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Characterisation of Mal d 1-related genes in *Malus*

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Abstract

It has been suggested that there are at least 15 Mal d 1-related (PR10) genes in one genotype of apple (*Malus* × *domestica* Borkh.). We sequenced cDNA libraries of cultivar 'Royal Gala' and identified 12 members of the Mal d 1 family, including the previously reported Mal d 1b and Mal d 1d, an allelic variant of the previously reported Mal d 1a. Eight Mal d 1 gene products were expressed in tree-ripened fruit, in either the cortex or the skin, and most of these were also expressed in leaves in response to challenge with *Venturia inaequalis*—a fungal disease of apple. Mal d 1 gene products were identified from a large number of different tissues. Degree of ripeness as measured by standard parameters was shown not to predict either the amount of protein able to bind to a specific monoclonal antibody 5H8, previously shown to bind to an allergenic epitope in Mal d 1b and a/d, or the amount of Mal d 1 mRNA present. Mal d 1d and Mal d 1b were the most highly expressed isoforms in 'Royal Gala', particularly in the skin of fruit, and these isoforms were also predominant in other cultivars and species of apple. Genotypes, however, differed in relative predominance of Mal d 1b and Mal d 1d. The predominantly expressed Mal d 1 genes in ripe apple fruit were translated *in vivo* into proteins and proteins binding to the antibody were found in all cultivars and species examined. New Mal d 1 proteins were identified that bound to the 5H8 antibody. At least two new subfamilies have been identified, and while some structural differences are predicted between groups of isoforms, the P-loop motif is identical in all except two isoforms. A role in intracellular signalling in plants is suggested and *in vitro* expression of the isoforms should help in assessing their relative roles in disease, allergic responses, senescence and nucleotide-, cytokinin- and brassinosteroid-binding.

Abbreviations: bp, base pairs; BSA, bovine serum albumin; CHAPS, 3(3-[3-chloramidopropyl] dimethyl-ammonio)-1 propanesulfonate; 2-D, two-dimensional; 3-D, three-dimensional; DAFB, days after full bloom; DTT, dithiothreitol; ESI, electrospray ionisation; EST, expressed sequence tags; FW, fresh weight, HPLC, high performance liquid chromatograph; IPG, immobilised pH gradient; kD, kilodaltons; LC-MS, liquid chromatograph-mass spectrometer; PAGE, polyacrylamide gel electrophoresis; NBT/BCIP, a precipitating alkaline phosphatase substrate; PCR, polymerase chain reaction; PDA, piperazine diacrylamide; PEG, polyethylene glycol; pI, isoelectric point; PR, pathogenesis related; PVDF, polyvinylidene fluoride; PVPP, polyvinyl polypyrrolidone; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulphate; SSC, salt, sodium citrate; TBS, Tris-buffered saline; UV, ultra violet; v/v, volume per volume; w/v, weight per volume

Introduction

The percentage of the world's population suffering from Type I allergic reactions has shown a dramatic increase in recent years (Knox and Suphioglu, 1996). One of the main causes of such allergies is pollen from the white birch (*Betula verrucosa* Ehrh.). A 17 kD protein, Bet v 1, expressed in the pollen was shown to cause allergen-specific IgE binding and is responsible for more than 95% of the allergies to birch pollen (Breiteneder *et al*., 1989). An allergic reaction to apples that consists mainly of oral itching and swelling is very common in people who are also allergic to birch pollen (Lahti *et al.,* 1980; Dreborg, 1988; Ebner *et al*., 1991; Vieths *et al*., 2002). Immunoblotting studies show sharing of allergenic epitopes leading to the cross-reactivity of IgE antibodies (van Ree and Aalberse, 1993; Fahlbusch *et al.,* 1995).

An 18 kD protein, Mal d 1, was identified as a major allergen associated with Type 1 allergies in apple. N-terminal sequencing of Mal d 1 showed that the sequence shared 62% (over 25 amino acids; Vieths *et al*., 1994a) and 67.6% identity (over 37 amino acids; Fahlbusch *et al*., 1995) to Bet v 1. Furthermore, antibodies to Bet v 1 also cross-reacted with this protein (Ebner *et al*., 1991, 1996; Vieths *et al*., 1994b). Subsequently, two cDNAs coding for Mal d 1 were isolated by PCR from apple. Their predicted amino acid sequences were shown to have either 63% (GenBank Accession X83672, Schöning *et al*., 1996; Mal d 1a, Son *et al*., 1999) or 64.5% identity (GenBank Accession Z48969, Vanek-Krebitz *et al*., 1995; Mal d 1c, Son *et al.,* 1999) to Bet v 1. In our laboratory, another cDNA sequence (AP15, GenBank Accession L42952, Atkinson *et al*., 1996) was isolated from an apple cDNA library that coded for a further Mal d 1 isoform (named Mal d 1b by Son *et al*., 1999). Northern hybridisation analysis indicated that expression of this gene and its paralogues increased during fruit ripening (Atkinson *et al*., 1996).

