

RESEARCH PAPER

Dissecting the role of climacteric ethylene in kiwifruit (*Actinidia chinensis*) ripening using a 1-aminocyclopropane-1-carboxylic acid oxidase knockdown line

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Abstract

During climacteric fruit ripening, autocatalytic (Type II) ethylene production initiates a transcriptional cascade that controls the production of many important fruit quality traits including flavour production and softening. The last step in ethylene biosynthesis is the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by the enzyme ACC oxidase (ACO). Ten independent kiwifruit (*Actinidia chinensis*) lines were generated targeting suppression of fruit ripening-related ACO genes and the fruit from one of these lines (TK2) did not produce detectable levels of climacteric ethylene. Ripening behaviour in a population of kiwifruit at harvest is asynchronous, so a short burst of exogenous ethylene was used to synchronize ripening in TK2 and control fruit. Following such a treatment, TK2 and control fruit softened to an 'eating-ripe' firmness. Control fruit produced climacteric ethylene and softened beyond eating-ripe by 5 d. In contrast, TK2 fruit maintained an eating-ripe firmness for >25 d and total volatile production was dramatically reduced. Application of continuous exogenous ethylene to the ripening-arrested TK2 fruit re-initiated fruit softening and typical ripe fruit volatiles were detected. A 17 500 gene microarray identified 401 genes that changed after ethylene treatment, including a polygalacturonase and a pectate lyase involved in cell wall breakdown, and a quinone oxidoreductase potentially involved in volatile production. Many of the gene changes were consistent with the softening and flavour changes observed after ethylene treatment. However, a surprisingly large number of genes of unknown function were also observed, which could account for the unique flavour and textural properties of ripe kiwifruit.

Key words: ACC oxidase, ethylene, kiwifruit, ripening, softening, volatiles.

Introduction

The plant hormone ethylene regulates many important aspects of plant growth and development as well as responses to the environment (Wang *et al.*, 2002). The ethylene biosynthetic pathway has been well described by Yang and Hoffman (1984). In this pathway, S-adenosylmethionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS), and ACC is converted to ethylene by the enzyme ACC oxidase (ACO). In most biological processes, ethylene production is

auto-inhibitory, where perception of ethylene by the plant inhibits further ethylene biosynthesis (Type I). During ripening in fruits such as tomato (*Solanum lycopersicum*), apple (*Malus domestica*), and banana (*Musa acuminata*), there is a rapid increase in ethylene production that is accompanied by a 'climacteric' burst of respiration (Lelievre *et al.*, 1997; Alexander and Grierson, 2002; Giovannoni, 2004). The increase in ethylene production results from an autocatalytic (Type II) stimulation of ethylene synthesis.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; GPPS, geranylgeranyl pyrophosphate synthase; PME, pectin methyltransferase; PME_i, PME inhibitor; SAM, S-adenosylmethionine; SSC, soluble solids concentration; VPD, vapour pressure deficit.

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Type II ethylene production triggers a transcriptional cascade (Solano *et al.*, 1998) that regulates the expression of many genes involved in softening, texture, flavour, aroma, and colour—characteristics that ultimately determine the consumer acceptability of the fruit.

The use of ethylene response inhibitors such as aminoethoxyvinyl glycine (AVG; Saltveit, 2005) and 1-methylcyclopropene (1-MCP; Sisler and Serek, 2003), and genetic manipulation targeting ethylene biosynthetic genes has identified distinct roles of ethylene in controlling different ripening characteristics in different fruits. In tomato, reduced expression of *ACS* and *ACO* genes using antisense RNA strategies (Hamilton *et al.*, 1990; Oeller *et al.*, 1991; Picton *et al.*, 1993) revealed that a reduction in ethylene production delayed colour development, loss of acidity, and sugar accumulation, but did not affect the softening rate (Murray *et al.*, 1993). In *ACO* knockdown melon fruit, ethylene was shown to control yellowing of the rind, softening of the flesh, development of peduncular abscission, and climacteric respiration, whilst pulp colouration, accumulation of sugars, and loss of acidity were ethylene-independent processes (Ayub *et al.*, 1996; reviewed in Pech *et al.*, 2008). In apple, transgenic silencing of *ACO* and/or *ACS* showed that ethylene regulated fruit softening and the synthesis of esters and α -farnesene, while production of volatile aldehydes and alcohols were only marginally repressed. Starch breakdown and loss of acidity acted independently of ethylene, but ethylene could accelerate starch breakdown (Dandekar *et al.*, 2004; Schaffer *et al.*, 2007; Johnston *et al.*, 2009). Recent reports have also highlighted the role of ethylene in non-climacteric fruit such as grape (*Vitis vinifera*; Chervin *et al.*, 2004), citrus (*Citrus paradisi*; McCollum and Maul, 2007), and strawberry (*Fragaria ananassa*; Tian *et al.*, 2000; Bower *et al.*, 2003). Together these results indicate that ethylene controls many, but not all aspects of fruit ripening; that the different processes show different sensitivity and dependence on ethylene; and that the control differs among fruit crops.

Kiwifruit (*Actinidia* spp.) are classified as a climacteric fruit (Pratt and Reid, 1974) with the major commercial varieties coming from two species, *Actinidia deliciosa* and *Actinidia chinensis*. The key physiological events in post-harvest ripening of kiwifruit have been described in relation to four distinct softening phases (see Fig. 1). The fruit are harvested firm, when they are high in starch and produce few volatile aroma compounds (phase 1) and then enter a period of rapid softening (phase 2). The start of phase 3 is marked by the onset of autocatalytic ethylene production in which the fruit soften to ‘eating-ripe’ firmness. Fruit continue to produce ethylene as they become over-ripe (phase 4). Climacteric ethylene production in phase 3 is associated with a respiratory burst of CO₂ production and the development of the characteristic flavours and aromas of ripe fruit. *A. deliciosa* cultivars are characterized by green aroma notes (hex-E2-enal and C6 alcohols) whilst *A. chinensis* cultivars are noted for tropical, fruity aromas (ethyl butanoate, butyl butanoate, and eucalyptol) reminiscent of melon, mango, and banana (Jaeger *et al.*, 2003). Control of ethylene

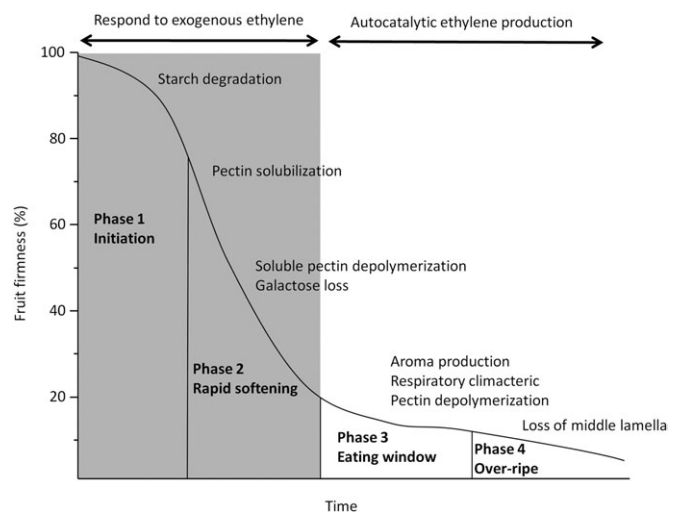


Fig. 1. Schematic representation of postharvest kiwifruit ripening in relation to the timing of key physiological events (1–4 are softening phases) based on Schröder and Atkinson (2006). Fruit at harvest do not produce endogenous ethylene but are highly sensitive to the application of exogenous ethylene (phase 1). After a period of rapid softening (phase 2), phase 3 starts with the onset of endogenous ‘autocatalytic’ ethylene production. Fruit in phase 3 are considered to be in the eating-ripe window for consumers—the fruit are soft and produce characteristic ripe fruit aroma volatiles. Fruit in phase 4 are unacceptably soft and often exhibit ‘off flavour’ notes. The duration of the softening phases depends on species, environmental conditions, and harvest time (early or late season). Application of exogenous ethylene in phase 1 accelerates and synchronizes fruit ripening in phases 1 and 2.

is important in the postharvest handling of kiwifruit. Fruit at harvest are considered ‘competent’ to ripen and are highly sensitive to exogenous ethylene, which both accelerates and synchronizes fruit ripening. The use of 1-MCP and other ethylene inhibitors has been shown to delay softening (Boquete *et al.*, 2004; Koukounaras and Sfakiotakis, 2007; Iliina *et al.*, 2010; Mworio *et al.*, 2010).

