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# The use of ethylene-suppressed lines to assess differential sensitivity to ethylene of the various ripening pathways in Cantaloupe melons

F. Flores<sup>a</sup>, M. Ben Amor<sup>b</sup>, B. Jones<sup>b</sup>, J. C. Pech<sup>b,\*</sup>, M. Bouzayen<sup>b</sup>, A. Latché<sup>b</sup> and F. Romojaró<sup>a</sup>

<sup>a</sup>CEBAS-CSIC Apto. de Correos 4195, E-30080 Murcia, Spain

<sup>b</sup>ENSAT UMR INRA 990, Avenue de l'Agrobiopole, BP 107 Auzeville, F-31326 Castanet-Tolosan, France

\*Corresponding author, e-mail: pech@flora.ensat.fr

Physiological characterization of ethylene-suppressed Cantaloupe Charentais melons (*Cucumis melo* var. *cantalupensis* Naud cv. Védraçais) revealed that some ripening-associated events, like degreening of the rind and cell separation in the peduncular abscission zone, are totally dependent on ethylene. By contrast, some other ripening events, like softening and membrane deterioration, depend only partially on ethylene and display some ethylene-independent components. Application of increasing levels of exogenous ethylene on these antisense 1-aminocyclopropane-1-carboxylic acid oxidase fruits enabled the determination of the gradual sensitivity of various ripening pathways to the hormone. The threshold level of ethylene capable of physiological activity varied from 1 ppm for degreening of the rind to 2.5 ppm for softening, membrane deterioration and cell separation in the peduncular abscission zone. Up to a saturating dose of 5 ppm, the extent of rind degreening was proportionally related to the level of applied

ethylene. The saturating levels of ethylene for flesh softening (2.5 ppm) and for membrane deterioration and cell separation in the peduncular abscission zone (5 ppm) were much lower than the internal ethylene found at the climacteric peak of wild-type fruit (over 100 ppm). The cessation of ethylene treatment resulted in a complete arrest of the rind degreening and peduncular cell separation indicating that both ripening pathways are completely dependent on ethylene. On the contrary, softening and membrane deterioration, though significantly slowed upon removal of ethylene treatment, continued to proceed in the absence of the hormone, thereby unmasking the ethylene-independent component of softening and membrane deterioration. The presence of ethylene-independent components in the regulation of individual pathways indicates that the ripening of climacteric fruit involves a substantial portion of non-climacteric regulation.

## Introduction

The plant hormone ethylene plays a major role in the ripening process of climacteric fruits. Other hormonal and/or developmental factors are also involved (Lelièvre et al. 1997), but the nature of these factors remains largely unknown. Significant progress has recently been made in the discrimination of ethylene-dependent and -independent ripening pathways through the use of ethylene-inhibited transgenic tomatoes (Murray et al. 1993, Picton et al. 1993, Theologis et al. 1993) and melons (Ayub et al. 1996, Guis et al. 1997). In the transgenic antisense 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) melon, coloration of the flesh, accumulation of sugars and loss of acidity are not altered by ethylene suppression, indicating that these processes are not regulated by the hormone (Guis et al.

1997). In contrast, ripening parameters such as degreening of the rind, flesh softening, detachment of the peduncle (Guis et al. 1997) and the production of aromas (Bauchot et al. 1998) are strongly reduced by the absence of ethylene in transgenic fruit. Treating ethylene-suppressed fruit with increasing amount of exogenous ethylene enables an evaluation of the sensitivity of the various ripening pathways to the hormone. Differential sensitivity to ethylene of the various components of ripening has long been recognised, but few experimental data are available in support of the concept (Wang and Hansen 1970, Kader 1992). One of the major difficulties in studying the role of ethylene regulation of individual ripening processes is that the effects of a pre-climacteric ethylene treatment are masked by the initial

*Abbreviations* – ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; AS, ACO antisense; WT, wild-type untransformed.

tion of autocatalytic ethylene production. Therefore, ethylene-suppressed transgenic fruit, unable to generate autocatalytic ethylene, represent a unique and valuable material for addressing the differential sensitivity to ethylene of the different ripening associated pathways. Moreover, since the sensitivity to ethylene is not affected in the ethylene-inhibited lines, the ripening process can be controlled through exogenous application of the hormone. As a result, individual ethylene-controlled ripening processes can be studied for their dose dependence and time course of their response to the hormone.

