

RESEARCH PAPER

Co-ordination of early and late ripening events in apples is regulated through differential sensitivities to ethylene

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Abstract

In this study, it is shown that anti-sense suppression of *Malus domestica* 1-AMINO-CYCLOPROPANE-CARBOXYLASE OXIDASE (*MdACO1*) resulted in fruit with an ethylene production sufficiently low to be able to assess ripening in the absence of ethylene. Exposure of these fruit to different concentrations of exogenous ethylene showed that flesh softening, volatile biosynthesis, and starch degradation, had differing ethylene sensitivity and dependency. Early ripening events such as the conversion of starch to sugars showed a low dependency for ethylene, but a high sensitivity to low concentrations of ethylene ($0.01 \mu\text{l l}^{-1}$). By contrast, later ripening events such as flesh softening and ester volatile production showed a high dependency for ethylene but were less sensitive to low concentrations (needing $0.1 \mu\text{l l}^{-1}$ for a response). A sustained exposure to ethylene was required to maintain ripening, indicating that the role of ethylene may go beyond that of ripening initiation. These results suggest a conceptual model for the control of individual ripening characters in apple, based on both ethylene dependency and sensitivity.

Key words: ACC oxidase 1 (*MdACO1*), apple, ethylene, fruit ripening, *Malus*.

Introduction

The importance of ethylene for fruit ripening has made the biosynthetic pathway for this hormone an attractive target for studies aiming to manipulate and understand the control of fruit ripening. Among the first transgenic fruit ever produced targeted ethylene synthesis in tomato. These included the reducing levels of the enzymes *ACC SYNTHASE (ACS)* (Oeller *et al.*, 1991) and *ACC OXIDASE (ACO)* (Picton *et al.*, 1993), and over-expressing *ACC DEAMINASE* to remove ACC from the ripening fruit (Klee *et al.*, 1991). In other fruit species, researchers have targeted *ACO* in melon (Ayub *et al.*, 1996), and *ACO1* (Ross *et al.*, 1992) in the apple cultivars, ‘Greensleeves’ and ‘Royal Gala’ (Dandekar *et al.*, 2004; Schaffer *et al.*, 2007). These studies demonstrated reducing ethylene in the climacteric fruit reduces fruit ripening, an effect reversed by adding exogenous ethylene.

Measuring the sensitivity of a tissue to a hormone gives insight into how the hormone acts. Due to the ambiguous nature of the word ‘sensitivity’, Firn (1986) proposed five measures of sensitivity. In this study, the term ‘sensitivity’ is used for category III sensitivity, namely ‘the minimum

concentration needed to achieve a significant response’, ‘dependency’ is used for the category II sensitivity, namely ‘the ratio of the maximum response relative to the response to in the absence of the hormone’, and ‘responsivity’ is used to describe category I sensitivity ‘the magnitude of a maximum response which can be evoked’.

The significance of different sensitivities to the ripening hormone ethylene was not appreciated in early studies, as dependency was not considered alongside sensitivity. ACS tomatoes, demonstrating less than 0.5% of peak ethylene production, showed a delay in many ripening attributes, including chlorophyll loss and reddening (Oeller *et al.*, 1991). It was also found that levels of the cell wall gene *POLYGALACTURONASE (PG)* were of a similar level to control fruit suggesting that this was regulated independently to ethylene (Oeller *et al.*, 1991). This expression result was later found to be caused by the sensitivity of *PG* to ethylene and it has now been established that this *PG* is actually strongly regulated by ethylene in tomatoes, as suppression of ethylene production to 0.5% of the controls

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was still sufficient to induce 100% expression of this gene (Sitrit and Bennett, 1998). While the *PG* is obviously highly sensitive to low concentrations of ethylene, the *ACS* mutant tomatoes demonstrated a slower loss of chlorophyll (10–20 d) than the controls and, finally, they never turned bright red, suggesting that the colour response is less sensitive to ethylene. Work on the transgenic melon has demonstrated that many ripening attributes appear to be independent of ethylene (Ayub *et al.*, 1996; Guis *et al.*, 1997; Pech *et al.*, 2008). In melon, it was found that the production of aroma volatiles and rind yellowing are considered to be ethylene-dependent ripening events (Guis *et al.*, 1997), while starch breakdown, sugar accumulation, loss of acidity, and flesh colouration are considered ethylene-independent events. Fruit softening showed both dependent and independent effects with the knockout *ACO* fruit showing a significant softening albeit at a reduced rate. This ethylene-independent softening has been explained by the fact that some cell wall-modifying enzymes are strongly regulated by ethylene while others appeared to have an ethylene-independent expression (Nishiyama *et al.*, 2007).

Currently, there is little known about the ripening sensitivity of apples to different ethylene concentrations. There is some evidence that the ripening sensitivity to ethylene increases during on-tree maturation, but these studies were limited to measurements of endogenous ethylene production and respiratory activity (Harkett *et al.*, 1971; Knee *et al.*, 1987). Ethylene concentrations inside the core cavity of apples increase from $<0.1 \mu\text{l l}^{-1}$ to $>500 \mu\text{l l}^{-1}$ during ripening (Johnston *et al.*, 2001), but it is not known if this entire range of concentrations is physiologically active or if production is in excess of that required during the late stages of ripening. Apple ripening consists of the conversion of starch to sugars, reduced acidity, yellowing of the skin, reduced flesh firmness, and an increase in flavour volatiles. Inhibitors of ethylene action such as 1-MCP have been shown to reduce softening and volatile production, but have inconsistent or no effect on the conversion of starch to sugars (Fan *et al.*, 1999). If similar processes were occurring in apple as in melon, this variation in efficacy could be explained by each ripening component having differing dependencies or sensitivities to ethylene.

The controlled application of different concentrations of ethylene to determine ripening thresholds is not possible using standard apple cultivars, as most cultivars produce too much endogenous ethylene. This makes it difficult to determine if ripening responses are due to the added ethylene, or to changes in endogenous ethylene. The application of inhibitors of ethylene biosynthesis and action also do not sufficiently suppress endogenous production in apple to allow such studies. The present study circumvents this problem by using a recently developed ‘Royal Gala’ *ACO1* antisense line that only produces background levels of ethylene (Schaffer *et al.*, 2007). Using this system, the present study determines the sensitivity, responsiveness, and dependency of early and late ripening events in apple. New knowledge is presented on the ethylene ripening thresholds for individual ripening characters of apples, and a new

conceptual model is presented for the ethylene-mediated co-ordination of early and late ripening events in apples.