We have produced a transgenic *ACO* knockdown kiwifruit line that does not produce detectable levels of climacteric ethylene. Fruit ripening is arrested in phase 3, significantly extending this phase of ripening. Application of exogenous ethylene to transgenic fruit arrested in phase 3 was sufficient to re-initiate fruit softening and for production of aroma volatiles. Gene expression changes were monitored during this period on oligonucleotide microarrays representing 17 472 genes. The results from these physiological and molecular experiments allow ethylene ripening processes in kiwifruit to be dissected and compared with those of apple, tomato, and melon.

Materials and methods

Sequence identification and analysis

The *A. deliciosa* (*A. Chev.*) C.F. Liang et A.R. Ferguson var. *deliciosa* ‘Hayward’ *ACO* sequence (pKIWIAO1, accession number M97961; MacDiarmid and Gardner, 1993) was used to identify

ACO genes in the *Actinidia* expressed sequence tag (EST) collection of >130 000 sequences (Crowhurst *et al.*, 2008) using BLAST. Homologous *A. deliciosa* and *A. chinensis* Planch. contigs (P values $< \exp^{-20}$) were identified and the most 5' EST was selected for full-length sequencing. Sequence alignments were constructed using ClustalX (version 1.8).

Generation of transgenic kiwifruit lines

A 547-bp fragment of AcACO1 (nucleotides 201–747; Supplementary Fig. S1 available at *JXB* online) was used to produce an RNAi hairpin construct in the binary vector pTKO2 (Snowden *et al.*, 2005). This vector utilizes the CaMV 35S promoter and the transcriptional terminator of the *Arabidopsis ACT2* gene. pTKO2_ACO1 was electroporated into *Agrobacterium tumefaciens* strain EHA105 and transgenic *A. chinensis* 'Hort16A' plants were regenerated and rooted as described by Wang *et al.* (2007). Independent transgenic lines were established in a containment greenhouse. *A. chinensis* vines have a long juvenile period, are large in size, and have difficulty flowering under standard containment greenhouse conditions (Wang *et al.*, 2006). To overcome these issues, ACO lines TK1 and TK2 were transferred to the New Zealand Controlled Environment Laboratory, Plant and Food Research (PFR), Palmerston North, and grown alongside two control lines (CK1 and CK2) under optimal lighting and climatic control conditions conducive for flower and fruit set.

Mature potted vines were pruned to give two main leaders (~2.5 m long) and chilled for 2 months at 7.0 ± 0.5 °C, with a vapour pressure deficit (VPD) of 0.2 ± 0.1 kPa and low photosynthetic activity lighting with an 8-h photoperiod. Over the chilling period, the vines were hand-watered fortnightly and prior to bud-break repotted into 50-l planter bags containing Dalton's fertilized DB Potting Mix (top-dressed with Osmocote® Plus NPK 15-5-11+1.2 Mg). For their growing period, vines had a mean photosynthetic photon flux at mid-canopy height of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$, ambient carbon dioxide, and twice daily alternate feeds via drippers of half-strength Hoagland's nutrient (Brooking, 1976) and water. Over the first month, they received a 14-h photoperiod at $18.0/10.0 \pm 0.5$ °C and VPDs of $0.8/0.1 \pm 0.1$ kPa, day/night, respectively. One month later, the photoperiod and temperature increased to 16 h and $22.0/12 \pm 0.5$ °C day/night, providing VPDs of $1.1/0.1 \pm 0.1$ kPa, respectively. Flowers were hand-pollinated using compatible pollen. On completion of pollination, the vines were lightly pruned and the temperature increased to $22.0/14.0 \pm 0.5$ °C supplying VPDs of $1.1/0.2 \pm 0.1$ kPa day/night, respectively, up until fruit harvest.

Fruit quality measurements

Fruit from transgenic and control lines were harvested when softening could be detected by hand (start of phase 2, ~24 weeks post anthesis). Individual fruit were weighed and packed into trays with plastic pocket packs and polyliners. *A. chinensis* fruit at harvest display considerable variation in the progression of fruit ripening. To synchronize fruit ripening, both control and transgenic fruit were treated with a short burst of exogenous ethylene ($100 \mu\text{l l}^{-1}$, 24 h), then held in an ethylene-free environment at 20 °C.

Endogenous climacteric ethylene production of individual fruit was monitored by placing fruit in 529-cm³ respiration containers for 1 h. Samples (1 cm³) were then withdrawn from the container headspace and ethylene measured by flame ionization chromatography (PU 4500 Chromatograph; Phillips, UK) as described in Johnston *et al.* (2009). Ambient ethylene concentrations in empty respiration jars were used as controls. CO₂ production was measured by injecting 1-cm³ samples into a gas analyser fitted with a miniature infrared detection cell (Servomex 01514/701 infrared transducer; Servomex PLC, Crowborough, East Sussex, UK). Fruit firmness was measured non-destructively using a KIWIFIRM device (Hopkirk *et al.*, 1996), or destructively using an Instron Universal Testing Machine (Model 4301; Instron, Inc. Canton,

MS, USA). Soluble solids concentration (SSC) was measured using a digital refractometer (Atago, model PAL-1; Japan). Colour was assessed in lightness, L, chroma, C, and hue angle, H, by chromameter (Minolta, model CR-300; Japan).

Semi-quantitative PCR and western analysis

Total RNA was extracted from rapidly expanding leaf tissue according to Chang *et al.* (1993) and treated with 10 U of DNase I (Roche Applied Science, Mannheim, Germany) prior to cDNA synthesis. First-strand cDNA was synthesized from 2 μg of total RNA with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and diluted 30-fold prior to use. Semi-quantitative PCR reactions (50 μl) were run using the Expand High Fidelity PCR System (Roche Biochemicals) according to the manufacturer's instructions and the gene-specific primers in Supplementary Table S1 at *JXB* online. Reactions were denatured for 2 min at 94 °C, then given 30 cycles at 94 °C for 15 s (denaturing), 60 °C for 30 s (annealing), and 72 °C for 90 s (extension) before a final extension of 5 min.

Proteins were extracted from fruit tissue ground to powder using a mortar and pestle under liquid nitrogen. Powder (700 mg) was extracted using hot SDS buffer (Sonnewald *et al.*, 1993) then cleared by centrifugation (16 000 \times g, 10 min). Proteins were separated on precast 12% NuPAGE® Bis-Tris gels according to the manufacturer's instructions (Invitrogen). Electroblothing and immunolocalization using an apple ACO3 polyclonal antibody (1:500, w/v) were performed as described in Nieuwenhuizen *et al.* (2007). For detection, the PVDF blots were incubated with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) and ACO binding visualized using 1-Step BCIP NBT Liquid Substrate System (Thermo Scientific).

Volatile measurements

Intact fruit were placed into sealed 500-ml flasks. Dried air was introduced to sweep the headspace (25 ml min⁻¹) for 1 h onto a volatile absorbent trap (80 mg Chromosorb™ 105). Traps were dried with an N₂ flow at 10 psi, 35 °C for 15 min before analysis by gas chromatography/mass spectrometry (GC/MS). The collected sample was thermally desorbed at 175 °C onto a 30 m \times 0.32 mm internal diameter, 0.5 μm film DBWax GC column (J&W Scientific, Folsom, CA, USA) in a HP6890 GC (Agilent Technologies, Santa Clara, CA, USA). Peaks were identified by time-of-flight mass spectrometry (TOF-MS, Leco Pegasus III; St Joseph, MI, USA) according to Nieuwenhuizen *et al.* (2009). The oven temperature programme was 35 °C for 2 min, then ramped at a rate of 2 °C min⁻¹ to 60 °C followed by 5 °C min⁻¹ to 100 °C, 8 °C min⁻¹ to 140 °C, 10 °C min⁻¹ to 200 °C, and held for 6 min. Component identification was based on calculation of retention indices, mass spectra of authentic standards, and comparison with library spectra (NIST 98; Wiley 7 and in-house). Amounts of each chemical were calculated as ng g⁻¹ with an average detector response factor based on a standard containing ethyl acetate, ethyl butanoate, methyl butanoate, hexanal, myrcene, cineole, hex-E2-enal, ethyl hexanoate, hexanol, linalool, ethyl benzoate, and methyl salicylate in pentane.