In this study, ethylene-suppressed transgenic melons were used to assess the ethylene sensitivity of a number of important ripening processes: degreening of the rind; flesh firmness; loss of the semipermeability of cell membranes; and the development of the peduncular abscission zone. The following parameters were estimated: (1) the threshold concentration of ethylene capable of initiating individual pathways; (2) the saturating level of ethylene above which no additional effect could be observed; (3) the most effective combination of concentration and duration of treatment for completely restoring the wild-type (WT) phenotype; and (4) the effect of the cessation of ethylene treatment at various time intervals.

## Materials and methods

### Plant material

Plants from the sixth generation of the B-17 transgenic ACO antisense line (AS) of Cantaloupe Charentais melon (*Cucumis melo* var. *cantalupensis*, Naud cv. Védraçais) were used (Ayub et al. 1996). Fruit from this line exhibit an inhibition of ethylene production of over 99.5% compared to the WT untransformed. WT melons have been used as controls. Plants were grown under standard hydroponic conditions in a greenhouse. Self-pollination was performed by hand in order to tag the pollination date with accuracy. Only two fruits were left per vine.

### Harvest and exogenous ethylene treatments

Fruit were harvested at 38–40 days after pollination, i.e. 2 days before the onset of the climacteric rise in ethylene production in WT fruit. Fruit were treated with various levels of ethylene at 22°C in 200-l tanks containing potassium hydroxide in order to trap respiratory CO<sub>2</sub>. The tanks were flushed every day with fresh air during the whole treatment and the ethylene concentration was adjusted daily by injecting ethylene through a septum. Two different ethylene treatment experiments were carried out. During the 1998 season, five levels of ethylene were assayed (0.1, 1, 2.5, 5 and 50 ppm). In 1999, fruit were treated with 5 ppm only. WT and AS fruit were stored in air in the absence of exogenous ethylene. Five fruit were analysed for the various parameters at each ethylene concentration. Samples were taken after 0, 1, 3 and 5 days of ethylene treatment, immediately upon removal from the treatment containers (1998 experiments) or after 0, 2 and 4 additional days in the air (1999 experiments).

## Determination of physical and biochemical parameters

Flesh firmness was evaluated using a Penefel-type penetrometer (Copa Technologie, 13103 St-Etienne du Gres, France) and measured as the force required for the penetration of a 0.5-cm<sup>2</sup> calibrated rod into the pulp of a half-cut melon. Four measurements were performed at a distance of 1.5 cm from the external edge of each fruit cut transversely. The colour of the rind was monitored with a reflectance meter (Minolta CR-300 chromameter, Osaka, Japan) according to the manufacturer recommendations. Measurements were made in the L\*a\*b\* colour system. The b parameter (chromacity co-ordinates on a yellow-blue axis) was chosen as most representative of the changes from the green to the yellow colour of the rind. Four equidistant and independent measurements were taken in the equatorial zone of each fruit. The correlation coefficient was calculated between 'b' colour parameter and ethylene levels in the range of 0–5 ppm after 5 days of treatment. Chlorophylls from the rind were extracted using 80% acetone and measured according to Hill et al. (1985). Electrolyte leakage of the tissues was determined by incubating ten discs of rind tissue from the equatorial region of the melon in 50-ml flasks containing 40 ml of 0.4 M mannitol. The flasks were shaken for 2 h at 120 cycles per min and the conductivity of solution (EC<sub>0</sub>) was measured afterwards with a microdigital conductivity meter. Total conductivity (EC<sub>T</sub>) was measured after freezing the mannitol solution and the tissues at –20°C for 24 h followed by additional heating at 121°C for 20 min. Ion leakage was calculated as (EC<sub>0</sub>/EC<sub>T</sub>) × 100 (Ben-Amor et al. 1999). The development of cell separation in the peduncular detachment zone was measured by estimating the portion showing cell separation at the point of attachment of the fruit to the peduncle. Data are given as a percentage of total cell separation of the peduncle. The results are expressed as the average ± SE values of independent measures taken in five fruits at each day and ethylene concentration.