Materials and methods

Apple growth conditions

A single transgenic *Malus×domestica* ‘Royal Gala’ tree, containing the *ACC OXIDASE 1* gene (Ross *et al.*, 1992) in an antisense orientation (*ACO1as*), was selected as it produced only 0–0.13 ng l⁻¹ levels of ethylene in its fruit at full climacteric (Schaffer *et al.*, 2007). Seven scions were grafted onto ‘M9’ rootstocks and grown next to three ‘Royal Gala’ untransformed lines that act as a control in a greenhouse. At full bloom, flowers were pollinated by hand with *M.×domestica* ‘Granny Smith’ pollen.

Three hundred and forty three *MdACO1as* apples and 56 control apples were harvested at the same time based on the background colour of the control apples. The *MdACO1as* apples were randomly allocated into 14 groups of 14 apples, with 13 of these groups allocated to the ripening treatments described in Table 1, and one group assessed for maturity characteristics at harvest. The control apples were randomly allocated into four groups of 14 apples, with three of these groups used for ripening treatments (Table 1), and one group assessed for maturity at harvest. Fruit ripening assessments were performed 14 d after harvest for all ripening treatments.

Post-harvest treatments

Each group of apples was subjected to a different treatment (Table 1) each in a separate single-layered tray. Ethylene treatments were conducted at 20 °C in 340 l ripening bins with continuous air movement and lime to absorb CO₂. Nil ethylene treatments were also carried out in sealed bins with lime and Purafil™ (Multimix MM-1000, Circul-Aire, Montreal, Canada) to remove any residual atmospheric ethylene. Ethylene was injected into the injection ports and assessed for correct concentration 1 h after injection. 1-Methylcyclopropane (1-MCP) (SmartFresh™, AgroFresh Ltd) treatments at a concentration of 600 nl l⁻¹ were also performed for 24 h in 340 l sealed bins containing lime. In all treatments, the 340 l bin contained a maximum of 14 apples (a combined mass of 1100–1200 g).

Assessment of fruit maturity and ripening

For each fruit, flesh firmness was evaluated using a Texture Analyser TAXT plus (Stable Microsystems, United Kingdom) fitted with a 7.9 mm Effegi penetrometer probe. The probe was driven into the flesh at 4 mm s⁻¹ to a depth of 9 mm, and the maximum force recorded as flesh firmness. Two readings were made on opposite sides of each fruit with the skin removed. Soluble solids concentration was used to estimate the sugar content, and was determined by placing an aliquot of the juice released during firmness measurement onto a digital refractometer (Atago, model PAL-1, Japan). Starch pattern index was determined by

Table 1. Summary of ripening treatments for *MdACO1as* and control 'Royal Gala' apples

Ripening treatments included ethylene dosage, duration of ethylene treatment, 1-methylcyclopropene (MCP) treatment, and ripening temperature. Assessments for each treatment were performed 14 d after harvest.

Treatment no.	Genotype	Ethylene dosage ($\mu\text{l l}^{-1}$)	Ethylene treatment duration	Ripening temperature ($^{\circ}\text{C}$)	600 nl l^{-1} MCP treatment
1	Control	–	–	20	No
2		–	–	20	Yes
3		–	–	4	No
4	ACO1as	–	–	20	No
5		–	–	4	No
6		–	–	20	Yes
7		0.01	14 d	20	No
8		0.1	14 d	20	No
9		1	14 d	20	No
10		10	14 d	20	No
11		100	14 d	20	No
12		1000	14 d	20	No
13		100	30 min	20	Yes
14		100	4 h	20	Yes
15		100	1 d	20	Yes
16		100	7 d	20	Yes

placing the cut surface of an apple into iodine solution (2.5 g l^{-1} iodine and 10 g l^{-1} potassium iodide in distilled water) for 60 s and then rating the staining pattern according to the ENZAFRUIT New Zealand International scale from 0 (cut surface completely stained, high starch) to 6 (no surface staining, low starch). Longitudinal quarter sections of fruit were held at -20°C for the determination of titratable acidity. Titrations were performed according to Harker *et al.* (2002). Background skin colour was measured using a chromameter (Minolta, model CR-300, Japan), with two readings made per fruit on blush-free areas of skin. Internal ethylene concentration was determined by extracting a 1 ml core cavity gas sample and injecting it into a gas chromatograph (Hewlett Packard, 5890 series II) equipped with an injector at 160°C , an activated alumina F1 column (Alltech, glass 1.5 m \times 6 mm \times 2 mm, mesh 80/100) set isothermally at 130°C , N_2 as the carrier gas (20 ml min^{-1}), a flame ionization detector set at 200°C (H_2 at 20 ml min^{-1} , air at 200 ml min^{-1}), and an integrator (Hewlett Packard, model 3395) calibrated with certified gas standards. Volatiles were measured as described in Schaffer *et al.* (2007).

Data analysis

The significance of treatment effects were determined using analysis of variance, and mean separation using a protected least significant difference (5%). Ethylene response data were analysed by non-linear regression and a logistic function:

$$\text{Ripening response} = \frac{A - B}{1 + \left(\frac{[\text{ethylene}]^D}{C}\right) + B}$$

where A is the lower asymptote, B is the upper asymptote, C is the [ethylene] for 50% response, and D is the rate of change. Parameter estimates and standard errors for C were interpreted as sensitivity, and parameter D as

responsivity. All statistical procedures were performed using Genstat, 9th edition.

Ethylene dependency was calculated as:

$$\text{Dependency (\%)} = \frac{1000 \mu\text{l l}^{-1} \text{ treatment} - 0 \mu\text{l l}^{-1} \text{ treatment}}{1000 \mu\text{l l}^{-1} \text{ treatment}} \times 100$$

Results

Maturity characteristics at harvest

At harvest, the control fruit had internal ethylene concentrations ranging from 0.05 to 528 $\mu\text{l l}^{-1}$, while the *MdACO1as* fruit had levels ranging from undetectable to 0.08 $\mu\text{l l}^{-1}$ (Fig. 1A). Both the control fruit and *MdACO1as* fruit on exposed parts of the trees developed red blush at harvest (Fig. 2A, B), although the intensity and skin coverage of reddening was more pronounced on control fruit. Both fruit types had similar flesh firmness, but the *MdACO1as* fruit had lower concentrations of soluble solids and titratable acidity, less starch clearance, and a greener background skin colour (Fig. 1B–E). The starch pattern index for control fruit ranged from 0 to 6 and 0 to 3 in *MdACO1as* fruit.

