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Characterization of the 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase multigene family of *Malus domestica* Borkh

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ABSTRACT

Two 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) genes have been cloned from RNA isolated from leaf tissue of apple (*Malus domestica* cv. Royal Gala). The genes, designated *MD-ACO2* (with an ORF of 990 bp) and *MD-ACO3* (966 bp) have been compared with a previously cloned gene of apple, *MD-ACO1* (with an ORF of 942 bp). *MD-ACO1* and *MD-ACO2* share a close nucleotide sequence identity of 93.9% in the ORF but diverge in the 3′ untranslated regions (3′-UTR) (69.5%). In contrast, *MD-ACO3* shares a lower sequence identity with both *MD-ACO1* (78.5%) and *MD-ACO2* (77.8%) in the ORF, and 68.4% (*MD-ACO1*) and 71% (*MD-ACO2*) in the 3′-UTR. Southern analysis confirmed that *MD-ACO3* is encoded by a distinct gene, but the distinction between *MD-ACO1* and *MD-ACO2* is not as definitive. Gene expression analysis has shown that *MD-ACO1* is restricted to fruit tissues, with optimal expression in ripening fruit, *MD-ACO2* expression occurs more predominantly in younger fruit tissue, with some expression in young leaf tissue, while *MD-ACO3* is expressed predominantly in young and mature leaf tissue, with less expression in young fruit tissue and least expression in ripening fruit. Protein accumulation studies using western analysis with specific antibodies raised to recombinant *MD-ACO1* and *MD-ACO3* produced in *E. coli* confirmed the accumulation of *MD-ACO1* in mature fruit, and an absence of accumulation in leaf tissue. In contrast, *MD-ACO3* accumulation occurred in younger leaf tissue, and in younger fruit tissue. Further, the expression of *MD-ACO3* and accumulation of *MD-ACO3* in leaf tissue is linked to fruit longevity. Analysis of the kinetic properties of the three apple ACOs using recombinant enzymes produced in *E. coli* revealed apparent Michaelis constants (K_m) of 89.39 μM (*MD-ACO1*), 401.03 μM (*MD-ACO2*) and 244.5 μM (*MD-ACO3*) for the substrate ACC, catalytic constants (K_{cat}) of 6.6×10^{-2} (*MD-ACO1*), 3.44×10^{-2} (*Md-ACO2*) and 9.14×10^{-2} (*MD-ACO3*) and K_{cat}/K_m (μM s^{-1}) values of 7.38×10^{-4} μM s^{-1} (*MD-ACO1*), 0.86×10^{-4} M s^{-1} (*MD-ACO2*) and 3.8×10^{-4} μM s^{-1} (*MD-ACO3*). These results show that *MD-ACO1*, *MD-ACO2* and *MD-ACO3* are differentially expressed in apple fruit and leaf tissue, an expression pattern that is supported by some variation in kinetic properties.

1. Introduction

Ethylene is a gaseous plant hormone that regulates many processes associated with plant growth and development (Abeles et al., 1992). In higher plants ethylene is synthesized *via* two committed enzyme-catalyzed steps from *S*-adenosyl-L-methionine (SAM). The first of these, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) catalyzes the cyclization of SAM to ACC and subsequently ACC oxidase (ACO) catalyzes the oxidative conversion of ACC to ethylene (Adams and Yang, 1979; Yang and Hoffman, 1984; Kende, 1993). ACS is a pyroxidal phosphate-requiring enzyme while ACO is a non-haem iron enzyme which requires Fe^{2+} and CO_2 as co-factors as well as ascorbate as a co-substrate in a reaction that proceeds in the presence of O_2 (Ververidis and John, 1991; Bouzayen et al., 1991; Dong et al., 1992a,b). In many studies published thus far, ACS is considered the rate-determining step in the committed ethylene biosynthetic pathway, and in all plant species examined in any detail thus far, ACS is comprised of a larger gene family. Indeed, in *Arabidopsis,* 12 genes have been identified in the family. In contrast, ACO has been characterised as a smaller differentially expressed multigene family in many plant species including tomato (Bouzayen et al., 1993; Barry et al., 1996; Nakatsuka et al., 1998), peach (Ruperti et al., 2001; Rasori et al., 2003), melon (Lasserre et al., 1996, 1997) and in white clover (Hunter et al., 1999; Chen and McManus, 2006).

For apple (*Malus domestica* Borkh), the first ACO gene identified was designated *MD-ACO1* [clone p*AP4*, (Ross et al., 1992); clone p*AE12*, (Dong et al., 1992a,b)]. Subsequently, three further *MD-ACO* genes have been identified (Wiersma et al., 2007). *MD-ACO1* expression and its role in fruit ripening has been a major research

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focus for many years in apple, including the analysis of the gene promoter and the phenotypes of anti-sensed ACO plants (Dong et al., 1992a,b; Ross et al., 1992; Dilley et al., 1995; Zhu et al., 1995; Bolitho et al., 1997; Atkinson et al., 1998; Tan and Bangerth, 2000; Costa et al., 2005; Cin et al., 2005; Park et al., 2006). Further, ACO activity has been extracted from apple fruit tissue and the enzyme extensively characterized both as a purified protein (Dong et al., 1992a,b; Fernández-Maculet and Yang, 1992; Kuai and Dilley, 1992; Poneleit and Dilley, 1993; Fernández-Maculet et al., 1993; Pirrung et al., 1993; Dilley et al., 1993; Dupille et al., 1993; Mizutani et al., 1995) and as a recombinant protein (Charng et al., 1997; Charng et al., 2001; Yoo et al., 2006).

The focus on *MD-ACO1* and the coded enzyme reflects an intensive research interest into the role of ethylene in fruit ripening and for apple, particularly. However, given the evidence of differential expression of ACO multigene families in many other plant species, and the realisation that a small multi-gene family is also present in apple (Wiersma et al., 2007), there are no studies on the expression of other members of the gene family or biochemical characterization. Further, ethylene plays an important role in leaf development and, as yet, there have been fewer studies that focus on the differential expression of ACO genes during leaf ontogeny (Hunter et al., 1999; Chen and McManus, 2006).

In this paper, therefore, we aim to isolate expressed ACO genes from apple leaf tissue, to examine their expression during leaf and fruit ontogeny in apple as well as to functionally express these *MD-ACO* cDNAs in *E. coli* to characterize each gene product, biochemically.

2. Results

2.1. Nucleotide and deduced amino acid sequence comparison of MD-ACO1, MD-ACO2 and MD-ACO3

RT-PCR approaches, using RNA isolated from developing, mature-green and senescent leaf tissue of apple, amplified cDNAs of *ca.* 850 bp. Subsequent sequencing revealed that two distinct ACO sequences were identified from RNA isolated from mature-green leaf tissue (designated *MD-ACO2* and *MD-ACO3*), which were also distinct from *MD-ACO1*. All three *MD-ACO* sequences were then aligned against an apple EST library maintained at the Horticultural and Food Research Institute of New Zealand (HortResearch). The relevant ESTs are now publicly available on Genbank with the accession numbers of *MD-ACO1* (X61390), *MD-ACO2* (EB14037) and *MD-ACO3* (EB151006). Further, an alignment, at the nucleotide level, of the genes identified in this study (and verified in the HortResearch EST library) with those reported by Wiersma et al. (2007) show that *MD-ACO2* most closely aligns (98%) with *MD-ACO2* (AF015787) reported in Wiersma et al (2007) an *MD-ACO3* is equivalent (99% identity) with *MD-ACO3* (AF086888) reported in Wiersma et al (2007).

Comparison of nucleotide sequences of the putative *MD-ACO* multi-gene family revealed that the coding sequences of *MD-ACO1* and *MD-ACO2* are quite highly identical (93.9%) with less identity between *MD-ACO1* and *MD-ACO3* (78.5%) and *MD-ACO2* and *MD-ACO3* (77.8%). When the 3́-UTR sequences are compared, then the degree of identity between *MD-ACO1* and *MD-ACO2* decreases (69.5%) while yet lower identities are observed between *MD-ACO1* and *MD-ACO3* (68.4%) and *MD-ACO2* and *MD-ACO3* (71%). The observation of lower identities between the 3′-UTR sequences when compared with the coding sequences of ACO multi-gene families is consistent with other studies (e.g., white clover) where greater identity was found over the coding region (>90%) when compared with the 3′-UTR (~70%) (Hunter et al., 1999). Likewise, in peach, where identity between the coding regions of *PP-ACO1* and *PP-ACO2* (77.7%) is higher than for the 5′ and 3′-UTRs of 44% and 50.4%, respectively (Ruperti et al., 2001).

Identities between the *MD-ACO* amino acid sequences broadly reflected the percentage identities observed for the nucleotide coding sequences with highest identity observed between *MD-ACO1* and *MD-ACO2* (92.04%), with less identity between *MD-ACO1* and *MD-ACO3* (80.57%) and *MD-ACO2* and *MD-ACO3* (75.16).

