sensitivity to chilling injury [50,51]. Treating transgenic melons with ethylene resulted in the restoration of chilling sensitivity. The tolerance to chilling injury was associated with a lower accumulation of ethanol and acetaldehyde, reduced membrane deterioration and higher capacity of the fruit to remove active oxygen species through activated oxygen scavenging enzymes.

Inhibition of ethylene sensitivity by MCP also resulted in higher tolerance to chilling injury of avocados [52] and pineapples [53]. On the contrary, in some citrus varieties, such as Fortuna mandarin, ethylene treatments enhanced resistance to chilling injury [54]. The opposite is true for other types of citrus such as ‘‘Shamouti’’ oranges [55], indicating that the role of ethylene in chilling injury may vary from one fruit to another.

## 11. Conclusions

In conclusion, the data reported in this review show that the melon has been a good model fruit for addressing the role of ethylene in fruit ripening. Contrary to many other fruit species, including tomato, the melon comprises climacteric and non-climacteric type. This has allowed undertaking specific studies on the genetic control of the climacteric character. Although no

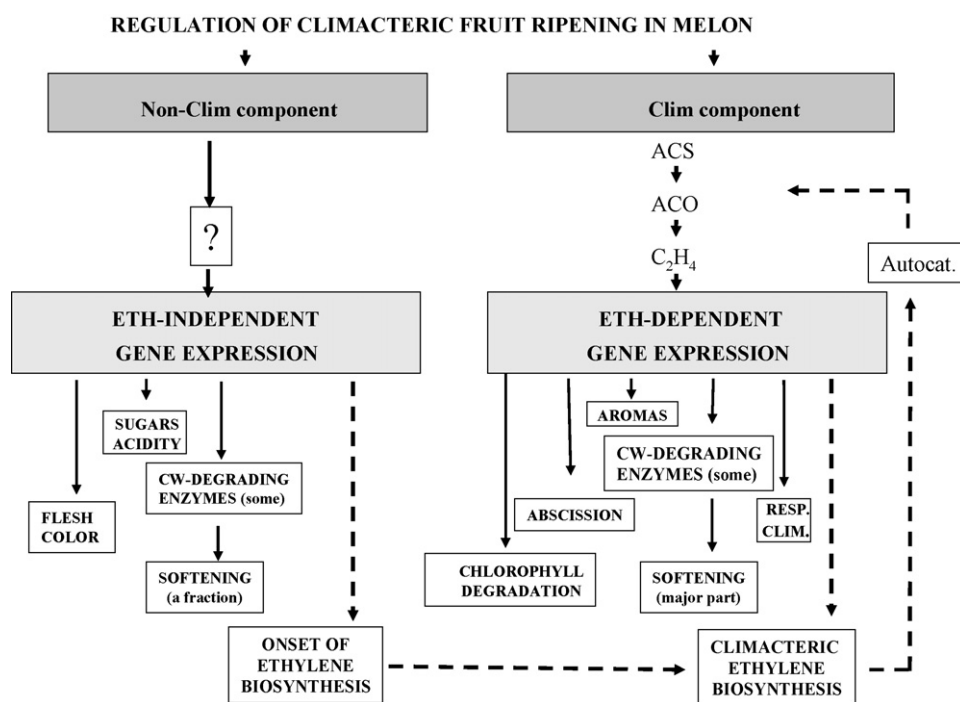


Fig. 2. General scheme showing the presence of ethylene-dependent and independent processes in ripening melon fruit. Clim, climacteric; RESP, respiration; CW, cCell wall; Autocat, autocatalytic regulation; ACS, ACC synthase; ACO, ACC oxidase.



clear picture is available today on the genetic determinism of the climacteric character, this topic is of crucial importance and deserves further research efforts. Inhibition of ethylene production through biotechnology has also allowed demonstrating that ethylene is controlling many aspects of ripening, but not all of them. The findings on cell wall degrading mechanisms and aroma biosynthesis are of special interest due to the fast softening rate of some types of melons and the high production of aroma volatiles. They exemplify the presence of ethylene-dependent and independent events that are under the control of ethylene-regulated and ethylene-independent genes. In other words, climacteric and non-climacteric regulation coexist during climacteric fruit ripening as shown in Fig. 2.

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