## Results

### Effects of ethylene on flesh firmness

Figure 1A shows that firmness of WT fruit left for 5 days at 22°C underwent a sharp decrease from 5 to 0.85 kg cm<sup>-2</sup>, while AS fruit similarly treated exhibited a much lower softening rate, from 5 to 2.8 kg cm<sup>-2</sup>. Therefore, the softening process is inhibited by about 50% in the absence of ethylene. After 5 days, the firmness of AS fruits remained above the limit of acceptable quality evaluated by Alavoine et al. (1988) (1–1.5 kg cm<sup>-2</sup>). Treating AS melons with 0.1 or 1 ppm ethylene did not result in any additional softening. However, at 2.5 ppm, softening was considerably accelerated and progressed at the same rate as WT. A further increase in the ethylene concentration up to 50 ppm did not result in any additional softening, indicating that 2.5 ppm was the saturating level for softening. It is noteworthy that the rate of softening was much higher for AS during the first day and thereafter similar to WT fruit.

The effect of the cessation of ethylene treatment was studied on fruit that were subjected to 5 ppm ethylene for 1,

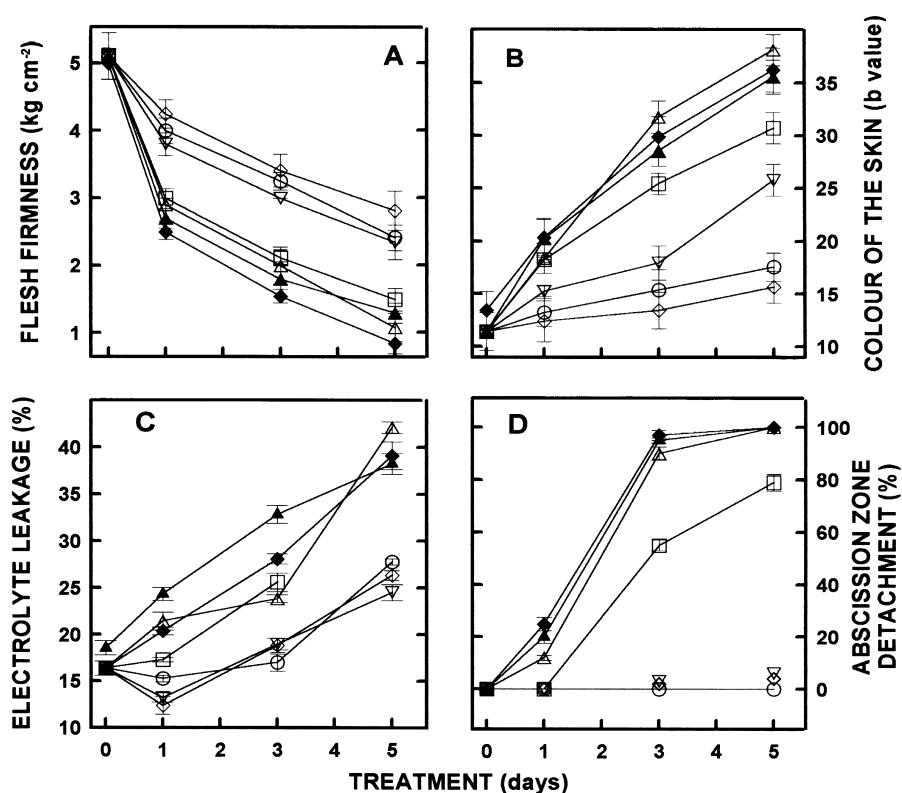


Fig. 1. Effect of treating AS fruit with different ethylene concentrations on flesh firmness (A), colour of the rind (B), electrolyte leakage of the rind (C) and detachment of the peduncule (D) of transgenic melons. (◆) WT + air; (◇) AS + air; (○) AS + 0.1 ppm; (▽) AS + 1 ppm; (□) AS + 2.5 ppm; (△) AS + 5 ppm; (▲) AS + 50 ppm. Each data represents the average of 5 independent measures  $\pm$  SE (1998 experiment).