Microarray analysis

Outer pericarp tissue was snap frozen in liquid nitrogen before storage at -80 °C. Total RNA was extracted as described by Chang *et al.* (1993) and cleaned using an RNAeasy cleanup kit (Qiagen) according to the manufacturer's protocol. Microarray construction, and all labelling and hybridizations followed the methods described by Schaffer *et al.* (2007). Microarrays contained 45- to 55-mer oligonucleotides with similar T_m s, and were designed to non-redundant contigs in the PFR *Actinidia* EST database (Crowhurst *et al.*, 2008). The oligonucleotides were combined to create a microarray representing 17 472 genes. Gene ontology

(GO) analysis of all genes on the kiwifruit microarray can be found in Supplementary Table S7 of Walton *et al.* (2009).

Microarray analysis used direct comparisons of samples collected at 0 h (T0) with samples collected at 4, 12, 24, and 168 h after ethylene treatment. There were two biological replicates, each with two technical replicates. Data from each comparison were normalized using global loess normalization, without background correction Schaffer *et al.* (2007). Each experiment was then analysed separately using the Linear Models for Microarray Analysis (Limma) package in Bioconductor (www.bioconductor.org), incorporating between-gene information. Gene lists were obtained for each comparison. Differential expression was determined using a false discovery rate threshold of 0.05 and lists were filtered to remove genes that had a <2-fold change in expression. Genes were identified using TBLASTN against GenBank with $P < \exp^{-10}$.

Real-time RT-PCR analysis

Total RNA was extracted from outer pericarp tissue and first-strand cDNA synthesized as described for semi-quantitative PCR. Relative quantification real-time gene expression analysis of each gene and the housekeeping gene *EFT α* were performed as described in Atkinson *et al.* (2009a) on a LightCycler® 480 platform using the LightCycler 480 SYBR Green master mix and results were analysed using LightCycler 480 software (Roche). Programme: 5 min at 95 °C; 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C; followed by melting curve analysis: 95 °C 5 s, 65 °C 60 s then ramping at 0.18 °C s⁻¹ to 95 °C. The primer pairs used for real-time PCR analysis (Supplementary Table S1 at *JXB* online) were designed using Vector NTI software (Invitrogen) to have similar T_m s, produce products of similar size, and to avoid hairpins and primer-dimer combinations. Standard errors of the means were generated using GenStat® version 10.1.0.71.

Results

ACO genes in *A. chinensis* and *A. deliciosa*

A. chinensis is a diploid species ($n=29$) that is believed to have contributed one or more genomes to the hexaploid species *A. deliciosa* (Atkinson *et al.*, 1997). The high haploid chromosome number of *A. chinensis* suggests that it is a palaeo-polyploid (He *et al.*, 2003), which means that multiple gene copies of varying homology are often expressed in the same tissue. To identify the number of ACO genes potentially expressed during *A. chinensis* fruit ripening the PFR *Actinidia* EST collection of >130 000 sequences was screened for sequences that were similar to the ACO cDNA clone pKIWIAO1 from *A. deliciosa* (accession number M97961; MacDiarmid and Gardner, 1993). ESTs representing six contiguous sequences (contigs) showed homology to pKIWIAO1 with at least 80% nucleotide identity (Table 1). The most homologous contig (designated ACO1) contained a sequence from *A. deliciosa* that was identical to the pKIWIAO1 sequence and an orthologous gene from *A. chinensis* that showed 99.3% identity. A second contig (ACO2) contained *A. deliciosa* sequences identical to a second partial ACO gene from *A. deliciosa* (KWACCOX1, accession number AB003514) reported by Xu *et al.* (1998). Contigs ACO3–5 contained sequences from both *A. chinensis* and *A. deliciosa* whilst the ACO6 contig was specific to *A. chinensis*. These sequences

Table 1. ACO genes in *A. chinensis* and *A. deliciosa*. The PFR *Actinidia* EST database was BLAST searched using the sequence of the *A. deliciosa* cDNA clone pKIWIAO1 (accession number M97961) to identify homologous sequences. Six ACO contigs were identified from *A. chinensis* and *A. deliciosa* and representative clones were completely sequenced and deposited in GenBank (accession numbers HQ293204–HQ293212). The percentage nucleotide identity (% id) of each clone in the open reading frame to pKIWIAO1, the source library for each clone, and the number of ESTs (No. ESTs) identified for each clone are given.

Contig	Species	Accession Nos	% id	Source	No. ESTs
ACO1	<i>A. chinensis</i>	HQ293204	99.3	fruit	34
	<i>A. deliciosa</i>	M97961	100	fruit	8
ACO2	<i>A. deliciosa</i>	HQ293205	94.6	fruit	12
		AB003514	94.0		
ACO3	<i>A. chinensis</i>	HQ293206	94.6	fruit	55
	<i>A. deliciosa</i>	HQ293207	94.6	fruit	56
ACO4	<i>A. chinensis</i>	HQ293208	89.9	fruit, leaf	10, 1
	<i>A. deliciosa</i>	HQ293209	89.3	bud	1
ACO5	<i>A. chinensis</i>	HQ293210	80.0	bud	2
	<i>A. deliciosa</i>	HQ293211	80.3	bud, flower	15, 1
ACO6	<i>A. chinensis</i>	HQ293212	89.6	fruit	4

showed from 80% to 95% identity to pKIWIAO1 at the nucleotide level (aligned in Supplementary Fig. S1 at *JXB* online). Clones in contigs ACO1–4 and ACO6 were found in fruit libraries, whilst clones in contig ACO5 were derived from flower and bud libraries (Table 1).

Generation and analysis of ethylene knockdown lines

A fragment of the *A. chinensis* ACO1 gene (accession number HQ293204) was used to produce an RNAi hairpin construct in the binary vector pTKO2. This fragment targeted a region of high homology within the five ACO genes (ACO1–4 and ACO6) expressed in fruit tissues. Ten independent transgenic *A. chinensis* lines were generated and screened by semi-quantitative PCR for suppression of ACO gene expression in leaf tissue. Seven of the eight transgenic lines showed some suppression of ACO gene expression (Supplementary Fig. S2A at *JXB* online) and the two most suppressed lines (TK2 and TK4) were selected for further analysis. The selected lines were transferred to the New Zealand Controlled Environment Laboratory, PFR, Palmerston North to obtain flowering and fruiting alongside two control lines (CK1 and CK2).

The first batch of control and transgenic *A. chinensis* fruit was treated with a short burst of exogenous ethylene (100 μ l l⁻¹, 24 h) to synchronize fruit ripening, then held in an ethylene-free environment at 20 °C. Following this treatment endogenous climacteric ethylene production was detected in control fruit 48, 72, 96, and 144 h after exogenous ethylene treatment (range 0.52–1.06 μ mol kg⁻¹ s⁻¹). No endogenous ethylene production was measured in the

transgenic ACO TK2 line, whilst very low levels (range 0.001–0.008 $\mu\text{mol kg}^{-1} \text{s}^{-1}$, representing <1.5% of the lowest control) were detected in the TK4 line (Fig. 2A). CO_2 production was also measured at the same time points, with transgenic TK2 and TK4 lines showing 60% and 75%, respectively, of the production of the lowest control (Fig. 2B). No differences in fruit SSC or colour were observed between the control and transgenic lines during this time course (data not shown). Western analysis using an apple ACO3 polyclonal antibody indicated that ACO protein expression was undetectable in extracts from TK2 and TK4 fruit taken 168 h after exogenous ethylene treatment. At this time point, ACO protein expression reached maximum levels in extracts from a ‘Hort16A’ ripening fruit series (Supplementary Fig. S2B at *JXB* online).