MdACO1as fruit have improved water retention and reduced incidence of ripening-related rots

Fourteen *MdACO1as*-suppressed apples and 14 'Royal Gala' untransformed fruit (control fruit) were kept at 20°C for 30 weeks. These were weighed and visually inspected every 2 weeks for shrivel and ripening-related rots. After 6–8 weeks the control fruit began to show signs of shrivel much earlier than the *MdACO1as* fruit that began to shrivel 20–26 weeks during this treatment (Fig. 2C, D). By 20 weeks five of the control fruit had completely rotted while there were no visual

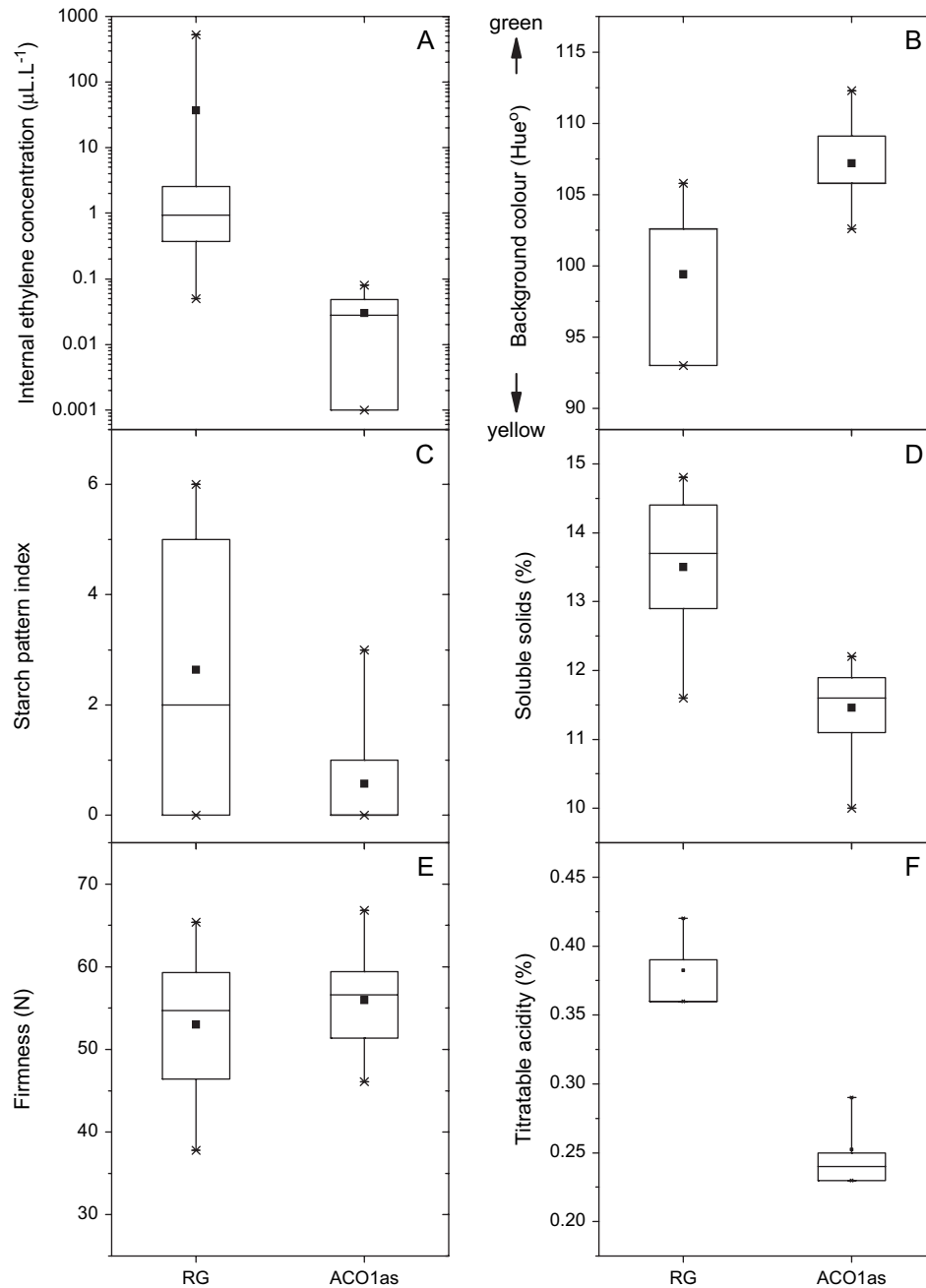


Fig. 1. Untransformed 'Royal Gala' (RG, wild type) and *MdACC oxidase1* (*ACO1as*) fruit at harvest. Concentration of internal ethylene in the core cavity (A), background skin colour (B), starch pattern index (C), soluble solids concentration (D), flesh firmness (E), and titratable acidity (F).

rots on any of the *MdACO1as* fruit for the whole of this period. In both the *MdACO1as* lines and control lines the water loss from the fruit was linear (Fig. 2E), with a slower rate of water loss from the *MdACO1as* lines (average $1.6 \text{ mg g}^{-1} \text{ FW d}^{-1}$) compared to the controls ($2.2 \text{ mg g}^{-1} \text{ FW d}^{-1}$).