2.2. Evidence of a multi-gene family in apple by genomic Southern analysis

DNA isolated from leaves of apple was digested with either *Eco*RI, *Hind*III or *Xba*I, and probed with sequences comprising the 3′ region of the coding sequence and a contiguous region of the 3'-UTR to generate gene-specific probes. The Southern analysis demonstrates that the *MD-ACO3* sequence is encoded by a gene distinct from both *MD-ACO1* and *MD-ACO2*, as the hybridization patterns following restriction digests with *Eco*RI, *Hind*III and *Xba*I, clearly differ (Fig. 1). However, each one of the restriction digested fragments which hybridises with the *MD-ACO2* probe are of the same approximate size as fragments which hybridize with the *MD-ACO1* probe, although the relative intensity of many of the bands differs in each digest when probed with either *MD-ACO1* or *MD-ACO2*. An example can be seen in the *Hind*III digest, where fragments of 1.5 and 1.9 kb strongly hybridize with *MD-ACO1* compared with the one fragment of 1.9 kb which hybridizes with *MD-ACO2* (Fig. 1).

2.3. Analysis of the deduced translational sequences of each of the ACO proteins

From a deduced translation of each of the three ACO open reading frames (ORFs), amino acid sequences were aligned and compared (Fig. 2). From previous studies with recombinant protein

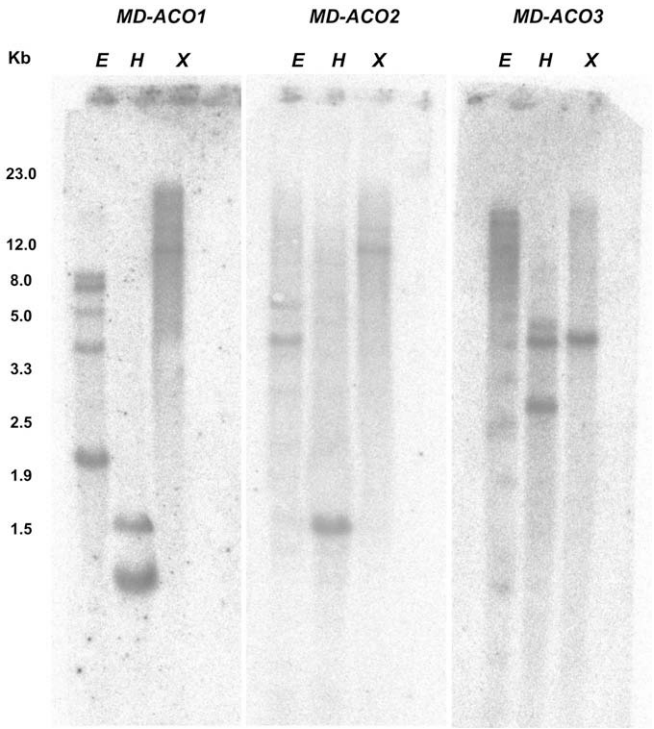

Fig. 1. Southern analysis of *Malus domestica* genomic DNA. The hybridisation pattern of ^{32}P labelled *MD-ACO1*, ^{32}P labelled *MD-ACO2*, ^{32}P labelled *MD-ACO3*, as indicated, with genomic DNA digested with *Eco*RI (E), *Hind*III (H) and *Xba*I (X). Molecular sizes are indicated.

Fig. 2. Comparison of the deduced amino acid sequences of the open reading frames of *MD-ACO1*, *MD-ACO2* and *MD-ACO3*, as indicated. Amino acids important for apple ACO activity are in bold, and numbered, and the conserved lysine and cysteine residues are italicized, and numbered.

expressed from clones *pAP4* and *pAE12* (equivalent to MD-ACO1 in this study), activity requires that some specific amino acid residues be conserved. Specifically, residues H^{177}, D^{179} and H^{234} are required to bind iron in the active site (Shaw et al., 1996; Kadyrzhanova et al., 1997; Kadyrzhanova et al., 1999), while R^{244}, S^{246} and T^{157} as binding sites for the ACC carboxyl group (Kadyrzhanova et al., 1999; Dilley et al., 2003; Seo et al., 2004). Also, the lysine residues K^{158}, K^{230} or K^{292} (K^{293} in *MD-ACO3*) are strongly implicated for ascorbate activation (Kadyrzhanova et al., 1999; Dilley et al., 2003) and residue R^{175} has been proposed to bind directly to $NaHCO_3$ (Dilley et al., 2003). Eight lysine residues [K^{72}, K^{144}, K^{158}, K^{172}, K^{199}, K^{230}, K^{292} and K^{296} (K^{293} and K^{297} in *MD-ACO3*)] observed in *pAP4* and *pAE12* are conserved in other plant species (Kadyrzhanova et al., 1999; Dilley et al., 2003), and in common with K^{158}, K^{230} or K^{292} (K^{293} in *MD-ACO3*), the other five lysine residues [K^{72}, K^{144}, K^{172}, K^{199}, K^{296} (K^{297} in *MD-ACO3*)] may also be involved in ascorbate binding. In the C-terminus, E^{297}, R^{299} and E^{301} (E^{298}, R^{300} and E^{302} in *MD-ACO3*) are important for enzyme activity, particularly R^{299} (R^{300} in *MD-ACO3*) which may be involved in the mechanism of CO_2 activation (Kadyrzhanova et al., 1999). Three conserved cysteine residues C^{28}, C^{133} and C^{165} have been observed of which the C^{28} residue only is important for catalysis (Kadyrzhanova et al., 1999; Dilley et al., 2003). In common with MD-ACO1, the two other sequences, MD-ACO2 and MD-ACO3 also contain the specific residues outlined. Further, MD-ACO3 contains a fourth cysteine residue at position 60 (C^{60}).

2.4. Confirmation of MD-ACO differential gene expression in vivo

Semi-quantitative (sq)RT-PCR analysis of *MD-ACO1*, *MD-ACO2* and *MD-ACO3* gene expression is shown as Fig. 3, and the amplification products of the expected sizes are indicated for ribosomal

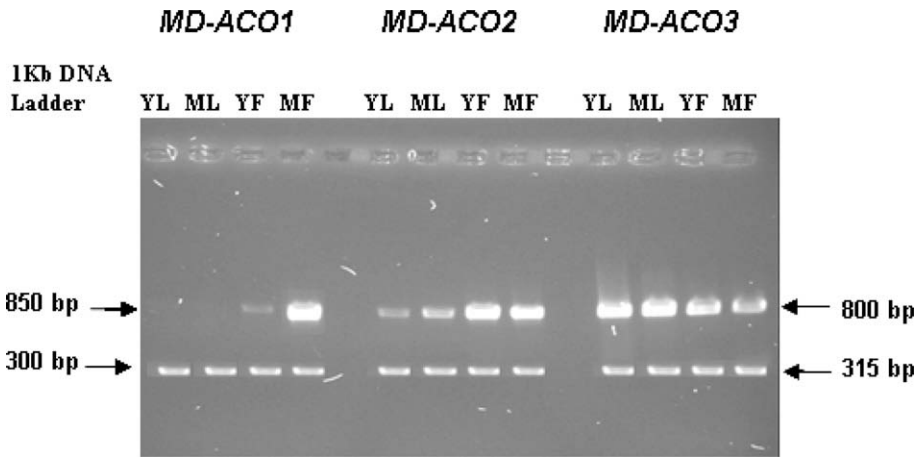

Fig. 3. Semi-quantitative (sq)RT-PCR analysis of *MD-ACO1*, *MD-ACO2* and *MD-ACO3* expression *in vivo*, as indicated. RNA was extracted from pooled young leaf (YL), mature leaf (ML), young fruit (YF) and mature fruit (MF) tissues, harvested from 15 trees, as indicated. The molecular weights (1 Kb DNA ladder, Gibco BRL) of two of the standards are indicated on the left of the figure. The approximate size of the amplification products are indicated by arrows to the right of the figure (18S ribosomal RNA *ca*. 315 bp; putative MD-ACOs *ca*. 790–800 bp). The PCR products were visualized using ethidium bromide staining.

RNA (315 bp), *MD-ACO1* (800 bp), *MD-ACO2* (790 bp) and *MD-ACO3* (800 bp). Primers specific for *MD-ACO1* amplified products from mature apple fruit tissue (17 WAFB) and less intensely from young apple fruit tissue (7 WAFB), with no visible band for either young or mature green leaf tissue. By contrast, primers specific for *MD-ACO2* amplified products from both apple leaf and fruit tissue, but with a greater intensity in the fruit tissue, whilst *MD-ACO3* gene-specific primers amplified products in all tissues, although less intensely in tissue from young fruit and less again from mature fruit. No expression, using (sq)RT-PCR, was determined in senescent leaf tissue (data not shown).