As expected, all fruit rapidly lost firmness (~40%) within 48 h of exogenous ethylene treatment as the fruit passed through phase 2 and entered phase 3. Control fruit entered the over-ripe phase (phase 4, <3 KIWIFIRM units, ~4 N firmness) by 144 h (see Fig. 3). The transgenic line ACO TK4 maintained a phase 3 firmness of ~3 KIWIFIRM units for >15 d, whilst the firmness of the transgenic TK2 line held at >3 KIWIFIRM units for >25 d (Fig. 3). Firmness measurements were ended at these times because the fruit started to show the first signs of desiccation, making firmness measurements by KIWIFIRM unreliable.

Fruit volatiles were identified and semi-quantified by GC/MS 144 h after exogenous ethylene treatment (Fig. 4). The major volatiles produced in control fruit were esters: in particular ethyl butanoate, methyl butanoate, and ethyl acetate (see Supplementary Table S2 at *JXB* online for individual compounds and semi-quantification). These compounds form part of the typical ripe fruit volatile profile for *A. chinensis* ‘Hort16A’ entering phase 4 ripening (Marsh *et al.*, 2006; Friel *et al.*, 2007). Total volatile production in the transgenic TK2 and TK4 lines was reduced to <3% (7–20 ng g^{-1}) of the lowest control level (815 ng g^{-1}). TK2 fruit produced essentially no esters (0.1 ng g^{-1}), whilst TK4 fruit produced very low levels of esters (13 ng g^{-1}), including trace amounts of ethyl butanoate and methyl butanoate.

Re-initiation of the ripening process in TK2 fruit

A second independent batch of fruit from the ACO knockdown TK2 line and the control CK1 were treated with exogenous ethylene (100 $\mu\text{l l}^{-1}$) for 24 h and held at 20 °C for 144 h. At this point, the control fruit were entering phase 4 ripening (2.77 ± 0.16 KIWIFIRM units) and produced climacteric ethylene ($0.62 \pm 0.08 \mu\text{mol kg}^{-1} \text{s}^{-1}$) and aroma volatiles (Table 2, column 10), whilst the TK2 line was arrested in phase 3 with fruit that were firmer (4.41 ± 0.10 KIWIFIRM units) and did not produce climacteric ethylene or aroma volatiles (Table 2, column 4). To establish whether ripening could be re-initiated in the TK2 line, the phase 3-arrested fruit were then placed in sealed chambers and treated with continuous exogenous ethylene for 4, 12, 24, 96, and 168 h. Two treatments were held as controls without addition of ethylene for 96 and 168 h.

Softening in the phase 3-arrested TK2 fruit was re-initiated within 24 h of continuous exogenous ethylene treatment and after 96 h and 168 h fruit had lost 35% and 60% of their initial firmness, respectively (Fig. 5). After 96 h treatment, fruit had entered phase 4 and were in the over-ripe stage (<3 KIWIFIRM units). Fruit held without exogenous ethylene treatment softened slowly, losing only 15% and 20% of their initial firmness after 96 h and 168 h, respectively.

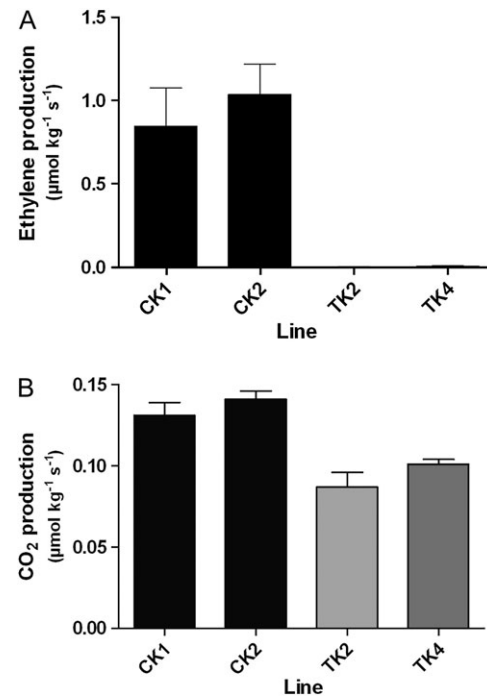


Fig. 2. Reduced ethylene and CO_2 production in ACO knockdown *A. chinensis* lines. Endogenous ethylene (A) and CO_2 (B) production was measured by gas chromatography in fruit from two control lines (CK1, CK2) and two transgenic lines (TK2, TK4). Measurements shown were taken 48 h after treatment with exogenous ethylene. Error bars are SEM, $n=9-20$ fruit.

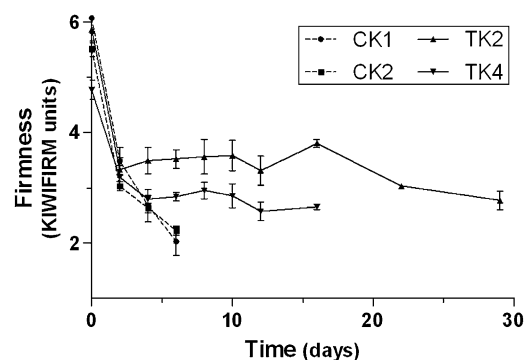


Fig. 3. Arrest of phase 3 softening in ACO knockdown *A. chinensis* lines. Fruit firmness was measured non-destructively using a KIWIFIRM device from two control lines (CK1, CK2) and two knockdown lines (TK2, TK4). Fruit with firmness of <3 KIWIFIRM units are considered over-ripe. Error bars are SEM, $n=9-20$ fruit.

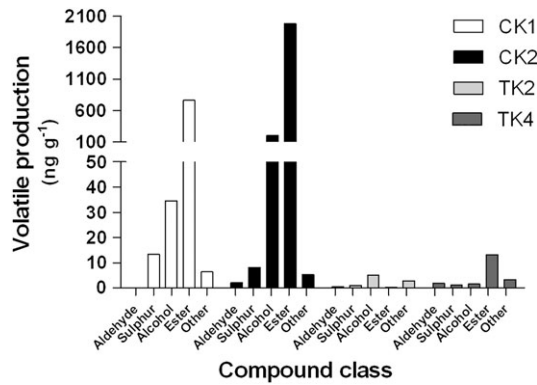


Fig. 4. Reduced volatile production in ACO knockdown *A. chinensis* lines. Volatile production was measured by GC/MS in fruit from two control lines (CK1, CK2) and two knockdown lines (TK2, TK4). Measurements were taken 144 h after treatment with exogenous ethylene. Individual semi-quantified compound data (Supplementary Table S2 at *JXB* online) were sorted into five compound classes representing the key volatiles present in ripe fruit. Data were derived from two or three independent ethylene treatments with each treatment consisting of two replicates of two fruit.

Volatile production in the phase 3-arrested TK2 fruit was also initiated by exogenous ethylene treatment (Table 2). The majority of esters observed in ripe control fruit (Table 2, column 10) were detected in TK2 fruit 168 h after exposure to exogenous ethylene. Total ester production in the TK2 fruit (830 ng g^{-1}) was higher than phase 3 control CK1 fruit in this experiment (190 ng g^{-1})—perhaps reflecting the exposure of the TK2 fruit to continuous high levels of exogenous ethylene. Like esters, the production of sulphur-related compounds also peaked after 168 h. The production of alcohols and aldehydes typically peaked earlier, with 2-propanol and 2-ethyl 1-hexanol peaking after 4 h, nonanal, and decanal within 12–24 h, and hexanal, octanal, and 1-hexanol after 96 h. TK2 fruit held for 168 h without exposure to exogenous ethylene emitted very low levels of volatiles, very similar to TK2 fruit at T0.

Ethylene-induced gene expression changes

Microarrays representing 17 472 non-redundant contigs were used to investigate changes in the abundance of RNA transcripts from phase 3-arrested TK2 fruit after exogenous ethylene treatment of 4, 12, 24, or 168 h. Changes in gene expression were compared with phase 3-arrested TK2 fruit that had not been treated with ethylene (T0+168 h) or CK1 fruit in phase 3. By 4 h, the expression of 25 genes had significantly changed (>2-fold with an adjusted P value of <0.05), after 12 h this had increased to 81 genes, by 24 h 183 genes had changed, and by 168 h the expression of 284 genes had changed. In total 401 genes were identified as significantly changing.