3 and 5 days. Figure 3A shows that upon return to the air (dotted lines), softening was sharply reduced except for fruit held in ethylene for 5 days which had already undergone considerable softening. After the cessation of the ethylene treatment, the rate of softening, which was now ethylene-independent, was similar to that of untreated AS fruit.

#### Effects of ethylene on the rind colour

Figure 1B shows the strong retention of the green colour of AS fruit while WT fruit exhibited rapid yellowing corresponding to a sharp increase in the b value. Treating AS fruit with ethylene resulted in a linear increase in yellowing over 5 days that was proportional to the concentration of ethylene within the range of 0.1–5 ppm. Within this range, a linear correlation ( $r = 0.98$ ) exists between the ‘b’ values and ethylene concentrations (between 0 and 5 ppm) after 5 days of treatment. Beyond 5 ppm, a saturation level was reached with a rate of yellowing similar to WT fruit and a complete recovery of the WT phenotype. The rate of changes in colour during the 5-day experiment was correlated linearly with the concentration of ethylene up to the saturation level (5 ppm). Yellowing of the rind was in good correlation with a loss of chlorophylls (Fig. 2). The chlorophyll content of the rind remained totally unchanged in untreated AS fruit, indicating a total dependence on ethylene. On the contrary, the b value was slightly increasing, indicating that some yellow components were synthesized at a slow rate in the absence of ethylene.

Moving ethylene-treated AS fruit to the air resulted in no further increases in the b value and a stabilisation of the

colour at the values reached at the end of the ethylene treatment, i.e. after 1, 3 or 5 days (Fig. 3B).

#### Effects on electrolyte leakage of the rind

A continuous increase of electrolyte leakage of rind tissues was observed over the 5 days of storage at 22°C of WT melons, indicating a degradation of membrane integrity (Fig. 1C). This phenomenon was almost twofold lower in AS melons kept in the air. Levels of up to 1 ppm of ethylene were unable to stimulate electrolyte leakage of AS fruit.

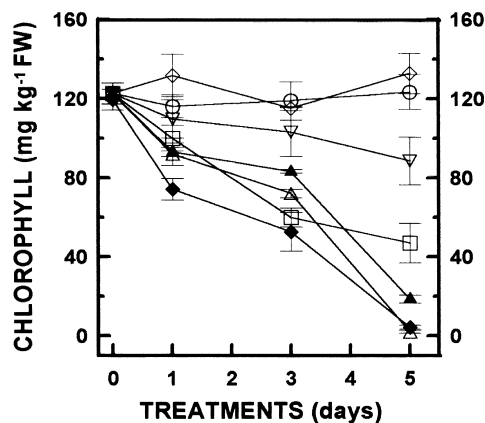
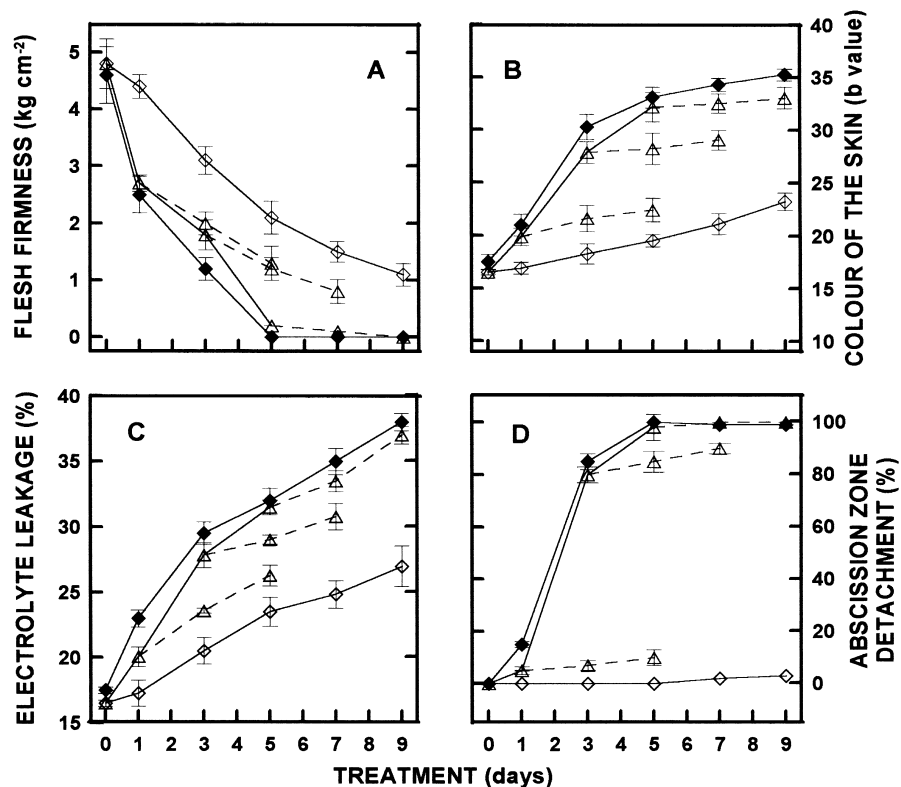