2.5. Phylogenetic analysis

A phylogenetic tree constructed from an alignment of 85 ACO sequences obtained from NCBI protein database with the deduced amino acid sequences for *MD-ACO1*, *MD-ACO2* and *MD-ACO3* over the entire open reading frame is shown as supplementary material (S1) including the full name of each entry. The localised groupings around *MD-ACO1* and *MD-ACO2* shown as Fig. 4A, and the groupings around *MD-ACO3* shown as Fig. 4B. The analysis suggests that *MD-ACO1* and *MD-ACO2* are distinct genes as these sequences have split on the tree and have diverged some time ago (Fig. 4A). The analysis also confirms that *MD-ACO3* is encoded from a gene distinct as the major splits of the tree are far removed from those of both *MD-ACO1* or *MD-ACO2* (Fig. 4B). *MD-ACO1* and *MD-ACO2* are grouped with *PP-ACO2* (peach fruit, unspecified developmental state), while *MD-ACO3* is grouped with *PP-ACO1* (from softening, ripening and wounded fruit). Interestingly, *PC-ACO1* (European pear) and *JP-AOX1* and *JP-AOX4* (Asian pear), which are expressed in immature and unripe mature fruit, associate with *MD-ACO1* and *MD-ACO2*. In contrast, *PP-AOX2A*, *PP-AOX2B* and *JP-AOX3* (Asian pear), which are expressed in ripe fruit tissue, associate with *MD-ACO3*.

2.6. Protein accumulation of MD-ACO1 and MD-ACO3 in apple tissue

Western analysis was performed on extracts of developing apple leaf, mature-green, fully expanded apple leaf, young fruit (7 WAFB) and mature fruit (17 WAFB), using antibodies raised against the gene products of *MD-ACO1* and *MD-ACO3* as recombinant proteins. The results indicate that in apple, the accumulation of ACO is tissue-specific, such that antibodies raised against MD-ACO1 recognised protein predominantly in the mature apple fruit extract (Fig. 5; lane 3). This is consistent with the gene expression studies (Fig. 3) in which *MD-ACO1* is expressed almost exclusively in the mature fruit tissue. A low level of detection of gene expression is observed in the younger fruit tissue but protein accumulation is not detected using western analysis. This may reflect the differences in sensitivity between the two techniques or may suggest that some post-transcriptional of the *MD-ACO1* gene or posttranslational modification of the MD-ACO1 protein occurs in the younger fruit tissue. In contrast, antibodies raised against MD-ACO3 recognised protein predominantly accumulating in young leaf tissue (Fig. 5; lane 4), and also in young fruit and mature leaf tissue, but to a lesser extent (Fig. 5; lanes 2 and 5). This again broadly correlated with the expression studies shown as Fig. 3, but did suggest more clearly that the accumulation of the MD-ACO3 protein is more directed towards the younger of the two tissues examined. Again, this discrepancy between the expression data and protein accumulation may reflect the differences in (sq)RT-PCR and western analysis, or could suggest that some degree of post-transcriptional or post-translational control is operating (although this was not investigated directly). No detection of MD-ACO1 or MD-ACO3 was observed in senescent leaf tissue (data not shown).

2.7. Seasonal expression of MD-ACO3 during apple leaf and fruit development

The expression of *MD-ACO3* and accumulation of MD-ACO3 in leaf tissue revealed in Figs. 3 and 5 was investigated *in vivo* over the summer and autumn using orchard-grown apple trees (Fig. 6). In the example shown, MD-ACO3 was shown, using western analysis, to accumulate in late-summer, but once fruit was strip-picked from the sampled trees (end of March), and no further accumulation was observed. Likewise, (sq)RT-PCR revealed that expression of *MD-ACO3* occurred in tissues in late summer, but again after fruit was strip-picked from the trees, then expression ceased. The detection of actin expression in these tissues post-stripping served to confirm that mRNA was successfully isolated from the senescent leaf developmental stages.

2.8. Kinetic properties of MD-ACO1, MD-ACO2 and MD-ACO3

Each of the three apple ACO cDNAs were over-expressed in *E. coli* and purified by His-tag-based affinity chromatography.

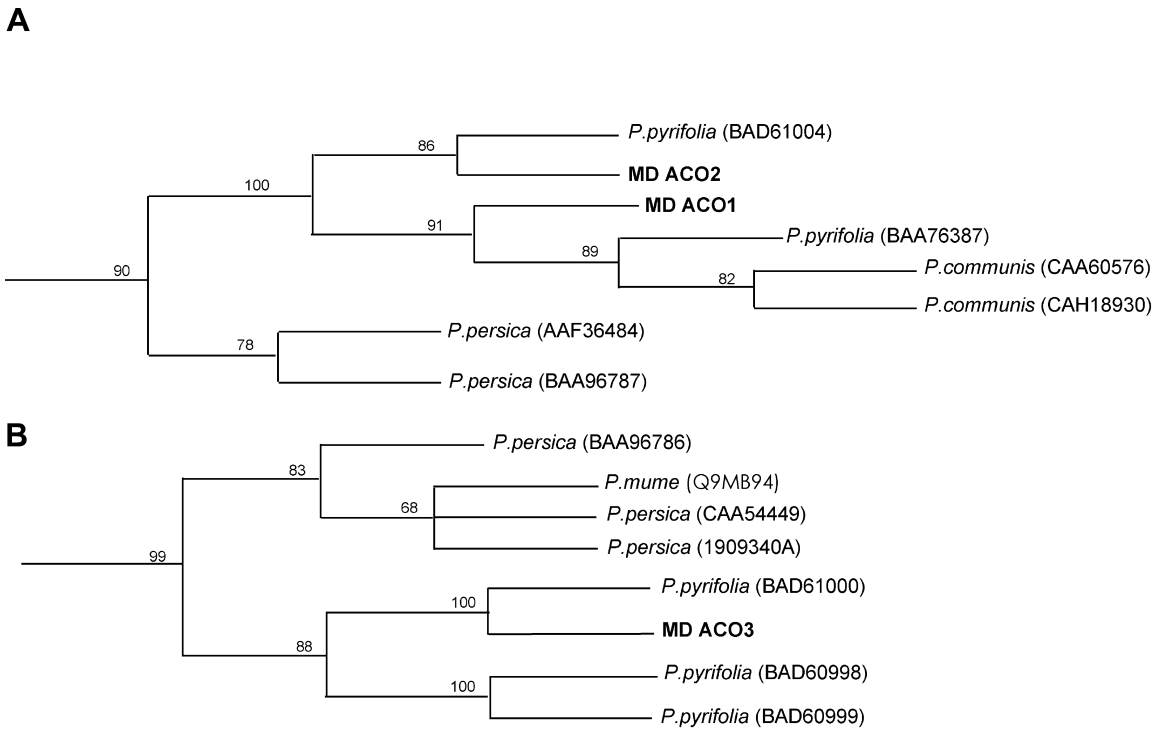

Fig. 4. Partial phylogenetic analysis of ACO amino acid sequences most closely associated with *MD-ACO1*, *MD-ACO2* (A) and *MD-ACO3* (C). The full phylogenetic tree with the full name for each entry is shown as Supplementary, Fig. 1. A. *P. pyrifolia* (BAD61004 = *PP-AOX4*) mRNA from immature fruit; *P. pyrifolia* (BAA76387 = *JP-AOX1*) mRNA from fruit; *P. communis* (CAA60576 = *PC-ACO1*) mRNA from unripe mature fruit cortical tissue; *P. communis* (CAH18930 = *ACO1*) mRNA from fruit mesocarp; *Prunus persica* (AAF36484 = *PP-ACO2*) mRNA from fruit; *P. persica* (BAA96787 = *PP-ACO2*; partial sequence) mRNA from fruit. B. *P. persica* (BAA96786 = *PP-ACO1*; partial sequence) mRNA from fruit; *Prunus mume* (Q9MB94 = *ACO1*) mRNA from fruit; *P. persica* (190934OA = *ACO*) mRNA from softening and wounded fruit; *P. persica* (CAA54449 = *ACO*) mRNA from ripening fruit; *P. pyrifolia* (BAD61000 = *JP-AOX3*) mRNA from ripe fruit; *P. pyrifolia* (BAD60998 = *PP-AOX2A*) mRNA from ripe fruit; *P. pyrifola* (BAD60999 = *PP-AOX2B*) mRNA from ripe fruit.

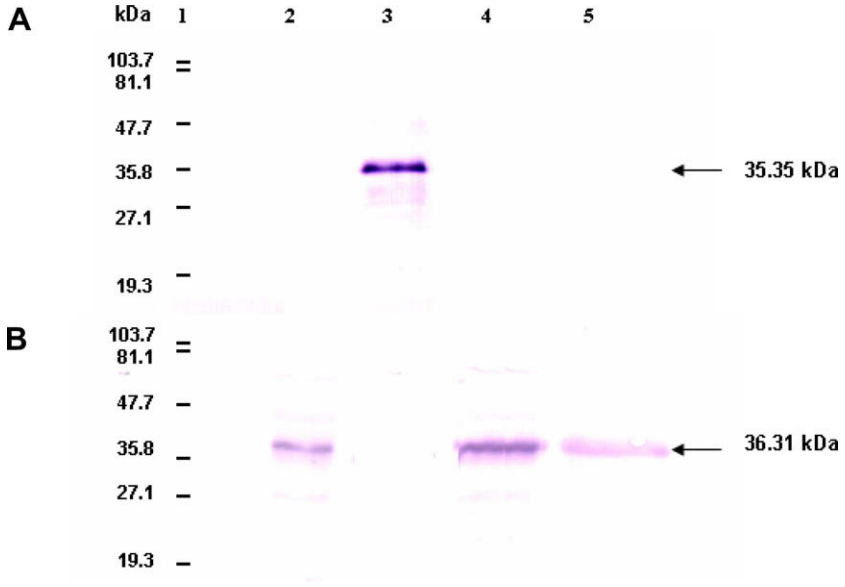

Fig. 5. Accumulation of MD-ACO1 and MD-ACO3 in various tissues of apple determined by Western blot analysis using antibodies raised against *MD-ACO1* (A) and *MD-ACO3* (B). Lane 1: Prestained low range molecular markers, lane 2: young fruit tissue (7 WAFB), lane 3: mature fruit tissue (17 WAFB), lane 4: young leaf tissue, lane 5: mature leaf tissue. The protein extracts were made from pooled tissues harvested at the developmental stages, as indicated, from 15 trees.