Comparison of ripening characters between *MdACO1as* and untransformed fruit

Three groups of 14 control fruit and three groups of 14 *MdACO1as* fruit were assessed following a 14-d period of

ripening. The first group of control and *MdACO1as* apples were stored with no treatment at $20 \text{ }^{\circ}\text{C}$. After this time the 'Royal Gala' control fruit had a higher internal ethylene concentration, had softened by approximately 20 N, had near-complete starch clearance, a more yellow background colour, and decreased titratable acidity (Fig. 3). The *MdACO1as* fruit had no detectable increase in internal ethylene concentration after 14 d at $20 \text{ }^{\circ}\text{C}$, with ethylene levels ranging from undetectable to $0.094 \text{ } \mu\text{L l}^{-1}$, and only changed slightly in skin background colour and firmness. Both the untransformed fruit and the *MdACO1as* fruit had a large degree of starch clearance, an increase in soluble

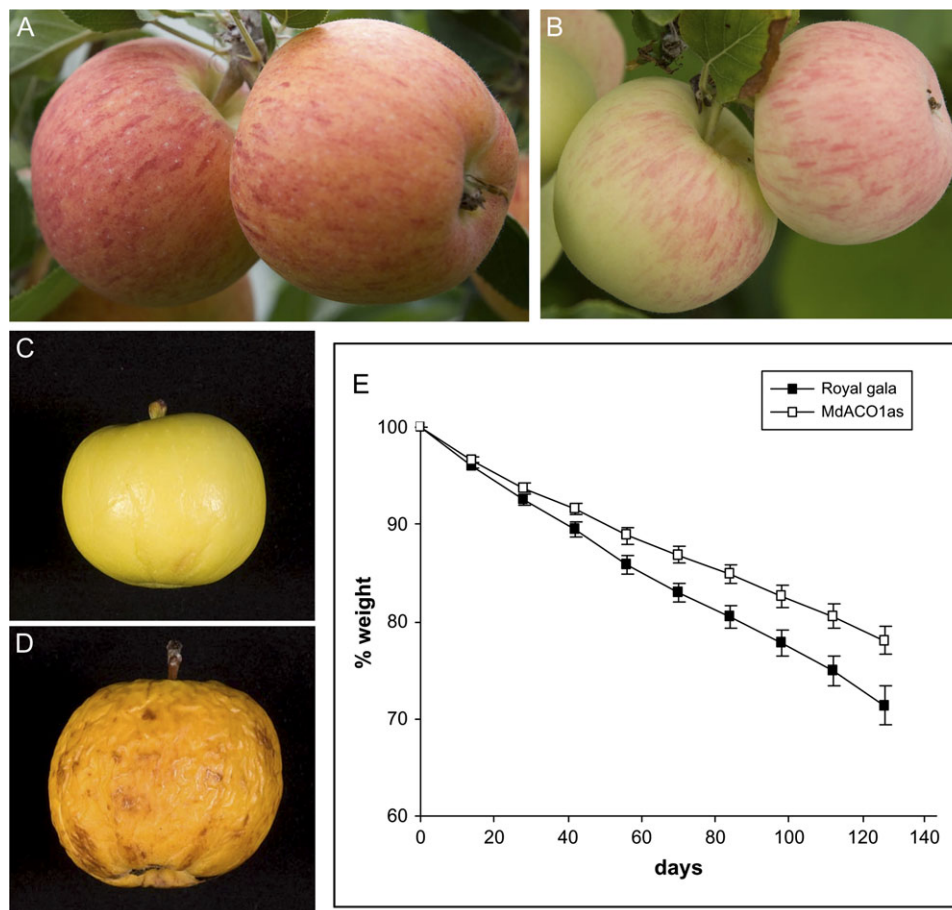


Fig. 2. Apples with *MdACC oxidase1* (*MdACO1as*) knocked out and untransformed 'Royal Gala'. 'Royal Gala' apples on tree at harvest (A). *MdACO1as* fruit at harvest (B). *MdACO1as* apple after storage at room temperature for 6 months (C). 'Royal Gala' apples after storage at room temperature for 6 months (D). Weight loss from 'Royal Gala' (filled boxes) and *MdACO1as* apples (open boxes) over a 14 week period (E).

solids concentration, and a loss of titratable acidity during the 14 d ripening period.

To assess whether there were any ripening effects caused by the very low ethylene concentrations detected in some of the *MdACO1as* fruit, the second group of *MdACO1as* fruit were treated with the inhibitor of ethylene action, 1-methylcyclopropene (1-MCP) before being left to ripen for 14 d at 20 °C. A group of control fruit were also treated. Treatment of the control fruit with 1-MCP reduced the internal ethylene concentration to between 0.023 and 4.2 $\mu\text{l l}^{-1}$. There was also reduced loss of firmness compared with the untreated fruit but 1-MCP had little effect on the clearance of starch and the associated increase in soluble solids concentration. *MdACO1as* fruit treated with 1-MCP showed no significant differences from the untreated fruit suggesting that the *MdACO1as* lines have a complete ethylene biosynthesis knockout with respect to ripening (Fig. 3).

The third group of *MdACO1as* and control apples were cold-stored at 4 °C for 14 d to assess the effect of cold storage on ripening characters. *MdACO1as* fruit showed no significant differences in ripening compared with the fruit stored at 20 °C for internal ethylene concentration, firm-

ness, and skin colour. Cold-stored *MdACO1as* fruit had slightly less starch clearance and an increase in soluble solids concentration (Fig. 3). Cold storage of the control fruit suppressed internal ethylene concentrations to 0.091–27.2 $\mu\text{l l}^{-1}$, and reduced softening, skin yellowing, and starch clearance compared with fruit stored at 20 °C.

Ethylene sensitivity for individual ripening characteristics

To establish the level of ethylene needed to induce a ripening response, *MdACO1as* lines were exposed continuously to a range of ethylene concentrations from 0.01 to 1000 $\mu\text{l l}^{-1}$ for 14 d at 20 °C. Ethylene response curves were then produced for the six main ripening traits and for the six dominant volatiles (Fig. 4). While there was no clear response to ethylene for titratable acidity and soluble solids concentration, distinct response curves were established for volatile production, firmness, skin background colour, and starch clearance. The general pattern for these curves was sigmoidal, with little or no change in ripening at low concentrations, followed by a rapid increase in response from 0.1 to 10 $\mu\text{l l}^{-1}$, and little or no change thereafter at which point the process was saturated. However, there were