The nearest homologous sequences to these 401 kiwifruit genes were identified using BLAST searches to GenBank. From this homology search, all genes were classified by

function (presented in Supplementary Table S3 at *JXB* online). The composition of each of these functional classes can be seen in Fig. 6. Of particular note from this analysis was the large number of genes of ‘unknown’ function (37%). Each group of functionally similar genes was clustered by expression pattern (Fig. 6). The clustering shows that phase 3-arrested TK2 fruit treated with continuous exogenous ethylene are most similar to CK1 fruit at eating-ripe firmness. In the ‘cell wall’ and ‘metabolism’ classes there are clusters of genes with differing expression patterns, whilst the majority of ‘photosynthesis’- and ‘starch’-related genes appear to be turned off by ethylene (Fig 6). The other clusters contained representations of genes that were both up-regulated and down-regulated with the application of endogenous ethylene.

The expression patterns of 13 genes that were identified as changing by microarray analysis were further characterized by qPCR (Fig. 7). Ten of these genes were selected from the ‘cell wall’ and ‘metabolism’ classes that were most likely to be those responsible for the observed physiological changes in fruit softening and volatile production. A summary of the putative functions and nearest homologues of all 13 genes analysed by qPCR is given in Table 3.

Discussion

This work adds to a growing understanding of biochemical changes underpinning fruit ripening in climacteric fruit. Our results using ethylene-suppressed kiwifruit (*Actinidiaceae*) lines are generally consistent with previous research using ethylene-suppressed tomato (*Solanaceae*), melon (*Cucurbitaceae*), and apple (*Rosaceae*) lines. The observation that the softening process and volatile production are ethylene-regulated in these four genetically distant plant species indicates that there is significant conservation of the ethylene-induced transcriptional cascade in fruit. However, our results also reinforce the importance of the differences that exist between these species. Total volatile production was more strongly inhibited in ethylene-suppressed apple and kiwifruit lines than in melon (Pech *et al.*, 2008), which may be the result of a weaker knockdown phenotype in melon (and the production of residual ethylene in the fruit) or a difference in ethylene sensitivity for volatile production in melon. Ester biosynthesis appeared to be strongly ethylene dependent in transgenic apple, melon, and kiwifruit lines. Aldehyde and alcohol production was almost completely inhibited in kiwifruit, melon, and in strong ethylene knockdown apple lines (Schaffer *et al.*, 2007); however, in partial ethylene knockdown apple lines alcohol and aldehyde production was still observed (Dandekar *et al.*, 2004). The strongest reduction in fruit softening has been observed in apple lines, though a small degree of ethylene-independent softening was observed in these lines (Dandekar *et al.*, 2004; Schaffer *et al.*, 2007; Johnston *et al.*, 2009). In melon, there was still significant residual softening, suggesting the presence of an ethylene-independent component to fruit softening (Guis *et al.*, 1997). In

Table 2. Induction of volatile production in the ACO knockdown. TK2 line treated with ethylene Fruit from the knockdown TK2 and the control CK1 lines were treated with exogenous ethylene ($100 \mu\text{l l}^{-1}$) and held at $20 \text{ }^\circ\text{C}$ for 144 h. Aroma volatiles were measured by GC/MS in the CK1 line (T0, column 10) and TK2 line (T0, column 4). The ripening process was re-initiated in the phase 3-arrested TK2 fruit using exogenous ethylene ($100 \mu\text{l l}^{-1}$). Aroma volatiles were semi-quantitatively measured (ng g^{-1}) by GC/MS after 4, 12, 24, 96, and 168 h of exposure to ethylene (E, columns 5–9); and in fruit held for 168 h without exposure to ethylene (column 3). RI, = retention index.

Compound (ng g^{-1})	RI	TK2 168 h 0	TK2 T0 0	TK2 4 h E	TK2 12 h E	TK2 24 h E	TK2 96 h E	TK2 168 h E	CK1 T0 0
Aldehydes									
Acetaldehyde	263	0.14	0.17	0.20	0.18	0.05	0.03	0.19	0.05
Nonanal	1448	0.10	0.03	0	0.48	0.12	0.07	0	0
Decanal	1593	0	0.05	0	0	0.21	0.07	0	0
Hexanal	726	0.22	0	0	0	0.08	0.28	0	0
Octanal	1254	0	0	0	0	0.09	0.20	0	0
Benzaldehyde	1620	0	0	0	0	0	0.02	0	0
Benzaldehyde, ethyl-	1810	0	0	0	0	0	0	0.47	0
Alcohols									
1-Hepten-3-ol	1535	0	0.18	0	0	0	0	0	0
2-Propanol	435	0.07	0	0.19	0.06	0.05	0.09	0	0.00
1-Hexanol, 2-ethyl-	1586	0	0.11	0.18	0	0.06	0.05	0	0.14
1-Hexanol	1391	0	0	0	0	0.10	0.14	0	0
Ethanol	444	0.95	0.58	0.41	0.46	0.12	0.82	19.23	3.72
1-Butanol	940	0	0	0	0.13	0.09	0.25	0.73	0.24
Esters									
1-Propen-2-ol, acetate	388	0	0	0	0	0	0.03	0	0
Acetic acid, methyl ester	324	0	0.02	0.02	0	0.01	0.01	0.65	0.32
Ethyl acetate	376	0.03	0.03	0	0.07	0.04	0	18.21	0.26
Propanoic acid, methyl ester	398	0	0	0	0	0	0	0.53	0.82
Propanoic acid, ethyl ester	471	0	0	0	0	0	0.05	1.81	0.22
Butanoic acid, methyl ester	522	0	0	0	0	0	1.02	112.38	74.14
Butanoic acid, ethyl ester	633	0	0	0	0	0	0	656.21	99.24
Acetic acid, butyl ester	708	0	0	0	0	0	0.01	0.65	0
Pentanoic acid, methyl ester	759	0	0	0	0	0	0	0.10	0
Butanoic acid, propyl ester	844	0	0	0	0	0	0	2.01	1.20
Pentanoic acid, ethyl ester	896	0	0	0	0	0	0	1.71	0.00
2-Butenoic acid, ethyl ester	967	0	0	0	0	0	0	0.24	0
Hexanoic acid, methyl ester	1022	0	0	0	0	0	0	0.08	0.30
Butanoic acid, butyl ester	1103	0	0	0	0	0	0	3.85	13.18
Hexanoic acid, ethyl ester	1138	0	0	0	0	0	0	30.79	0.93
3-Hexenoic acid, ethyl ester, (Z)-	1287	0	0	0	0	0	0	0.14	0
Benzoic acid, methyl ester	1729	0	0	0	0	0	0	0.57	0.05
Benzoic acid, ethyl ester	1772	0	0	0	0	0	0	0.78	0
Benzoic acid, butyl ester	1927	0	0	0	0	0	0	0.10	0
Sulphur compounds									
Dimethyl sulfone	1950	0	0	0.09	0	0	0	0	0
Dimethyl sulphide	281	0	0	0.48	0	0.12	0	1.45	13.21
Methane, thiobis-	1690	1.66	1.63	2.94	1.07	1.43	10.64	12.88	4.89
Other compounds									
2-Buteneone	450	0.08	0	0	0.01	0.07	0.05	0	0
Acetic acid	1538	1.96	0.95	0.87	0.72	0.73	0.92	1.67	0.35
2-Propanone	315	0.26	0.34	5.33	0.20	0.27	0.32	0.32	0.25
6-Methyl-5-hepten-2-one	1352	0	0.09	0	0.01	0.09	0.04	0	0
2-Butanone	390	0	0	0	0	0.04	0	0	0
2-Butanone, 3-methyl-	500	0	0	0	0	0	0.02	0	0
Furan, 2-methyl-	384	0	0	0	0.21	0.05	0.88	1.21	0.35
Formic acid	1617	0.11	0	0	0.07	0	0.02	0.65	0