However, over-expression of the recombinant MD-ACOs in *E. coli* resulted in degradation of the protein as judged by fragmentation using SDS–PAGE (data not shown). Therefore, anion exchange was used to separate active, full-length enzyme from the inactive forms of the protein (as well as from any other contaminating *E. coli* proteins) and these purified recombinant enzyme preparations were used for subsequent kinetic analysis.

Initially, the pH optima of each recombinant enzyme was determined at 1 mM ACC (Table 1) after which the optimal requirements of each isoform for co-substrate and cofactors were then

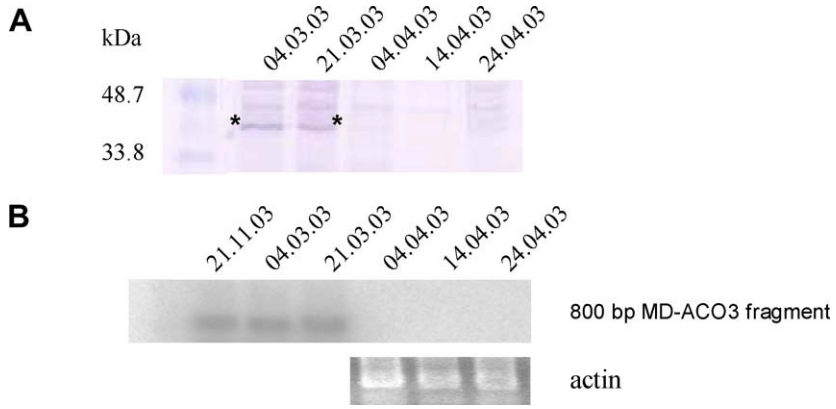

Fig. 6. (A) Changes in *MD-ACO3* protein accumulation using western analysis with antibodies raised against recombinant *MD-ACO3* on extracts (40 μg per lane) of pooled leaf tissue, harvested at 11.00 am on the dates indicated, from pre-tagged bourse shoots from 15 trees. (B) Changes in *MD-ACO3* gene expression using (sq)RT-PCR performed on RNA isolated from pooled leaf tissue, harvested at 11.00 am on the dates indicated, from pre-tagged bourse shoots from 15 trees. The PCR products were visualised using a ^{32}P labelled gene-specific *MD-ACO3* probe. The fruit was harvested on 24.03.03.

Table 1
Substrate and co-factor requirements for *MD-ACO1*, *MD-ACO2* and *MD-ACO3*, as indicated.

	MD-ACO1	*MD-ACO2*	*MD-ACO3*
pH			
Activity range	6.5–8.0[a]	6.5–8.5	6.5–8.0
Optima	7.2	7.8	7.5
Ascorbate			
Optimal concentration	30 mM	30 mM	30 mM
50% inhibition	40 mM	40 mM	40 mM
$FeSO_4 \cdot 7H_20$			
Optimal range	15–25 μM	15–25 μM	15–25 μM
Maximum activity	20 μM	15 μM	20 μM
⩾70% Inhibition	40 μM	40 μM	40 μM
$Na^+HCO_3^-$			
30–40% activity[a]	0 mM	0 mM	0 mM
Optimal range	20–40 mM	20–40 mM	10–40 mM
Maximum activity	30 mM	30 mM	30 mM
70–100% inhibition	60 mM	60 mM	60 mM

[a] Data is collected from six independent experiments.

determined at their optimal pHs and 1 mM ACC (Table 1). In terms of co-substrate and co-factor requirements, the three isoforms showed broadly similar requirements. In this study all *MD-ACO*s were maximally activated with 30 mM $NaHCO_3$, but were completely inhibited in the presence of 60 mM $NaHCO_3$, were activated by 30 mM ascorbate and were inhibited (50%) by 40 mM, and displayed an optimum for 15–25 μM $FeSO_4$.

In terms of the calculation of the Michaelis-Constant (K_m) and maxium velocity (V_{max}) of the *MD-ACO* isoforms, the activity of *MD-ACO1* approached saturation at an ACC concentration of 0.5 mM, while *MD-ACO2* and *MD-ACO3* were saturated at an ACC concentration of 1 mM (Fig. 7). The apparent K_m and V_{max} were determined from Lineweaver–Burk plots and these are summarised in Table 2. *MD-ACO2* has a lower V_{max} (12.94 nmol of ethylene $mg^{-1}\ min^{-1}$) than the V_{max} of *MD-ACO1* (15.15 nmol of ethylene $mg^{-1}\ min^{-1}$) and *MD-ACO3* (18.94 nmol of ethylene $mg^{-1}\ min^{-1}$) which are similar. These values are also consistent with the V_{max} and K_m values obtained using Eadie Hofstee regression (data not shown). Further, K_{cat} values [ethylene evolution ($mol\ s^{-1}$)/MD-ACO (mol)] (refer Table 2) revealed *MD-ACO3* (9.14×10^{-2}) to have the higher turnover rate compared to *MD-ACO1* (6.6×10^{-2}) and the very sluggish *MD-ACO2* (3.44×10^{-2}). The K_{cat}/K_m ($\mu M\ s^{-1}$) is a useful means for comparing activities between enzymes, and values of $7.38 \times 10^{-4}\ \mu M\ s^{-1}$ (*MD-ACO1*), $0.86 \times 10^{-4}\ \mu M\ s^{-1}$ (*MD-ACO2*) and $3.8 \times 10^{-4}\ \mu M\ s^{-1}$ (*MD-ACO3*) were calculated (Table 2).

In terms of thermal stability, no significant difference was found in this study between the *MD-ACO* isoforms. However, the activities of the enzyme differed significantly between each of the temperatures assayed over time (Table 3). ACO in apple is sensitive to high temperature. For example, in this study the half-life ($t½$) at 25 °C was determined to be ~29 min, at 35 °C, the $t½$ is ~13 min and at an incubation temperature of 45 °C, while the $t½$ is ~5 min 30 s at a temperature of 45 °C (Table 3).

3. Discussion

The characterisation of ACC oxidase gene expression, particularly during apple fruit ripening, has been extensively researched, such that the *MD-ACO1* gene and the *MD-ACO1* protein is perhaps the best characterized of all of the ACC oxidases. However, in common with all other plant species examined, ACO exists as a multigene family in apple, but apart from *MD-ACO1*, there are no studies reported that have examined either the expression of the other *MD-ACO* genes or the biochemical characterisation of their protein products. This is the aim of this study, and to do this, RNA was extracted from leaf tissues at the developing, mature-green and senescent leaf stage from the apple cultivar Royal Gala and degenerate primers used to isolate any ACC oxidase genes expressed during these leaf developmental stages. Two additional genes were identified, designated *MD-ACO2* [EB14037 equivalent to AF015787 in Wiersma et al. (2007)] and *MD-ACO3* [EB151005 equivalent to AF086888 in Wiersam et al. (2007)]. The expression of these genes, in addition to *MD-ACO1*, and the functional characterization of their protein products is thus described.