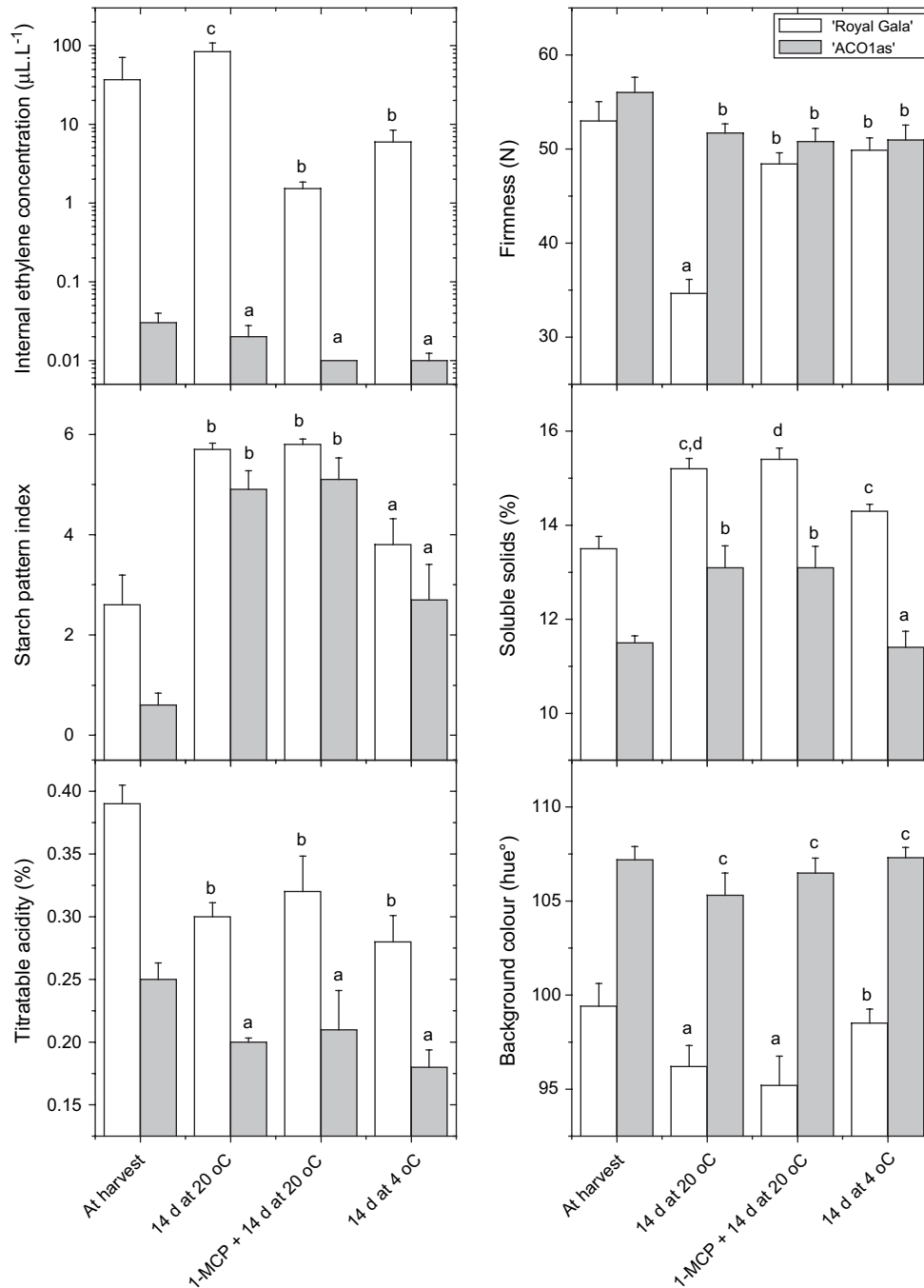


Fig. 3. Untransformed 'Royal Gala' (wild type, unfilled bars) and *MdACO1as* fruit (filled bars) at harvest and after ripening following three different storage treatments. Analysis of variance *P* values for all ripening characters were <0.001. Treatments labelled with the same letter are similar according to a 5% least significant difference.

subtle differences in the responses of individual ripening traits, where the majority of the response occurred between 0.1 and 10 $\mu\text{L l}^{-1}$ for softening, background colour and total volatiles, while in contrast a lower range of 0.01 to 1 $\mu\text{L l}^{-1}$ was required for starch clearance. Results from the 0.01 and 0.1 $\mu\text{L l}^{-1}$ ethylene concentrations were indistinguishable from the no-ethylene treatment controls for volatiles, firmness, and background colour suggesting that these concentrations are

below the response threshold for these processes. A small increase in starch degradation was observed for the 0.01 $\mu\text{L l}^{-1}$ ethylene-treated fruit, suggesting a lower response threshold for this trait.

The differential sensitivity of the different ripening traits was further quantified using non-linear regression to estimate the ethylene concentration required to induce a half-maximum response. This approach showed that the half-maximal response for starch clearance occurred at low ethylene