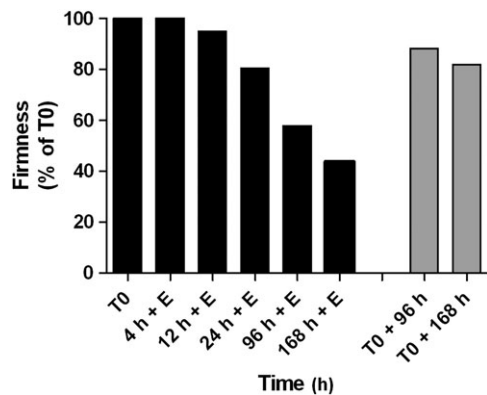


Fig. 5. Re-initiation of the softening process in the ACO knock-down TK2 line. Fruit from the TK2 line were placed in sealed chambers and treated with exogenous ethylene ($100 \mu\text{l l}^{-1}$) for 4, 12, 24, 96, and 168 h to re-initiate the ripening process (black bars). For the 96 h and 168 h treatments, chambers were aired for 1 h each 24 h period, resealed, and ethylene re-added ($100 \mu\text{l l}^{-1}$). Two treatments were held as controls without addition of ethylene for 96 h and 168 h (grey bars). Fruit firmness was measured non-destructively using a KIWIFIRM device using four to eight fruit per treatment. Firmness values for each treatment are expressed as a percentage of fruit firmness at the start of the experiment (T0).

knockdown tomato lines, there was no significant change in fruit softening until the fruit reached the over-ripe stage (Murray *et al.*, 1993). In kiwifruit, our results indicate that a reduction in climacteric ethylene also reduces fruit softening as fruit enter the late softening phases, with transgenic lines showing a significant delay in entering the over-ripe stage (phase 4, Fig. 1).

Microarray analysis revealed a large number of genes that were transcriptionally regulated by ethylene in ripening kiwifruit. The expression patterns of 13 genes that changed on the microarray were further validated by qPCR, with a focus on genes in the ‘cell wall’ and ‘metabolism’ classes (Table 3). Of the genes in the ‘cell wall’ class, a polygalacturonase (EST260725) has previously been shown to be ripening induced in kiwifruit (Bonghi *et al.*, 1996; Wang *et al.*, 2000). qPCR showed that polygalacturonase gene expression increased 100 000-fold and peaked 12 h after ethylene treatment. Polygalacturonases have been implicated in the depolymerization of pectin in kiwifruit (Redgwell *et al.*, 1992) and their down-regulation in tomato, strawberry, and apple has been shown to affect fruit firmness (Kramer *et al.*, 1992; Atkinson *et al.*, 2009b; Quesada *et al.*, 2009). A pectate lyase (EST245817) and a pectin methylesterase inhibitor (PMEi, 191682) peaked in expression 24 h after ethylene treatment (14 000-fold and 9-fold, respectively), by which time the softening process had clearly been re-initiated in the ethylene-treated transgenic fruit (Fig. 5). Pectate lyases have been implicated in the softening process in strawberry (Jimenez-Bermudez *et al.*, 2002; Santiago-Domenech *et al.*, 2008) and banana (Payasi and Sanwal, 2003), where they are involved in the degradation of pectins by a β -elimination reaction (in contrast to the hydrolytic mechanism of polygalacturonases).

De-esterification of pectin by pectin methylesterases (PMEs) is a common feature of fruit ripening that affects the rigidity of the pectin network and modifies the diffusion and activity of enzymes within the cell wall (Brummell, 2006). Down-regulation of the *Pmeu1* isoform in tomato resulted in an enhanced rate of softening during fruit ripening (Phan *et al.*, 2007). The kiwifruit PMEi protein has been shown to bind to PME in a non-covalent 1:1 complex (Di Matteo *et al.*, 2005) but its involvement in fruit softening has not been determined.

The expression of the ‘cell wall’ class genes EST228432 (encoding a xylan-degrading enzyme) and EST310095 (encoding an endo-1,3- β -glucosidase) peaked in expression by microarray analysis 168 h after ethylene treatment. qPCR confirmed this pattern of expression for EST228432 (100-fold increase in expression), whilst the qPCR expression pattern for EST310095 was less apparent, with highest expression at T0 and T0+168 h. Xylan-degrading enzymes comprising β -xylosidase or endo- β -xylanase are involved in the modification of xylans in fruit. Their activity has been linked to softening in banana and avocado (Ronen *et al.*, 1991; Prabha and Bhagyalakshmi, 1998). During avocado ripening, xylanase activity remains constant, whereas β -xylosidase activity reaches a peak at the climax of ethylene production. Evidence for β -xylosidase up-regulation during fruit softening has been shown in strawberry and pear (Itai *et al.*, 1999; Martinez *et al.*, 2004). β -1,3-Glucosidases/glucanases are cell wall-related enzymes that can be induced by ethylene during ripening but whose primary role may be in pathogen defence rather than fruit softening (Derckel *et al.*, 1998; Roy Choudhury *et al.*, 2009).

In the ‘metabolism’ class, expression of EST308930 [encoding a peptide methionine sulphoxide (PMS) reductase] was highest 24 h after ethylene treatment. PMS reductase enzymes are involved in the repair of inactive peptides and proteins caused by the oxidation of methionine residues. A fruit-specific PMS reductase, *Fapmsr*, has been isolated from strawberry where it is hypothesized to be responding to the oxidative stress conditions produced during ripening (Lopez *et al.*, 2006). EST102988 (encoding a quinone oxidoreductase) also increased 5-fold in expression, peaking 168 h after ethylene treatment. In strawberry, the quinone oxidoreductase *FaQR*, has been shown to be strongly ripening induced and auxin dependent (Raab *et al.*, 2006). The enzyme functions as an enone oxidoreductase in the biosynthesis of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furanol), which is an important strawberry flavour and aroma component. A related protein from tomato has also been shown to use the same flavour precursors (Klein *et al.*, 2007). In *Arabidopsis thaliana*, a ζ -crystalline quinone oxidoreductase catalyses the reduction of reactive alkenals (e.g. hex-E2-enal) to less reactive *n*-alkanals (e.g. hexanal) (Mano *et al.*, 2002). Hex-E2-enal and hexanal are important contributors to kiwifruit flavour, imparting a fresh, green, and grassy flavour (Wang *et al.*, 2010). Total aldehyde content in *A. deliciosa* ‘Hayward’ fruit is 100-fold greater than that found in *A. chinensis* ‘Hort16A’ fruit. In ‘Hayward’,