Southern analysis of the cv. Royal Gala genome suggests that two copies of *MD-ACO1* occur. In previous studies, the *pAP4* clone [cv. Golden Delicious; X61390; (Ross et al., 1992); designated as *MD-ACO1* in this study] was used as a RFLP marker to construct linkage maps of the progeny of a cross between the apple cultivars Prima and Fiesta (in common with Royal Gala, these cultivars have 34 somatic chromosomes and a haploid number of 17), and was found at two loci, one on chromosome 5 (*pAP4*a) and one on chromosome 10 (*pAP4*b) (Maliepaard et al., 1998). Also, Southern analysis using DNA extracted from cv. Golden Delicious, and digested with *Eco*RI, *Hind*III and *Bam*HI and then probed with *pAP4* (*MD-ACO1*) (Ross et al., 1992), revealed that two fragments hybridized with *pAP4* for each of the genomic digests. Similarly, for the Golden Delicious genomic DNA digested with *Eco*RI a fragment hybridised

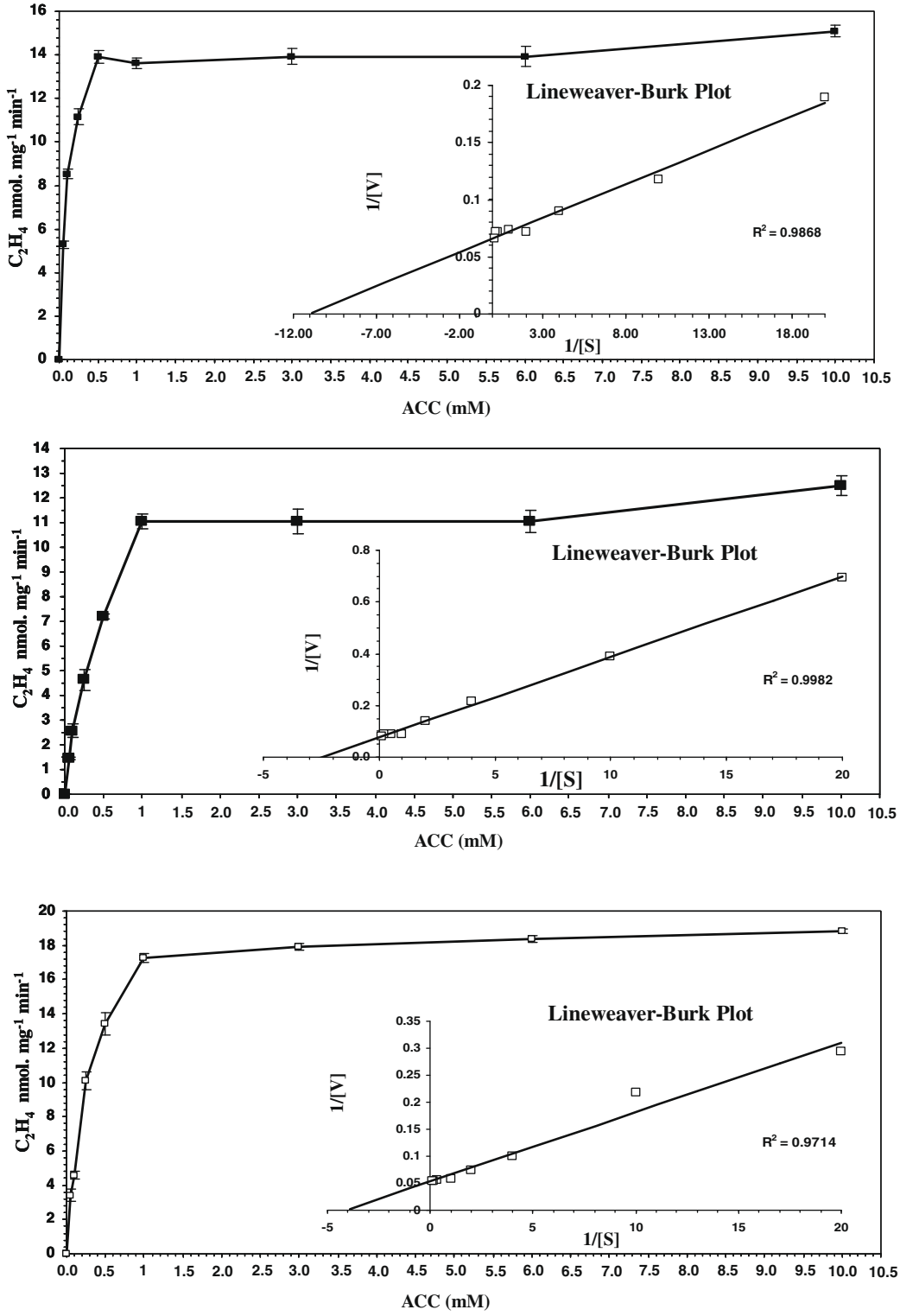

Fig. 7. Recombinant *MD-ACO1* (A), *MD-ACO2* (B) and *MD-ACO3* (C) activity as a function of ACC concentration. Each inset is the corresponding Lineweaver–Burk (double reciprocal) plot. Values and means ±SE ($n = 6$).

strongly with *pAP4* of *ca.* 2.5 kb and less intensely with a fragment of *ca.* 5.0 kb.

By contrast one copy of *MD-ACO2* appears to be present in the cv. Royal Gala genome. However, it is also interesting to observe that the restriction digested fragments which have hybridized with *MD-ACO2* are the same approximate size as fragments which have hybridized with *MD-ACO1* suggesting that *MD-ACO1* and *MD-ACO2* may either be tightly clustered on the genome and encoded by distinct genes, or they are alleles of the same gene.

From the Southern analysis performed, *MD-ACO3* is clearly a gene distinct from both *MD-ACO1* and *MD-ACO2*. Further, *MD-ACO3* is probably present in the cv. Royal Gala genome as a single copy, but the two fragments which hybridize with the probe following the *Hind*III digest also raises the possibility that two copies

Table 2
Summary of kinetic properties of *MD-ACO1*, *MD-ACO2* and *MD-ACO3*.

	MD-ACO1	*MD-ACO2*	*MD-ACO3*
K_m (μM)	89.39 ± 9.7[1]	401.03 ± 22.1	244.5 ± 14.2
V_{max} (nmol mg^{-1} min^{-1})	15.15 ± 0.73	12.94 ± 0.97	18.94 ± 0.69
K_{cat} s^{-1}	6.6×10^{-2}	3.44×10^{-2}	9.14×10^{-2}
K_{cat}/K_m (μM s^{-1})	7.38×10^{-4}	0.86×10^{-4}	3.8×10^{-4}

Table 3
Thermal stability of *Malus domestica* ACC oxidases at the incubation temperatures, as indicated.

Temperature	10 min	20 min	30 min	60 min
25 °C	15%[a,b]	30%	50%	75%
35 °C	40%	70%	80%	
45 °C	85–90%			

[a] The data is presented as a percentage of the enzyme activity lost at each temperature and at each time (min) of sampling and represents the mean of three experiments.
[b] Data is pooled for *MD-ACO1*, *MD-ACO2*, and *MD-ACO3*.

of *MD-ACO3* may be present. To determine the relationship of *MD-ACO1* and *MD-ACO2*, and to determine the copy number of *MD-ACO3* more unequivocably, Southern analysis using a greater array of restriction enzymes is required.

Expression studies with *MD-ACO1* showed that the gene is expressed in mature fruit, with negligible amounts in young fruit, and no transcripts were visible at all in leaf tissue. This is consistent with observations from many studies on *MD-ACO1* expression during fruit ripening. For example, the *pAP4* gene (*MD-ACO1*) from apple fruit was found to be up-regulated during the ripening of cvs Gala, Braeburn and Granny Smith. Expression in Gala was detected earlier than in Braeburn and Granny Smith cultivars (Atkinson et al., 1998), relative to the internal base-line levels of ethylene concentration for each of the cultivars. Further, mRNA (clone *pAE12*) encoding *MD-ACO1* accumulated to high levels over the course of the ethylene climacteric. For example, Ross et al. (1992) found *MD-ACO1* (clone *pAP4*) probes did not hybridize to young immature apple fruit tissue extract, but as ripening progressed the degree of hybridization increased. Such an increase in apple ACO mRNA transcripts corresponds with the climacteric rise in ethylene biosynthesis from the mature green stage (Dilley et al., 1995; Lelievre et al., 1997; Bleecker and Kende, 2000; Giovannoni, 2001). Finally, fragments of the *MD-ACO1* (clone *pAP4*) promoter (−1966 bp upstream of ATG) fused to the β-glucuronidase (GUS) reporter gene and transformed into tomato were found to be active only in the fruit in a ripening specific pattern (Atkinson et al., 1998).

MD-ACO1 in this study also appears to have a similar expression pattern in fruit tissue to *LE-ACO1* (Barry et al., 1996; Blume and Grierson, 1997) and to the *LE-ACO4*, both from tomato (Nakatsuka et al., 1998), as well as *PP-ACO1* from peach (Ruperti et al., 2001; Rasori et al., 2003; Moon and Callahan, 2004) and *PA-ACO1* from apricot (Mbéguié-A-Mbéguié et al., 1999). Both *LE-ACO1* and *LE-ACO4* are expressed in immature green and mature green fruit, with the abundance increasing greatly during the climacteric, particularly for *LE-ACO1* (Nakatsuka et al., 1998). In common with *MD-ACO1*, *LE-ACO4* is not detectable in leaf tissue (Nakatsuka et al., 1998), whereas *LE-ACO1* transcripts have been observed to increase (up to 27-fold) at the onset of leaf senescence (Barry et al., 1996). Given that both the tomato *LE-ACO1* and the peach *PP-ACO1* genes are expressed abundantly during the fruit climacteric and during leaf senescence, the prediction that either *MD-ACO1* or *MD-ACO2* are good candidates for the senescence associated gene (SAG) in apple leaf seems reasonable. However, from the results of this study neither *MD-ACO*s have been observed to fulfil this role in leaf tissue.