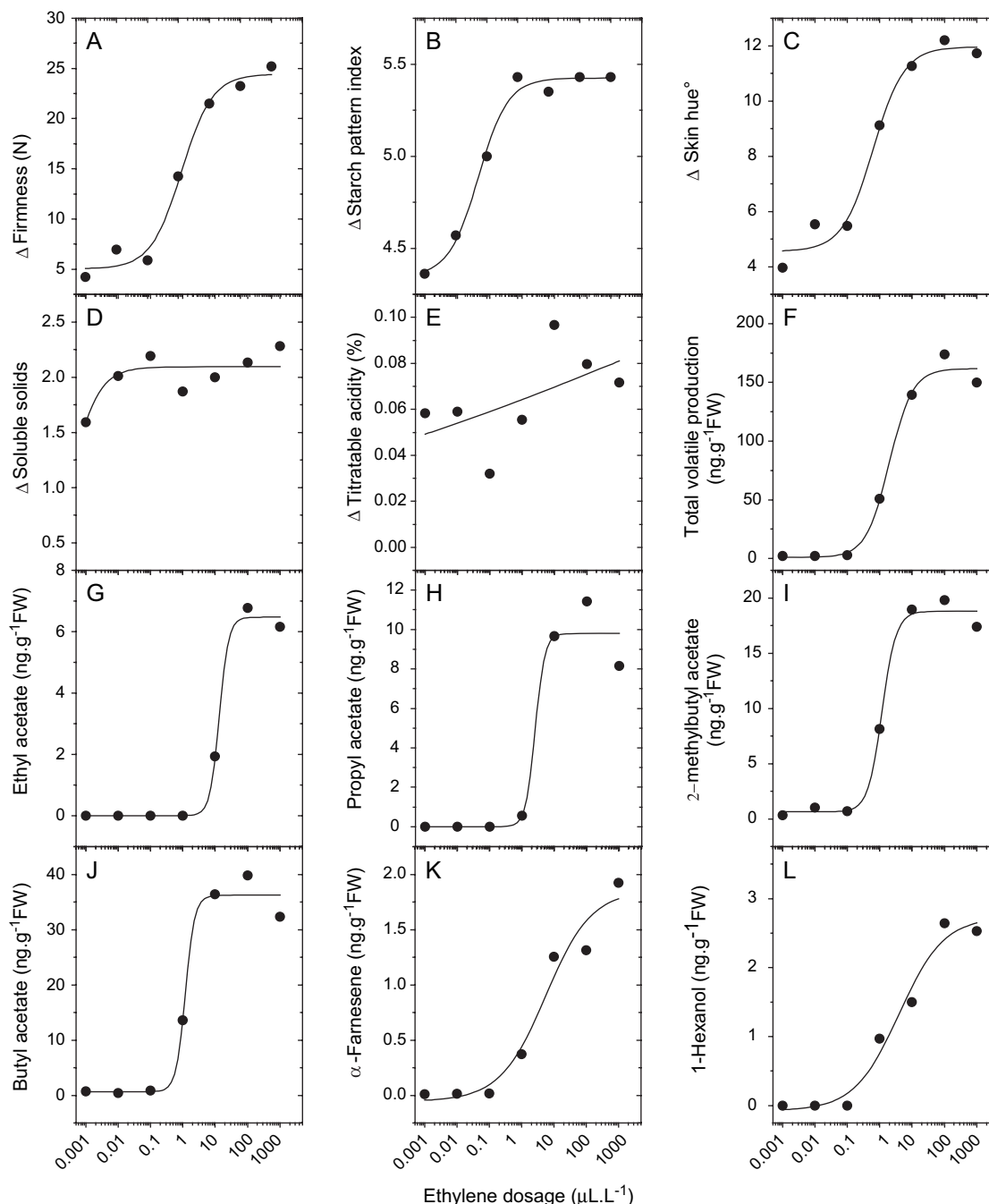


Fig. 4. Response curves of different ripening characters for *MdACO1as* fruit treated with different concentrations of ethylene. Flesh firmness (A) starch pattern index (B), skin background colour (C), soluble solids concentration (D), titratable acidity (E), total volatiles (F), and individual volatiles (G–L). Each point depicts the mean of 14 fruit.

concentrations, with skin yellowing, softening, and volatile production requiring progressively higher ethylene concentrations to induce a similar 50% response (Fig. 5A). The rate of change parameter estimated from this analysis was similar for all ripening characters (Fig. 5B), suggesting the responsiveness to ethylene was similar despite differences in sensitivity.

The ethylene response curves also differed between volatiles. The production of α -farnesene, butyl acetate, and 2-methylbutyl acetate occurred once the ethylene concentration exceeded $0.1 \mu\text{L l}^{-1}$, while the production of ethyl

acetate and propyl acetate only occurred once the ethylene concentration exceeded a higher concentration of $1 \mu\text{L l}^{-1}$ (Fig. 4). The esters tended to have an abrupt increase in production in response to increased ethylene dosage, whereas volatiles such as α -farnesene and hexanol had a more gradual increase and seemed to respond to a wider range of ethylene concentrations. Alpha-farnesene and 1-hexanol also showed a higher saturation threshold concentration of $>100 \mu\text{L l}^{-1}$, whereas most other volatiles were saturated at concentrations $>10 \mu\text{L l}^{-1}$.

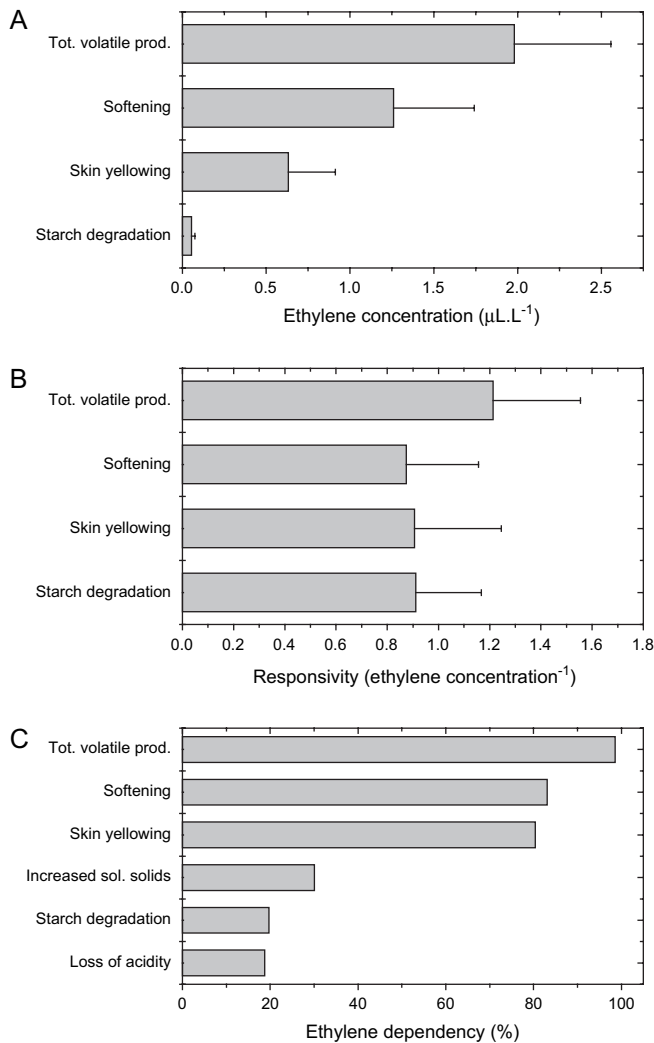


Fig. 5. Sensitivity (A), responsivity (B), and dependency (C) of the *MdACO1as* apples to exogenous ethylene. Sensitivity and responsivity was determined as the ethylene concentration required for a 50% response and the rate of change, respectively, using non-linear regression and a logistic function. Ethylene dependency (%) was calculated as the difference between the $0 \mu\text{l l}^{-1}$ and $1000 \mu\text{l l}^{-1}$ ethylene treatments divided by the $1000 \mu\text{l l}^{-1}$ ethylene treatment, and multiplying by 100 to give a percentage: $0\% = \text{ethylene independent}$, $100\% = \text{ethylene dependent}$.