Fig. 2. Effect of treating AS fruit with different ethylene concentrations on chlorophyll content of the rind. (◆) WT + air; (◇) AS + air; (○) AS + 0.1 ppm; (▽) AS + 1 ppm; (□) AS + 2.5 ppm; (△) AS + 5 ppm; (▲) AS + 50 ppm. Each data represents the average of 5 independent measures  $\pm$  SE (1998 experiment).

Fig. 3. Effect of treating with ethylene (continuous line) and subsequently transferring to air after 1, 3 and 5 days (dotted line) on flesh firmness (A), colour of the rind (B), electrolyte leakage of the rind (C) and detachment of the peduncule (D) of AS transgenic melons. (◆) WT + air; (◇) AS + air; (△) AS + 5 ppm. Each data represents the average of 5 independent measures  $\pm$  SE (1999 experiment).



With 2.5, 5 and 50 ppm, the phenomenon was greatly stimulated, with 5 ppm being sufficient to saturate the response of the tissues and bring it to the rate observed in WT fruit.

Upon transferring fruit treated 1 and 3 days with 5 ppm ethylene to air, the rate of electrolyte leakage was strongly diminished and paralleled the ethylene-independent rate displayed by untreated AS fruit (Fig. 3C). Transferring AS fruit to an ethylene-free atmosphere following 5 days of exposure to the hormone did not affect the rate of membrane deterioration, which was similar to that observed in WT fruit, indicating that the maximum of membrane deterioration was reached with this duration of ethylene treatment.

#### Effects on the detachment of the peduncule

The detachment of the peduncule is a good reference for the choice of harvest date and stage of ripening of cantaloupe melons. Our data (Figs 1D, 2D) clearly indicate that the inhibition of ethylene synthesis in AS fruit prevents the detachment of the peduncule while complete detachment can be observed after 3 days in WT fruit (at 22°C). Exogenous ethylene applied at levels up to 2.5 ppm was ineffective at restoring abscission (Fig. 1D), while at 5 and 50 ppm, the process proceeded at a similar rate to WT fruit. With 2.5 ppm ethylene cell separation proceeded at a high rate after a lag phase of 1 day.

The cessation of the ethylene treatment of AS melon after 1 and 3 days of treatment (Fig. 3D) resulted in the arrest of cell separation in the peduncular abscission zone. After 3

days, both control fruit and ethylene-treated fruit had fully developed their abscission zone.