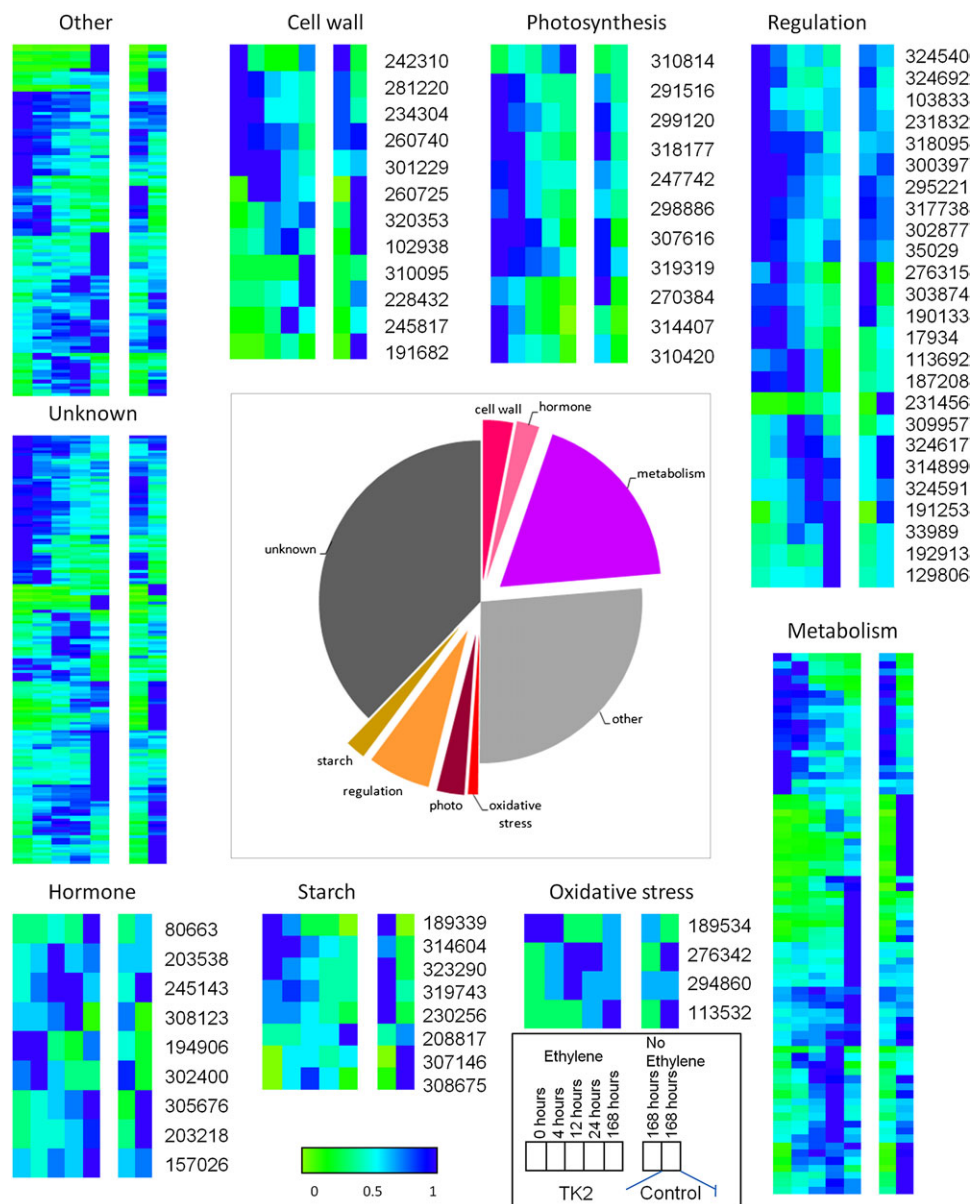


Fig. 6. GO classification of differentially expressed genes identified from microarray analysis. The centre pie chart indicates the percentage of genes in each ontology class. In each coloured panel, gene expression levels are shown with the highest expression shown in blue (maximum set to 1) and the lowest expression in green. The comparisons in each panel are given from left to right for: phase 3-arrested TK2 fruit at T0; fruit treated with ethylene for 4, 12, 24, and 168 h; phase 3-arrested TK2 fruit held without ethylene treatment for 168 h; and CK1 fruit at eating-ripe firmness. The order of genes in each ontology class follows the order of genes in Supplementary Table S3 at *JXB* online.

up to 85% of aldehydes were detected as hex-E2-enal, and only 6–10% as hexanal, whereas in ‘Hort16A’ a much lower proportion of hex-E2-enal (<47%) was found (Wang *et al.*, 2010). These results suggest that regulation of EST102988 might be important in determining the ratios of these flavour components in different kiwifruit cultivars.

Other genes that were identified with a potential role in flavour biosynthesis were EST225823 (encoding an esterase/lipase/thioesterase) and EST79391 (encoding a geranylgeranyl pyrophosphate synthase, GGPS). The expression of the putative esterase decreased in the first 12–24 h after ethylene treatment, and then increased again by 168 h.

Esterase enzymes can catalyse the hydrolysis of carboxylic esters into their corresponding alcohols and carboxylic acids. The increase in volatile esters observed late in kiwifruit ripening probably reflects a change in the balance between the production of esters by alcohol acyl transferases (Günther *et al.*, 2010) and their hydrolysis by esterases. The putative GGPS was up-regulated 9-fold, 12–24 h after ethylene treatment. GGPS enzymes are involved in the production of a wide range of structurally diverse terpenoid compounds (e.g. Schmidt *et al.*, 2010) with potential roles in fruit colour, flavour, senescence, and defence. These compounds include carotenoids, gibberellin

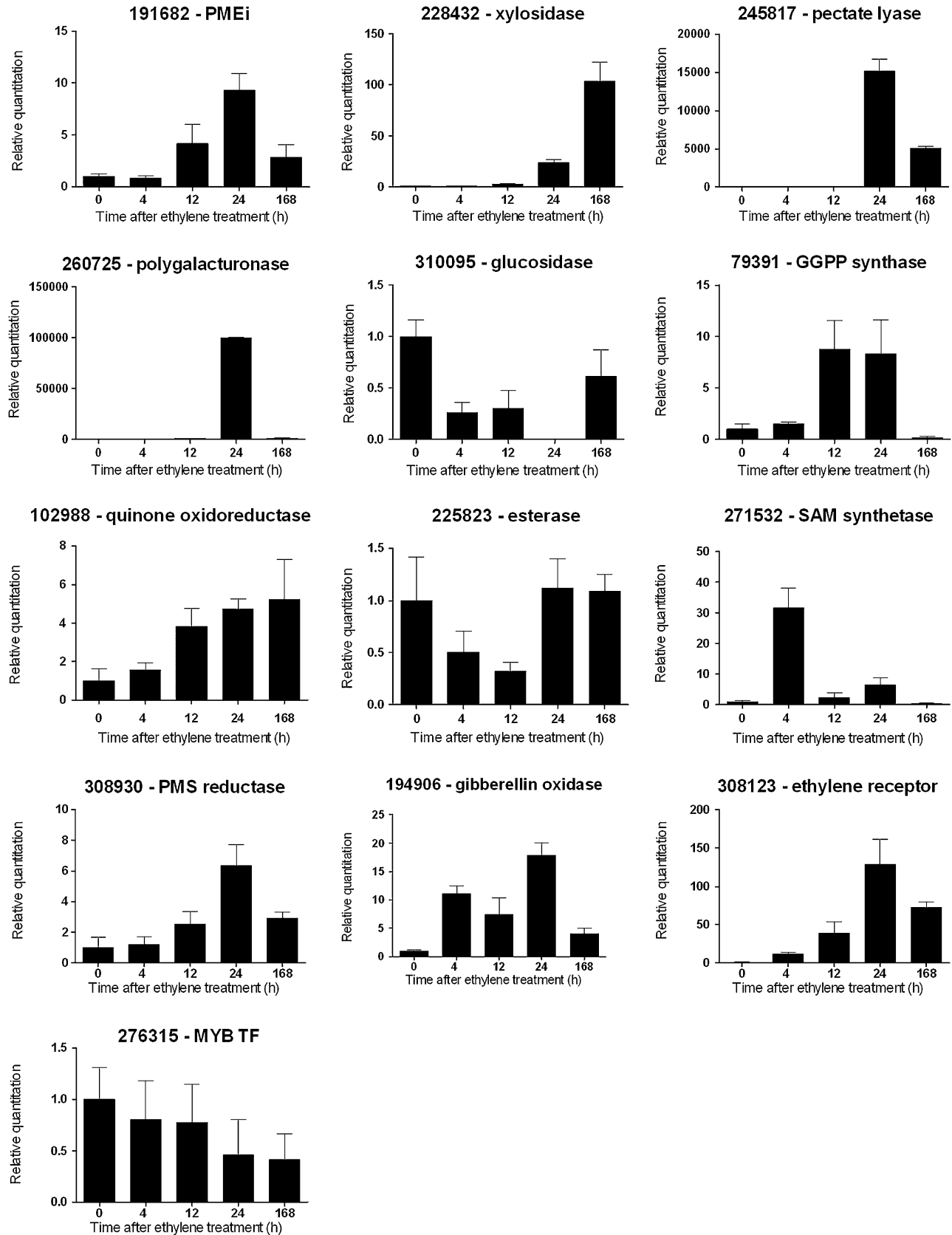


Fig. 7. qPCR expression profiles of 13 differentially expressed genes identified from microarray analysis. Gene expression was analysed by qPCR using RNA isolated from phase 3-arrested TK2 fruit treated with ethylene for 0, 4, 12, 24, and 168 h. Expression is given relative to the 0 h sample, which is set at 1. SEMs are based on eight samples (four technical replicates from two biological replicates). The putative function of each gene, ontology classification, and nearest homologue are given in Table 3.