Ethylene dependency for individual ripening characteristics

To establish the degree of ethylene dependence for each ripening trait, *MdACO1as* fruit treated with $1000 \mu\text{l l}^{-1}$ ethylene, and untreated fruit stored at 20°C , were compared with the harvest samples. Taking the value for each trait exposed to $1000 \mu\text{l l}^{-1}$ as 100% the percentage ripening for each trait in the absence of ethylene (ethylene-independent traits) could be determined (Fig. 5C). Two groups of ripening traits emerged, those that were strongly ethylene-dependent (increased total volatiles, loss of firmness and skin yellowing), and those that were only weakly ethylene-dependent (starch clearance, loss of titratable acidity, and increased soluble solids concentration).

A continuous exposure to ethylene is required for ripening

To assess if ethylene acts as a trigger for ripening or whether a continuous exposure to ethylene is required, *MdACO1as* fruit were exposed to $100 \mu\text{l l}^{-1}$ ethylene for different periods of time followed by treatment with 600 nl l^{-1} 1-MCP to stop further ethylene action. Fruit were exposed to ethylene for 30 min, 4 h, 1 d, and 7 d, and the same ripening characters as assessed above were measured after 14 d at 20°C . The results from these fruit were also compared with those from fruit that had been treated with $100 \mu\text{l l}^{-1}$ for 14 d in the previous experiment.

For the ripening traits showing a moderate-to-strong dependency for ethylene (background skin colour, firmness, and volatiles), the responses of fruit receiving a short period of ethylene (30 min, 4 h, and 1 d) exposure followed by treatment of 1-MCP were indistinguishable from the responses of no-ethylene treatment *MdACO1as* fruit. However, for starch clearance (a ripening processes with a weak dependence for ethylene), one day was sufficient to significantly accelerate starch loss. Fruit treated with ethylene for 7 d had an intermediate level of ripening characters compared with fruit receiving the 14 d treatment (Fig. 6), suggesting a continued exposure of ethylene is needed for ripening to occur.

Discussion

Ripening in apples is a complex progression of developmental steps. These steps are not completely controlled by ethylene, as there is always a degree of ethylene-independent progression. This suggests a model of ethylene action where ethylene is acting as a modulator of ripening rather than a ripening trigger. While the morphological changes that occur in apples during ripening are well documented, it is shown that these traits seem to follow an overlapping sequence of events, starting with the conversion of starch to sugars and loss of acidity, yellowing of the skin, softening of the flesh, and finally an increase in aroma volatiles. As this sequence progresses, the dependency for ethylene becomes stronger and the sensitivity to ethylene decreases (Fig. 7). For most apple cultivars, rather than having a constant level, there is an increase in ethylene during the ripening process (Johnston *et al.*, 2001). This increase in ethylene concentration may allow the different ripening characters to be co-ordinated so that starch conversion always occurs before excessive fruit softening or the production of attractant volatiles. The low ethylene dependency for starch clearance suggests that this process is mainly driven by other developmental factors, but the high sensitivity of this process to low ethylene concentrations may provide a backup stimulus should the non-ethylene developmental cues fail to develop.

When the *MdACO1as* apples are left for a considerable period of time, the greatest change that occurs is weight loss, albeit at a lower rate than the control apples. This