#### Discussion

In climacteric fruit, ethylene acts as a molecular trigger for the initiation of the ripening process. In ethylene-suppressed antisense ACO melons the production of ethylene, and consequently, the ethylene-dependent ripening processes are arrested. Comparing WT and AS ACO melons enables discrimination to be made between ethylene-dependent and -independent processes (Guis et al. 1997). In the melon, degreening of the rind, flesh softening, aroma production, climacteric respiration and abscission of the peduncule are considered as ethylene-dependent processes, whereas coloration of the flesh, sugar accumulation and loss of acidity are ethylene-independent. In this paper, we demonstrated that individual pathways such as softening and membrane deterioration comprise ethylene-dependent and -independent components (Fig. 1A,C). The presence of at least two regulatory systems in fruit softening is consistent with the observation that the expression of individual members of gene families, such as the cell wall-degrading polygalacturonases, can be either ethylene-dependent or -independent (Guis et al. 1999). The loss of semipermeability of cell membranes, a general feature of senescing organs and ripening fruit (Field 1981, Brady 1987, Lester and Stein 1993, Lacan and Baccou 1996) also comprises ethylene-dependent and -independent components. It is conceivable that the complex set of events involved in membrane deterioration is differentially regulated, and some of them by ethylene. It is known for

instance that the expression of lipoxygenase is under both developmental and ethylene regulation (Griffiths et al. 1999). Changes in colour of the rind include a loss of chlorophylls and an increase in yellow components including carotenoids. The present data and previous experiments show that both the loss of chlorophylls (Fig. 2) and the increase in carotenoids (Guis et al. 1997) are totally ethylene-dependent. Since the 'b' parameter exhibited a small increase in AS fruit (Fig. 1B), it can be assumed that yellow compounds other than carotenoids accumulate in AS fruit independently of ethylene. The nature of these compounds remains to be determined. The ethylene-dependent chlorophyll degradation is in accordance with the observation that one of the major genes involved in chlorophyll breakdown, chlorophyllase, is ethylene-regulated (Jacob-Wilk et al. 1999). Cell separation in the peduncular abscission zone also appears to be totally dependent on ethylene (Fig. 1D). This has already been observed by Kasmire et al. (1970) using the ethylene-releasing compound, ethephon (2-chloroethylphosphonic acid). Ethylene has long been recognised as being a major factor in abscission (Abeles et al. 1973, Lurssen 1985, Reid 1985). However, cell separation represents only the final phase of the process and it is possible that earlier steps are ethylene-independent.

Applying ethylene to AS fruit completely restored the WT phenotype for the parameters under study, indicating that sensitivity to ethylene was not affected by the genetic transformation (Fig. 1). As a result, it was possible to study the effect of exogenous ethylene on the ethylene sensitivity and time course of each individual ethylene-controlled ripening process. Taking advantage of this material, we evaluated the concentration of ethylene capable of initiating, with no lag phase, the individual processes. Rind coloration (1 ppm) was the most sensitive, with the other parameters (pulp softening, electrolyte leakage and development of the abscission zone [2.5 ppm]) (Fig. 1B), being similar and slightly less sensitive to the hormone (Fig. 1A,C,D). Kader (1992) has previously shown that coloration of the rind can be initiated by minute amounts of ethylene in Cantaloupe and 'Honey Dew' melons. The ethylene-sensitive component of fruit softening requires at least 2.5 ppm ethylene in the melon. In the pear, 0.1 ppm ethylene is sufficient to induce fruit softening before any other changes are observed (Wang and Hansen 1970). Treatment of AS fruit with 0.1 and 1 ppm ethylene does not alter the existing rate of softening, further indicating the presence of an ethylene-independent component of the process (Fig. 1A). No significant differences were observed in the rate of softening AS ACO tomatoes (Murray et al. 1993) as compared with WT fruit. In this case, the residual ethylene may have been above the threshold level required to stimulate ethylene-dependent softening in the tomato. Sitrit and Bennett (1998) have demonstrated the presence of residual ethylene production sufficient to stimulate endo-polygalacturonase (EC 3.2.1.15) mRNA accumulation in antisense ACC synthase tomatoes (Oeller et al. 1991).