In addition to *MD-ACO1*, neither *MD-ACO2* nor *MD-ACO3* appear to be expressed in senescing leaf tissue, and we were unsuccessful at cloning any other ACO gene (using RT-PCR and degenerate primers) from this tissue. Many studies have identified at least one member of the ACO gene family as a senescence associated gene (SAG), for example *TR-ACO3* and *TR-ACO4* in white clover (Hunter et al., 1999; Chen and McManus, 2006), *LE-ACO1* in tomato (John et al., 1995) and *CP-ACO2* in papaya (Chen et al., 2003). Both *MD-ACO1* and *MD-ACO2* are expressed predominantly in mature fruit which may be associated with senescence, and *MD-ACO3* is more likely to be a photosynthetic associated gene (PAG), as it is expressed predominantly in initial bourse shoot leaf tissue. However, *MD-ACO3* is also expressed in mature green fully expanded leaf tissue, and *MD-ACO2* is also expressed in initial bourse shoot leaf tissue and mature green fully expanded leaf tissue. Therefore *MD-ACO2* may also be a PAG.

In terms of phylogenetic analysis, it is interesting to note that *MD-ACO1*, *MD-ACO2* and *MD-ACO3* most closely align with pear (*Pyrus pyrifolia* and *Pyrus communis*) ACOs. *MD-ACO2* shares the closest alignment with an ACO (*PP-AOX4*) expressed in the immature fruit of pear, and *MD-ACO1* is also associated with ACO expressed in pear fruit (*JP-AOX1* and *ACO1*) and unripe mature pear fruit (*PC-ACO1*). In contrast, *MD-ACO3* shares the closest alignment with an ACO from ripe pear fruit (*JP-AOX3*, *PP-AOX2A* and *PP-AOX2B*). Unfortunately, the literature does not appear to contain data on pear ACO expression in leaf tissue, differential expression or even temporal expression in the fruit, but rather studies have focused on the role of ethylene, cold treatment and ACO accumulation postharvest in pear fruit ripening (Lelievre et al., 1997; Gao et al., 2002; Hiwasa et al., 2003; Fonseca et al., 2004). Broadly, the peach ACO (*PP-ACO1* and *PP-ACO2*) each fall into one of two groups, with *PP-ACO1* in the MD-ACO3 grouping, and *PP-ACO2* in the *MD-ACO1* and *MD-ACO2* grouping (Fig. 4). Interestingly *PP-ACO1* resembles *MD-ACO3* as they are both expressed in young fully expanded green leaf tissue, but *PP-ACO1* also resembles *MD-ACO1* and *MD-ACO2* in that they are all expressed abundantly during the climacteric necessary for fruit ripening. However, unlike any of the *MD-ACO* genes, *PP-ACO1* is a SAG gene as it is expressed abundantly in senescing leaf tissue.

In terms of protein accumulation, the antibody raised against *MD-ACO1* recognizes protein accumulated in climacteric apple, but does not recognize ACO protein accumulated in young fruit, initial leaf tissue or senescent leaf tissue extract (Fig. 5). These results are in agreement with Dilley et al. (1993) who report that antibodies raised against MD-ACO1 (clone *pAE12*) were unable to detect protein in the preclimacteric Golden Delicious cultivar (nor was ACO activity detected). Further, no MD-ACO proteins were detected during the preclimacteric stage in the Granny Smith cultivar, using antibodies raised against either recombinant *MD-ACO1* (clone *pAE12*) or against *MD-ACO1* purified from apple (Lara and Vendrell, 2000). In contrast to *MD-ACO1*, antibodies raised against recombinant MD-ACO3 recognize protein predominantly in young leaf tissue, but also in mature-green leaf tissue, and in young fruit tissue.

These protein accumulation studies confirm the gene expression studies presented. They also confirm that, again, none of the genes under study here display expression or protein accumulation in senescent leaf tissue. As the senescence associated genes *LE-ACO1*, *PP-ACO1* and *CP-ACO2* are expressed both in mature fruit tissue and in senescing leaf tissue, it is unusual that the *MD-ACO* (transcript and protein) which increase in abundance during the climacteric (*MD-ACO1* and *MD-ACO2*) do not also show a senescence-associated increase at the onset of senescence in the leaves of apple.

The lack of expression of these particular ACO genes during leaf senescence may be significant as fruit removal appears to negatively impact on subsequent *MD-ACO3* expression and *MD-ACO3* accumulation. From other studies it is well established that fruit removal does influence leaf physiology in apple trees (Tartachnyk and Blanke, 2004) and peach trees (Nii, 1997). For example, delayed apple fruit harvest has been observed to enhance autumn leaf photosynthesis, delay chlorophyll degradation and the nitrogen content declined less rapidly when compared with the leaves from the trees where the fruit had been harvested (Tartachnyk and Blanke, 2004). Such an apparent delaying of apple leaf senescence by delaying the fruit harvest, and conversely inducing the earlier onset of senescence by removing the fruit, may underlie the regulation of *MD-ACO3*, as *MD-ACO3* is expressed (and *MD-ACO3* accumulated) prior to fruit removal, but were undetectable following fruit harvest. The timing of ACO gene expression in leaf tissue as it relates to fruit longevity has not previously been reported, but does underline the classification of *MD-ACO3* as a PAG gene. It seems likely that, in agreement with many other studies, a senescence-associated *MD-ACO* gene does exist. However, using the screening procedures reported her, no such gene was identified.

This study has also investigated the kinetic properties of three *MD-ACO* isoforms expressed in *E. coli*. ACO extracted from apple fruit tissue has been extensively characterized biochemically (Dong et al., 1992a,b; Fernández-Maculet and Yang, 1992; Kuai and Dilley, 1992; Poneleit and Dilley, 1992; Pirrung et al., 1993; Dilley et al., 1993; Dupille et al., 1993; Mizutani et al., 1995). Further, *MD-ACO1* has also been expressed both in *E. coli* (clone *pAE12*) (Charng et al., 1997; Charng et al., 2001; Yoo et al., 2006), and in yeast (clone *pAP4*) (Wilson et al., 1993). However, the current study is the first to compare the multiple members of the apple gene family.

The kinetic properties of the three *MD-ACO* isoforms do not markedly differ in terms of co-factors and co-substrate requirements. However, less $NaHCO_3$ is required for optimal enzymatic activity for *MD-ACO* purified from mature apple fruit (Dong et al., 1992a,b; Pirrung et al., 1993) when compared with the concentration required for recombinant *MD-ACO*s expressed in *E. coli* in this study (30 mM). Similarly, although ACOs exhibit an absolute requirement *in vitro* for ascorbate, the amount required for optimal activity of the *MD-ACO*s purified from mature apple fruit (1–10 mM), is again lower when compared with the amount of ascorbate required for recombinant MD-ACOs (20–30 mM). It is also interesting that increased concentrations of ascorbate have been observed to decrease the *MD-ACO* activities (in this study), which is consistent with the *LE-ACO*s expressed in yeast (Bidonde et al., 1998), *LE-ACO1* expressed in *E. coli* (Zhang et al., 1995) and in the MGI and SE1 isoforms purified from white clover leaf tissue (Gong and McManus, 2000).

In terms of thermostability, all three recombinant isoforms appear equally unstable. However, in this study following incubation at 25 °C for 60 min, the loss of enzyme activity decreased ~75%, whereas a purified ACO from Golden Delicious fruit displayed loss of activity when incubated at a temperature of 23 °C, with a *t*½ of ~2 h (Bolitho et al., 1997). Further, an ACO purified to near homogeneity from pear fruit displays a *t*½ of 60 min at an incubation temperature of 28 °C (Vioque and Castellano, 1998). Thus, both of these ACOs purified from plant tissues appear to be more stable than the recombinant *MD-ACO*s expressed in *E. coli* in this study.

Examination of kinetic constants for the three isoforms did reveal some significant differences, and which may also allude to the function of these isoforms. The K_m^{ACC} value for *MD-ACO1* is significantly lower than that calculated for *MD-ACO2* and *MD-ACO3*, although the turnover number is broadly similar. However, when the K_m^{ACC}/K_{cat} is compared, then *MD-ACO1* emerges as the preferred isoform with ACC as substrate, particularly when compared to *MD-ACO2*. The differences in K_m^{ACC} between *MD-ACO1* and *MD-ACO2* may also be significant physiologically when the likely levels of ACC *in vivo* are considered. For example, ACC levels have been shown to increase from *ca.* 1 nmol g^{-1} DW in the peel and the pulp of cv. Golden Delicious prior to the climacteric to 57.32 nmol g^{-1} DW in the peel and 47.19 nmol g^{-1} DW in the pulp during the apple fruit climacteric (Tan and Bangerth, 2000; Lara and Vendrell, 2000), and it is the isoform with the highest affinity for ACC (*MD-ACO1*) that accumulates at the developmental stage in fruit that accumulates the higher ACC level. This suggests a mechanism to ensure maximum autocatalytic ethylene production during the climacteric without ACC becoming limiting. Of further interest is that two oxidase genes *(MD-ACO2, MD-ACO3*) are expressed in pre-climacteric fruit, with the protein product of at least one (*MD-ACO3*) accumulating at this developmental stage. The expression of *MD-ACO2* is higher than *MD-ACO3* suggesting that *MD-ACO2* is the prominent isoform in pre-climacteric fruit. However, the relatively low affinity for ACC and the lower ACC levels at this developmental stage may provide a mechanism by which only low levels of ethylene are produced – the system I ethylene – a phenomenon commonly observed in pre-climacteric fruit. In our expression studies, low expression of *MD-ACO1* was also detected at the pre-climacteric stage (although no protein accumulation was detected). This low level of expression, coupled with a very low accumulation of protein, contributes to a high likelihood that significant ethylene production will not occur. In other studies where the expression of a climacteric-associated ACC oxidase gene has been examined, the expression of this gene can often be induced by exogenous ethylene at the pre-climacteric stages of fruit ripening (Tonutti et al., 1997; Whittaker et al., 1997; Liu et al., 1999), and as such may resemble *MD-ACO1* in this study (although we have not determined whether the expression of *MD-ACO1* is induced by ethylene). However, where expression of different members of the ACC oxidase have been dissected during fruit development, a more complex pattern emerges. In tomato fruit development, *LeACO1, LeACO3* and *LeACO4* are proposed to be responsible for the system 1 (pre-climacteric) ethylene, while *LeACO1* and *LeACO4* are responsible for the autocatalytic climacteric-associated system 2 ethylene production (Cara and Giovannoni, 2008). This complexity is akin to the pattern in this study where the expression of other genes, in addition to *MD-ACO1,* is apparent in young and mature apple fruit.