Table 3. Putative function of 13 selected genes identified as changing by microarray analysis and characterized by qPCR. EST number, corresponding GenBank accession number (Acc. No.), and GO classification are from Supplementary Table S3 at *JXB* online. The putative function of each EST, expect (E) value, and homologues with accession number were obtained from BLAST searches in GenBank.

EST	Acc. No.	Ontology class	Putative function	E value	Homologue
191682	FG458520	Cell wall	PMEi-like	e ⁻⁵⁵	<i>Solanum phurej</i> , DQ822993
228432	FG483283	Cell wall	β-Xylosidase	e ⁻¹⁰⁶	<i>Camellia sinensis</i> , EU714121
245817	FG486828	Cell wall	Pectate lyase	0	<i>M. domestica</i> , AY376878
260725	FG508776	Cell wall	Polygalacturonase	e ⁻¹⁷⁵	<i>A. chinensis</i> , AF152756
310095	FG501191	Cell wall	Glucan endo-1,3-β-glucosidase/glucanase	e ⁻³⁰	<i>Ricinus communis</i> , XP_002515703
79391	FG435317	Metabolism	GGPS	e ⁻⁶⁶	<i>Hevea brasiliensis</i> , AB294711
102988	FG437074	Metabolism	Quinone oxidoreductase	e ⁻⁵⁷	<i>F. × ananassa</i> , AAO22131
225823	FG483222	Metabolism	Esterase/lipase/thioesterase	e ⁻⁸⁵	<i>Rheum australe</i> , EU931231
271532	FG490888	Metabolism	SAM synthetase	0	<i>A. chinensis</i> , U17240
308930	FG525417	Metabolism	PMS reductase msrA	e ⁻⁹¹	<i>F. × ananassa</i> , Z69596
194906	FG471283	Hormone	Gibberellin 2-oxidase	e ⁻¹³⁸	<i>Nerium oleander</i> , AAT92094
308123	FG524691	Hormone	Ethylene receptor	e ⁻¹³⁵	<i>A. deliciosa</i> , EU170629
276315	HS075842	TF	R3-type MYB transcription factor	e ⁻¹⁷	<i>Gossypium hirsutum</i> , FJ402933

hormones, triterpenes, and plant sterols. Interestingly, 194906 expression (encoding a gibberellin 2-oxidase) was also confirmed by qPCR to be induced between 4 and 24 h after ethylene treatment.

Two genes potentially involved in ethylene production and perception, EST271532 (encoding a SAM synthetase) and EST308123 (encoding an ethylene receptor), were strongly induced (30-fold and 120-fold, respectively) on the microarray and confirmed by qPCR analysis. SAM synthetase enzymes and ethylene receptors have previously been shown to be ethylene responsive in kiwifruit (Whittaker *et al.*, 1997; Yin *et al.*, 2008).

In the ‘regulation’ cluster, a number of transcription factors were identified, including MYB genes that encode transcription factors of interest as potential regulators of colour, aroma, and flavour pathways. Expression of EST276315 (encoding a R2R3-type MYB transcription factor) was confirmed by qPCR to decrease after ethylene treatment. In *Petunia hybrida* flowers, volatile benzenoids have been shown to be regulated by the R2R3-type MYB *ODORANTI* (Verdonk *et al.*, 2005), although the orthologue does not appear to function in the same way in apple fruit (Ban *et al.*, 2009). Three further R2R3-type MYB activators of anthocyanin accumulation (MYB10/MYB1/MYBA10) have been identified in apple fruit and other Rosaceae (Kui *et al.*, 2010). As yet, no transcription factors controlling fruit ripening traits have been reported in kiwifruit. EST276315 and the nine other putative transcription factors identified in Supplementary Table S3 at *JXB* online are clearly an exciting subset of genes to explore in more detail.

Conclusion

Transgenic ACO knockdown kiwifruit (*A. chinensis*) lines were produced that reveal the critical importance of climacteric ethylene in determining the ripening character-

istics of kiwifruit. In the absence of climacteric ethylene production, volatile production in the fruit was significantly reduced, a result consistent with previous reports characterizing ethylene knockdowns in apple and melon. However, unlike other reports, softening of kiwifruit was arrested when the fruit reached ‘eating-ripe’ firmness, thereby extending the length of time before fruit become over-ripe. This difference in softening behaviour is likely to reflect the separation of climacteric ethylene production (in phase 3) from the period of rapid fruit softening (in phases 1 and 2) in kiwifruit.

These transgenic plants and the microarray data provide a tremendous resource for further analysis of the ripening process in kiwifruit. The microarrays have generated a large number of candidate genes for further analysis including those that may contribute to changes in texture and flavour, but also changes in starch metabolism, photosynthesis, colour and defence. The transgenic lines can also be used to determine the role of ethylene during phase 1 and 2 softening in kiwifruit. The observation that ethylene can synchronize and accelerate ripening at harvest and reports that 1-MCP inhibits rapid fruit softening, strongly suggest that ethylene may also play an important role in the induction and regulation of ripening in pre-climacteric kiwifruit.

Supplementary data

Supplementary Fig. S1. Nucleotide sequence alignment of ACO genes from *A. chinensis* and *A. deliciosa*. GenBank accession numbers for ACO genes from *A. chinensis* (Ac) and from *A. deliciosa* (Ad) are HQ293204–HQ293212. Genes are shown aligned with two ACO genes previously reported in *A. deliciosa*: M97961 (pKIWIAO1) and AB003514 (KWACCOX1).

Supplementary Fig. S2. Molecular characterization of transgenic kiwifruit lines. (A) Suppression of ACO gene

expression was assessed by semi-quantitative PCR using RNA isolated from the leaves of eight *A. chinensis* ACO knockdown lines (TK) and two control lines (CK) using ACO primers RA118–RA120 (top). These primers were expected to amplify the five *ACO* genes identified in ripe fruit libraries (*ACO1–4* and *ACO6*). A constitutively expressed gene was amplified using primers RA308–RA309 (bottom) to correct for differences in the amount of RNA template used in each PCR reaction. Primer sequences are given in Supplementary Table S1. (B) Suppression of ACO protein expression was assessed by western analysis using an apple ACO3 polyclonal antibody. Proteins were extracted from *A. chinensis* ‘Hort16A’ (16A), TK4 and TK2 fruit treated with ethylene (100 $\mu\text{l l}^{-1}$, 24 h) and held at 20 °C for the time indicated. Prestained ladder = SeeBlue®Plus2 (Invitrogen); sizes in kDa. Kiwifruit ACO runs at 42 kDa as indicated by the arrow. (C) Endogenous ethylene production in *A. chinensis* ‘Hort16A’ fruit grown under commercial orchard conditions at the Plant and Food Te Puke Research Centre. Harvested fruit were treated with ethylene as described in (B). Ethylene production was monitored by gas chromatography after 48, 72, 120, and 168 h.

Supplementary Table S1. Primers used for PCR.

Supplementary Table S2. Semi-quantified volatile compound data. Volatile production was measured by GC/MS in fruit from two control lines (CK1, CK2) and two knockdown lines (TK2, TK4). Measurements were taken 144 h after treatment with exogenous ethylene. Semi-quantified data (ng g⁻¹) for individual compounds were sorted into five volatile classes (alcohols, aldehydes, ester, sulphur compounds, and ‘other’ compounds). Data were derived from two or three independent ethylene treatments with each treatment consisting of two replicates of two fruit. RI, retention index.

Supplementary Table S3. Differentially expressed genes identified from microarray analysis. Comparisons are shown for phase 3-arrested TK2 fruit (T0) treated with ethylene for 4, 12, 24, and 168 h and phase 3-arrested TK2 fruit held without ethylene treatment for 168 h and CK1 fruit at eating-ripe firmness. Included are: the EST number and corresponding GenBank accession number for each gene, the number of ESTs representing this gene in the PFR EST database, a brief description of the gene based on BLAST homology to genes in GenBank, the expression level of each gene on the microarray and a GO classification. Highlighted genes were monitored by qPCR.

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