Autocatalytic ethylene production follows a sigmoidal curve during ripening, with the level of ethylene production peaking 1–2 days after the initiation of ripening in WT melon fruit. Assuming that the most sensitive events are

induced at the very early stages of the climacteric phase, while those requiring higher levels of ethylene are initiated later on, it becomes possible to establish a chronological order for the initiation of the ripening events in the melon. Based on this assumption, degreening of the peel would precede a decrease in pulp firmness, development of the abscission zone and deterioration of cell membranes, although all these events would occur at the very early stages of the climacteric. Chlorophyll degradation has already been noted as beginning before or at the initiation of the rise in respiration in the melon (Reid et al. 1970).

The threshold values of ethylene required for inducing changes in individual ripening parameters are more than likely related to the level of ethylene required for inducing the expression of specific genes. Very few data are available on this subject; however, Lincoln and Fischer (1988) found that the expression level of a number of ethylene-inducible genes was a function of ethylene concentration and that each gene displayed a unique dose-response curve. The dose response to ethylene differs between the individual ripening processes measured in this study. The rate of change in rind colour is quantitatively related to the concentration of ethylene up to a saturation level of 5 ppm (Fig. 1B), whereas the velocity of change is already at its maximum when a threshold level of ethylene is reached for flesh firmness, membrane deterioration and detachment of peduncle (Fig. 1A,C,D). The physiological and/or molecular basis for such behaviour remains to be elucidated.

Although the kinetics were not identical, it was possible in all cases to fully restore the WT phenotype with ethylene treatment of AS fruit. In similar studies, ethylene treatment of AS ACO tomatoes was unable to fully restore WT colour (Picton et al. 1993) and softening (Murray et al. 1993). In contrast, Oeller et al. (1991) observed that long-term (7 days) ethylene treatment of AS ACC synthase (EC 4.4.1.14) tomato fruit was capable of inducing the onset of ripening and restoring the ripening process so that ethylene-treated AS fruit could not be distinguished from ripe WT fruit. It is possible that the duration and/or the level of applied ethylene were not sufficient in the tomato AS ACO experiments. Alternatively, the fruit may have been treated at a stage where their sensitivity to ethylene was too low.

Ethylene at 5 ppm was saturating for all the parameters under study, indicating that the high levels (80–100 ppm) of ethylene produced during the climacteric are far in excess. However, it cannot be excluded that other pathways require ethylene concentrations higher than 5 ppm for saturation (Wang and Hansen 1970). It has been suggested that high ethylene concentrations are required for the full integration of the ripening process (McMurchie et al. 1972). The cessation of the ethylene treatment resulted in stabilisation of the ripening parameters at the value reached at the end of the treatment, indicating that the continuous presence of ethylene is required for the completion of the ripening process (Fig. 2). From a practical point of view, it should be possible to adjust the characteristics of ripened AS melons with a specific level and length of application of the hormone.

In summary, recent work has shown that ripening of climacteric fruit comprises both ethylene-dependent and

Table 1. Ethylene dependence of several ripening pathways and characteristics of ethylene action

Ripening parameter	Presence of an ethylene-independent component	Characteristics of the ethylene-dependent component	
		Threshold level (ppm)	Saturation level (ppm)
Flesh softening	Yes	2.5	2.5
Membrane deterioration	Yes	2.5	2.5
Abscission zone detachment	No	2.5	5.0
Rind degreening	No	1.0	5.0

-independent processes (Guis et al. 1997, Lelièvre et al. 1997). We have refined this observation to show that sensitivity to the hormone varies between the individual ethylene-dependent processes, and that some of the processes are totally ethylene-dependent while others comprise both ethylene-dependent and -independent components (Table 1). Non-climacteric fruit are those in which the majority of the ripening processes are ethylene-independent. This work has reinforced the idea that fruit ripening is an orchestration of ethylene-dependent and -independent processes. Therefore, the degree of dependence on the hormone accounts, at least partly, for the difference between climacteric and non-climacteric fruit.

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