In leaf tissue, a burst of ethylene evolution has been observed at bud-break in many species (Osborne, 1991), with higher levels of ACC also reported in young leaf tissue, which then decrease as the leaf matures (Hunter et al., 1999). However, in mature leaf tissue, ethylene levels are generally lower (*ca.* 0.5–2.0 nL g^{-1} FW) before increasing again during leaf senescence (Hunter et al., 1999). It is likely, therefore, that the accumulation of *MD-ACO3*, with a higher K_m (lower affinity for ACC), coupled with the lower levels of ACC may result in a generally lower rate of ethylene production, generally indicative of mature leaves (although it should be noted that neither ACC nor the rate of ethylene evolution has been measured from these leaf tissues).

4. Conclusions

Two further ACO genes, designated *MD-ACO2* and *MD-ACO3,* have been isolated and the expression and accumulation of the protein products has been elucidated in addition to the well characterised fruit-ripening associated gene, *MD-ACO1.* The expression of *MD-ACO2* and *MD-ACO3* also occurs in fruit tissue, with *MD-ACO3* predominantly expressed in the pre-climacteric fruit. In leaf tissue, the expression of *MD-ACO3* is again associated with younger leaf tissues, and ceases with fruit harvest. In this study, no leaf

senescence-associated ACO has been identified. Kinetic analysis of the three enzymes expressed in *E. coli*, has identified differences in biochemical properties which is postulated to provide a further tier of control in terms of the regulation of ethylene biosynthetic during fruit maturation and leaf development.

5. Experimental

5.1. Plant material

Leaves and fruit at the appropriate developmental stage were harvested from randomly pre-tagged bourse shoots, at the appropriate time of day (11.00 am) and time of year, from fifteen *Malus domestica* Borkh. cv. Royal Gala (10 year old) trees which had been grafted onto M9 dwarfing rootstock and grown at latitude 30° 40′; longitude 176° 53′ at the Hawkes Bay Research Centre of HortResearch, Havelock North, New Zealand. Harvested leaf tissue was frozen in liquid nitrogen immediately, and stored at −80 °C until use. Total chlorophyll content was used to determine leaf development. Young leaf tissue was designated the developmental stage when chlorophyll content was increasing (typically October–November), the mature-green stage occurred when the chlorophyll levels had stabilised as a maxima (typically December–March), while senescent leaves were harvested when the chlorophyll levels were declining (typically April–June). Fruit tissues were harvested as young fruit (mean diameter 2 cm; 7 WAFB) and mature (at ripening; 17 WAFB). Mature fruit were picked at ripening as determined by the detection of maximum ethylene evolution (the climacteric).

5.2. RNA Isolation

Total RNA was isolated using a hot borate method adapted by Hunter and Reid (2001). Briefly, leaf tissue was ground to a fine powder in liquid nitrogen and the powder extracted in a hot (80 °C) borate buffer (0.2 M sodium borate, pH 9.0, containing 30 mM EDTA, 1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 10 mM DTT, 2% (w/v) polyvinylpyrollidone (PVP-40) and 1% (v/v) octylphenylpolyethylene glycol (IGEPAL)). After vortexing for 30 s, proteinase K was added and the mixture incubated, at 42 °C, with shaking, for 1.0 h. A volume (0.08) of 2 M KCl was then added and the extraction continued on ice, with shaking, for 45 min. Cellular debris and denatured proteins were collected by centrifugation at 26,000g for 20 min at 4 °C, and then the supernatant was adjusted to a final concentration of 2 M LiCl and the RNA precipitated overnight at 4 °C. The RNA was collected by centrifugation at 26,000g for 20 min at 4 °C, the pellet resuspended in sterile water at room temperature, an equal volume of chloroform: isoamyl alcohol (24:1) added, the aqueous and organic phases mixed by vortexing and then separated by centrifugation at 20,800*g* for 5 min at room temperature. The upper aqueous phase was collected, the RNA precipitated with ethanol and then resuspended in a small volume of water, until required.

5.3. Isolation and identification of MD-ACOs

Total RNA (5 μg) was subjected to reverse transcriptase (RT) treatment using an oligo $d(T)_{15}$ primer, with putative ACO cDNAs amplified by the polymerase chain reaction (PCR) using initially, degenerate oligonucleotide primers known to bind to highly conserved regions within ACO genes. Nested PCR primers were used to amplify ACO sequences in two rounds of PCR using ACOF1 and ACOR1 as first round primers and E101 and E102 as the second round primers (Table 4). The PCR products were cloned into the pGEM®-T Easy Vector system (Promega) using the manufacturer's instruction and plasmids with ACO inserts were sequenced with an ABI Prism DNA sequencer using standard procedures. Alignment of the nucleotide sequences were analyzed using MT Navigator 1.0.2b3 (Applied Biosystems Inc.), Sequencher™ 4.2 (Genes Codes Corp., Ann Arbor, MI, USA) and Gene Computer Group (GCG version 11.0) software programmes. Interrogation of both the National Centre for Biotechnology Information (NCBI) nucleotide comparison using the Basic Logical Alignment Search Tool (BLAST) of the Genbank database, and searches of the Expressed Sequence Tag (EST) library for apple at HortResearch were undertaken. Full-length cDNAs complementary to each gene were then obtained from HortResearch with the GenBank accession numbers of X61390 (*MD-ACO1*), EB14037 (*MD-ACO2*) and EB151006 (*MD-ACO3*).

Table 4
Primer sequences used in the study.

Oligo name	Primer sequence (5′–3′)
ACOF1	GT GAATTC GAY GCN TGY SAN AAY TGG GG
ECOR1	TCG TCTAGA TC RAA NCK MGG YTC YTT
E101	GTGAATTC GCN TGY GAR AAY TGG GGH TT
E102	TCG TCTAGA GYT CYT TNG CYT GRA AYT T
PX-MD1F	GAC GAC CAT ATG GCG ACT TTC CCA GTT GTT G
PX-MD1R	GAC GAC CTC GAG TCA GGC AGT TGC AAC AGG
PX-MD2F	GAC GAC CAT ATG GCA ACT TTC CCA GTT GTT G
PX-MD2R	GAC GAC CTC GAG TCA ATT TTG ATT TCT TTT TGT C
PX-MD3F	GAC GAC CAT ATG GAG AAC TTC CCA GTT ATC AAC C
PX-MD3R	GAC GAC CTC GAG TCA AGC AGT ACT TAT AAC TGG ACC
PBMF	GAY TAC ATG AAG CTS TAY KCT GG
PBM2F	GGA ACC CGA CAA AAA G
SQ-MD1F	GAA GAG TAC AGG AAG ACC ATG AAG
SQ-MD2F	GAA GAT TAC AGG AAG ACC ATG AAG
SQ-MD3F	GAT GAG TAC AGG AAT GTG ATG AAG
UTR-MD1R	TTG TTG AGC AAA CAT TTG
UTR-MD2R	CTA ATA ATT TTT ATT TTA TAT AGA TAG ATT TCA AC
UTR-MD3R	CAC ATT GGT TAC TTT CTA CAA CG
ActinF	GCG AAT TCT TCA CCA CYA CHG CYG ARC G
ActinR	GCG GAT CCC CRA TCC ARA CAC TGT AYT TTC C

5.4. Southern Analysis

Genomic DNA was extracted from young expanding apple leaves using a modified method of Junghans and Metziaff (1990) and Keb-Llanes et al. (2002). Aliquots containing 25 μg of DNA were digested to completion with *Eco*RI, *Hind*III and *Xba*I (Roche), and fractionated on a 0.8% (w/v) agarose gel. The DNA was transferred to a Hybond™-N^+ membrane (Amersham) by downward capillary transfer in 10 x SSC at pH 7, and probed with cDNA fragments from either ^{32}P-labelled *MD-ACO1*, ^{32}P-labelled *MD-ACO2* or ^{32}P-labelled *MD-ACO3*. To generate gene-specific probes, the following primer sets were used: PBMF/UTR-MD1R (*MD-ACO1*), PBM2F/UTR-MD2R (*MD-ACO2*) and PBMF/UTR-MD3R (*MD-ACO3*) (Table 4). Membrane hybridization and washing was carried out using the method of Church and Gilbert (1984), with hybridization at 65 °C overnight, and the final stringent wash with 0.1× SSPE at 65 °C.