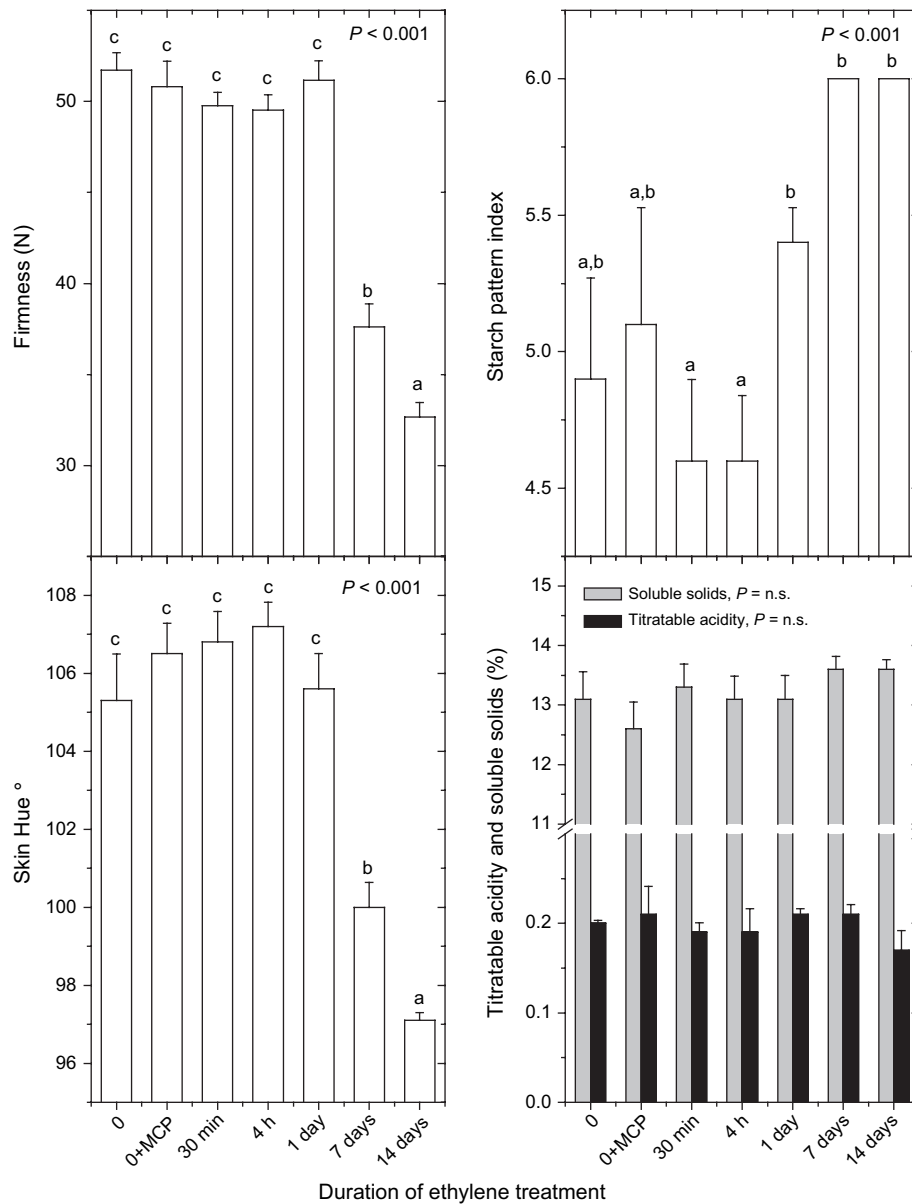


Fig. 6. Fruit ripening in *MdACO1as* fruit treated with $100 \mu\text{l l}^{-1}$ ethylene for different periods of time followed by treatment with 1-MCP. Fruit was measured after 14 d. Analysis of variance P values are displayed, treatments labelled with the same letter are similar according to a 5% least significant difference.

suggests that only a certain progression of ripening can be achieved in the absence of ethylene. The ethylene dependency for starch clearance appears less, as it would be likely that there would be a complete clearing of starch if the 2-week ripening period were extended.

Because of apple number constraints, a limitation to this study was that ripening was measured at a single time point in apple fruit development. It has been well documented that, as apples mature, they become more sensitive to ethylene (Harkett *et al.*, 1971). This means it is possible that the ethylene response curves may shift to the left or the right for fruit harvested at different maturities. It is also likely that these response curves may shift for different cultivars and for fruit exposed to different conditions during growth; factors which warrant further investigation. Never-

theless, despite these potential shifts in the response curve, it is likely that the relative sensitivities and dependencies of the different ripening characters for ethylene would remain in the same order, as most apple cultivars tend to follow the same sequence of ripening events (e.g. starch clearance is always an early ripening event). The present research also demonstrates that ethylene was most physiologically active for ripening between 0.01 and $10 \mu\text{l l}^{-1}$, which is similar to those concentrations measured in most apple cultivars during the harvest period. These concentrations also fit with storage recommendations for ethylene concentrations to be less than $0.1 \mu\text{l l}^{-1}$ to reduce ripening (Stow *et al.*, 2000). While research has been initiated to unravel the ethylene dependency of ripening pathways (Pech *et al.*, 2008), there is a lack of research on the molecular basis for ethylene

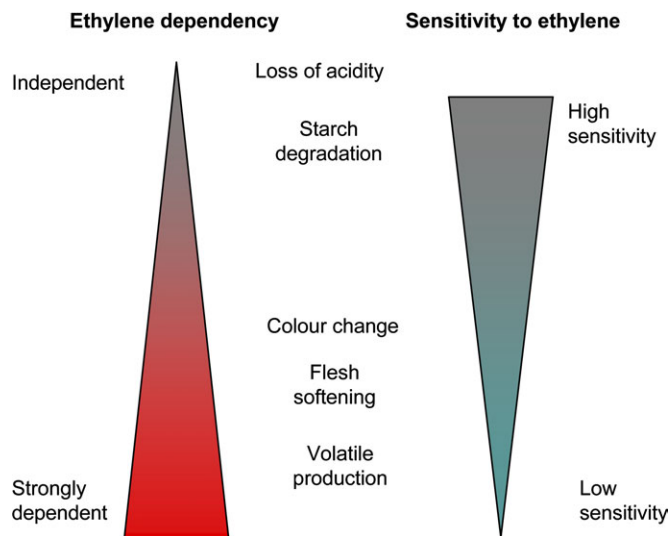


Fig. 7. Conceptual model for control of individual ripening characters in apples. Ethylene sensitivity calculated from the concentration of ethylene required for a 50% ripening response. Ethylene dependency measured by the amount of ripening measured in the absence of ethylene.

sensitivity. The differing ethylene sensitivities of individual ripening characters raises questions as to whether this is mediated through different signalling systems for each ripening pathway, or through differences in the numbers of ethylene-binding domains for key genes associated with each ripening process.

There is considerable variation in the ripening process in fleshy fruits, but when the individual traits are examined separately, there are remarkable consistencies. The starch to sugar conversion is common to many fruit. In some cultivars of apple it has been reported as ethylene independent, while in others it is more ethylene dependent (Thammawong and Arakawa, 2007). In melons, starch has also been suggested to be independent to ethylene (Ayub *et al.*, 1996), it would be interesting if this starch clearance could be accelerated in this fruit with exogenous ethylene. The dependency on ethylene for changes in skin colouration has been recorded in melon (Ayub *et al.*, 1996) and tomato (Oeller *et al.*, 1991). The ethylene dependency of fruit softening and volatile production has been extensively studied, with responses to ethylene by many candidate cell wall genes in apples responding to ethylene (Atkinson *et al.*, 1998; Wakasa *et al.*, 2003; Goulao *et al.*, 2008) and in other fruit, (Brummell, 2006), and by aroma biosynthesis genes (Defilippi *et al.*, 2005; Schaffer *et al.*, 2007). Interestingly, work with melon has shown that many of the cell-wall related genes are regulated independently of ethylene, in addition to those that are regulated by ethylene (Nishiyama *et al.*, 2007). While much progress has been made there is still much to learn about the molecular control of these genes.

Finally, it appears from this and previous work that the ethylene signal is constantly controlling the ripening process. As well as this study, it was shown in tomato (Oeller

et al., 1991) that ripening could be stopped if ethylene fruit were exposed to only a short period of ethylene. As ripening occurs through enzymic action, it is surprising that residual ripening does not occur even after a short period of ethylene exposure. This suggests that enzyme turnover is high during ripening or that there are additional layers of regulation for enzyme action to progress in the absence of ethylene.

Conclusion

This study has characterized for the first time the ethylene response curves for individual ripening characters in apple. It has revealed that ethylene-mediated ripening is controlled at two levels, firstly, through differing dependencies for ethylene, and, secondly, through differing sensitivities to ethylene. This dual control mechanism means that the sequence of individual ripening events is tightly coordinated so that early ripening events are less dependent on ethylene but are also highly sensitive to ethylene should the ethylene-independent factors fail to develop. This model warrants further investigation in relation to applicability to other fruits, particularly in relation to the ripening physiology of climacteric and non-climacteric fruit.

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