5.5. Expression studies using semi-quantitative (sq)RT-PCR

Reverse transcription of RNA was carried out using total RNA (5 μg) and random primers (10 μM) in a total volume of 10 μL, the mixture incubated for 3 min at 70 °C, quenched on ice for 2 min and collected by a brief pulse centrifugation at 4 °C. On ice, 4 μL of 5× Expand reverse transcriptase buffer (Roche), 2 μL of DTT, 2 μL of dNTP mix and 0.5 μL of RNase inhibitor were added, the contents mixed and pulse centrifuged briefly before incubating at 42 °C for 2 min. After which, 1 μL of Expand reverse transcrip-

tase (Roche) was added and the reaction incubated for 1 h at 42 °C. For PCR, each reaction contained 2 μL of the appropriate ACO or β-actin primers, 4 μL of a 1:4 ratio of 18S rRNA primers to competimers mix, 25 μL of Mastermix (Promega) in a total volume of 45 μL. Typically, 5 μL of the appropriate cDNA (the RT-reaction description described previously) was added and typical parameters for PCR were, typically, one cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a final incubation step at 72 °C for 5 min. To amplify each gene specifically, and to avoid any genomic DNA contamination, exon spanning primers were designed as follows: SQ-MD1F/UTR-MD1R (*MD-ACO1*), SQ-MD2F/UTR-MD2R (*MD-ACO2*) and SQ-MD3F/UTR-MD3R (*MD-ACO3*) (Table 4). To visual PCR product accumulation using radiolabelled probes, the DNA fragments generated by PCR were transferred to Hybond™-N$^+$ nylon membrane by the downward capillary transfer method, except the gel was not depurinated, denatured or neutralised before transfer, and the transfer time was 5 h. The membrane was probed with ^{32}P-labelled *MD-ACO1*, ^{32}P-labelled *MD-ACO2* or ^{32}P-labelled *MD-ACO3* generated using the same primer sets described for Southern analysis.

5.6. Expression and purification of recombinant proteins

PCR products, corresponding to each of the *MD-ACO* genes were generated using the following primer sets: PX-MD1F/PX-MD1R (*MD-ACO1*), PX-MD2F/PX-MD2R (*MD-ACO2*) and PX-MD3F/PX-MD3R (*MD-ACO3*) (Table 4). The PCR products were cloned into pGEM®-T Easy Vector system, digested with the appropriate restriction enzymes and directionally ligated into the corresponding sites of the pProEX-1 (Gibco-BRL) expression vector system. The resulting plasmid was introduced into *E. coli* (BL-21) competent cells and the cells grown in LB broth with 100 mg/mL ampicillin, initially at 37 °C until the OD_{600} of the culture reach *ca.* 0.6, and then the temperature was shifted to 20 °C for 15 min before adding ITPG to a final concentration of 0.6 mM. The culture was then incubated at 20 °C for approximately 16 h before the cells were harvested by centrifugation at 3000 *g* for 20 min at 4 °C, the supernatant removed and the cells resuspended in Lysis Buffer A (containing 50 mM sodium phosphate buffer, pH 8.5, containing 300 mM NaCl, 10 mM imidazole and 10% (v/v) glycerol). Following cell disruption using a Virsonic digital 475 Ultrosomic cell disrupter (The VirTis Company), the debris was removed by centrifugation at 12,000 *g* for 20 min at 4 °C. The His-tagged recombinant ACO proteins in the supernatant were purified by affinity chromatography using nickel nitrilotriacetic acid resin (Ni-NTA). To do this, the column was first pre-equilibrated with 8–10 volumes of Buffer A, the supernatant gently loaded onto the column, the column washed with ten volumes of Buffer A, two volumes of Buffer B (50 mM sodium phosphate buffer, pH 8.5, containing 300 mM NaCl, 20 mM imidazole and 10% (v/v) glycerol) and the His-tagged ACO fusion protein then eluted with ten volumes of Buffer C (50 mM sodium phosphate buffer, pH 8.5, containing 300 mM NaCl, 250 mM imidazole and 10% (v/v) glycerol). The eluate fractions containing proteins of interest were the pooled and exchanged into FPLC Buffer A (25 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 15 mM sodium ascorbate and 10 μM PA using Sephadex® G-25 resin (Pharmacia Biotech). In order to retain ACO activity, all steps necessary for the purification of the recombinant proteins were carried out within the same day and the extract stored at −20 °C overnight for further purification the next day. For FPLC, a Mono-Q prepacked HR 5/5 strong anion column (Amersham/Pharmacia Biotech) was equilibrated with at least 5 volumes of buffer A before sample loading and proteins were eluted within an linear salt gradient of 100% buffer A: 0% buffer B (25 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 15 mM sodium ascorbate, 10 μM PA and 1.0 M NaCl) to 0% buffer A: 100% buffer B. Fractions were collected on ice and assessed for the purity of recombinant ACO using SDS–PAGE and western analysis. Fractions of interest were stored at −20 °C prior to use for antibody production or for ACO assays. The protein content of extracts and column fractions, as appropriate was determined using the the method of Bradford (1979), and SDS–PAGE performed according to Laemmli (1970).

5.7. Protein extraction from apple tissue

ACO was extracted from apple leaf and fruit tissue using a procedure, based on that of Britsch and Grisebach (1986) for the extraction of flavanone-3-hydroxylase and modified by Fernández-Maculet and Yang (1992) for ACO extraction from the fruits of apple. Leaf tissue (free from the midrib) or apple fruit tissue was ground under liquid nitrogen to a fine powder, extraction buffer (100 mM Tris-HCl, pH 7.5, containing 30 mM sodium-ascorbate, 10% (v/v) glycerol, 4 mM DTT, 1.0% (v/v) Triton X-100) was added in a 3:1 (v/w) ratio to the ground frozen powder, and the mixture vortexed vigorously for 2 to 3 min before centrifugation at 20,800*g* for 20 min at 4 °C. The supernatant (crude extract) was then used for further experiments.

5.8. Production of primary polyclonal antibodies and western analysis

Polyclonal antibodies were raised, in New Zealand white rabbits, against affinity and anion exchange column chromatography His-tagged recombinant *MD-ACO* proteins. The primary immunisation comprised 400 μg of fusion protein emulsified with complete Freund's adjuvant, followed by three boosts, at 4–5 week intervals, with 300 μg of fusion protein emulsified with Freund's incomplete adjuvant. For western analysis, proteins separated by SDS–PAGE were electrophoretically transferred to polyvinylidine difluoride membrane (PVDF; Perkin Elmer™, Polyscreen®) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) in 25 mM Tris, 190 mM glycine and 10% (v/v) methanol, at pH 8.3. Antibody incubation and development of antibody recognition was carried out using standard procedures (Hunter et al., 1999).

5.9. ACC oxidase assays

ACO activity was measured essentially according to the method described by Ververidis and John (1991). The standard reaction mixture comprised 50 mM phosphate buffer, pH 7.5), containing 10% (v/v) glycerol, 1 mM ACC, 2 mM DTT, 30 mM sodium ascorbate, 20 μM $FeSO_4$ and 30 mM $NaHCO_3$. To start the reaction, 0.2 mL enzyme samples were pipetted into 4.5 mL capacity vacutainer tubes (Becton Dickinson) containing the reaction mixture (0.8 mL), pre-equilibrated at 30 °C, and the reactions incubated, with shaking, at 180 rpm for 20 min at 30 °C. After this time, 1 mL gas samples were removed, and the ethylene content determined using a Model GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan) fitted with a flame ionization detector. For ethylene analysis, a 2.5 × 3 mm I.D. glass column prepacked with Porapak-Q with a mesh size of 80/100 (Alltech Associates Inc., Deerfield, Il., USA) was used, with nitrogen as the carrier gas with a flow rate of 50 mL min^{-1} and the injector/detector temperatures were set at 85 and 150 °C, respectively. To determine pH optima, a range of pH values were constructed using a standard 100 mM triphosphate activity buffer adjusted to the appropriate pH with phosphoric acid. ACO assays were then performed as described previously. The optimal requirements for co-substrate and cofactors were determined at the optimal pH for each ACO assessed, and at saturating ACC concentrations. The apparent K_m and V_{max} values of each of the ACO isoforms was determined by triplicate measurements of initial velocity at different ACC concentrations, at the optimal pH for each isoform, with rate constants calculated using

ENZYPLOT (Walker,1997) and ENZFITTER version 2.0 (BioSoft, Cambridge, UK). The K_{cat} was determined from the equation: product (mol s^{-1})/enzyme (mol). To assess the thermal stability of each isoform, aliquots of each recombinant protein were incubated at either 25, 35 or 45 °C, as appropriate and aliquots removed at 10, 20, 30, 60, 90, 120 and 180 min intervals and ACO activity determined, as described.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.01.002.

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