There is some evidence that allergenicity to Mal d 1 is apple cultivar-dependent, resulting from differences in the quantity of the allergenic protein in each cultivar (Vieths *et al*., 1994b; Son *et al*., 1999). This may be due to differences in physiological background of the apple as suggested in experiments comparing storage and harvest times (Vieths *et al*., 1993; Hsieh *et al*., 1995). However, both Bet v 1 and Mal d 1 are members of large families consisting of at least 20 (Swoboda *et al*., 1995b) and 15 (Atkinson *et al*., 1996) isoforms, respectively. Differences in allergenicity may therefore be due either to minor sequence variations between the isoforms, leading to variation in IgE binding, or to differences in expression levels in the tissue tested. Variable expression has been demonstrated for 9 of the Bet v 1 isoforms (Ferreira *et al*., 1996). Furthermore, Ferreira *et al*. (1998) introduced point mutations at critical positions in the sequence of Bet v 1 and demonstrated reduced IgE binding. Similar results were found when mutations were introduced into Pru av 1 (Scheurer *et al*., 1999; Neudecker *et al*., 2003), and into the apple Mal d 1a and b isoforms (Son *et al*., 1999). Allergic patients have been shown to respond more strongly to apple skin than other fruit tissues (Fernández-Rivas and Cuevas, 1999), suggesting that expression of some isoforms might predominate in skin. Non-Mal d 1-like allergenic proteins have also been demonstrated in apple (relevant references are found at http://www.allergen.org) and some of these may also predominate in apple skin.

Many allergens are also pathogenesis-related (PR) proteins. Both Bet v 1 and Mal d 1 have been classified as belonging to the PR10 class of PR proteins because of their high homology to these proteins (Breiteneder *et al*., 1989; Matton and Brisson, 1989; Swoboda *et al*., 1996; Pühringer *et al*., 2000; Liu and Ekramoddoullah, 2003). The genes for these two allergens and PR10 proteins share the same intron positions (Hoffmann-Sommergruber *et al*., 1997). In birch, the PR10 protein family is expressed constitutively in the pollen, or induced by pathogens in cell suspension cultures and leaves (Swoboda *et al*., 1994, 1995a). In apple, the promoter for the gene encoding the Mal d 1b isoform was not only active in fruit and senescing leaves but was also stress- and pathogen-inducible (Pühringer *et al*., 2000).

The PR10 class of pathogenesis-related proteins have been shown to respond to a range of environmental signals (Atkinson *et al*., 1996; Walter *et al*., 1996; Poupard *et al*., 1998; Utriainen *et al*., 1998; Ekramoddoullah *et al*., 2000; Yu *et al*., 2000; Liu and Ekramoddoullah, 2003). They have also been shown to code for a protein with ribonuclease activity (Bufe *et al*., 1996; Swoboda *et al*., 1996; Bantignies *et al*., 2000) and cytokinin-

(Fujimoto *et al*., 1998; Gonneau *et al*., 2001; Mogensen *et al*., 2002), steroid- (Neudecker *et al*., 2001; Marković-Housley *et al*., 2003) or DNA- (Gajhede *et al*., 1996) binding activity. More recently a Mal d 1 homologue in St Johns Wort has been shown to be also involved in the formation of hypericin, a sterol derivative that has medical value (Bais *et al.,* 2003). The recent isolation of an interacting protein MdAP is the first clear evidence of a possible role for Mal d 1 proteins in signalling (Pühringer *et al.,* 2003). It is intriguing that no clear function has yet been defined for this class of proteins that has such far-reaching effects and is present in so many species.

The presence of many isoforms when sequencing ESTs in one genotype of apple led us to ask if there were differences in expression of these between apple tissues and in response to disease challenge. Some earlier experiments (Vieths *et al*., 1993; Vieths *et al*., 1994b; Hsieh *et al*., 1995) have indicated variation in levels of allergenic Mal d 1 according to apparent physiological state or by genotype. These studies gave no clear definitions of ripeness and harvest parameters so it has been difficult to interpret their data with respect to apple metabolic processes. We, therefore, used sixteen cultivars of *Malus* × *domestica* and 6 other species of apple, namely the putative ancestral apple species *M. baccata*, *M. sieversii* and *M. sylvestris* (Noiton and Alspach, 1996; Oraguzie *et al*., 2001) as well as *M. hupehensis*, *M. ioensis* and *M. niedzwetzkyana*, to compare Mal d 1-related expression during fruit ripening at both the mRNA and protein level (using a Mab that recognises an allergenic epitope in apple) to assess how much impact ripeness might have when comparing different genotypes for allergenicity and how many of the isoforms might be involved in ripening processes.

Materials and methods

Apple cultivars and species

The ripening fruit of 16 apple (*Malus* × *domestica* Borkh.) cultivars and six additional *Malus* species growing at the Havelock North Research Orchard, New Zealand, were selected for analysis. The apple cultivars were selected for their close relationship to the cultivars 'Golden Delicious' or 'Granny Smith'—both of which have been shown to have high levels of Mal d 1-like proteins and to cause strong allergic responses in some people (Son *et al*., 1999). Cultivars used (and their orchard reference) were: 'Akane' (Block 28), 'Baujade' (Block CC12), 'Braeburn' (Block CC20/9), 'Cortland' (Block 24), 'Cox's Orange Pippin' Bell (Block CC6/39), 'Fiesta' (Block CC26/9), 'Fuji' (Block CC20/12), 'Golden Delicious' Smoothie (Block 17), 'Goldsmith' (Block CC8/31), 'Granny Smith' (M45; Block 17), 'Jonagold' (Block CC14/16), 'Jonathan' (Block 30/966), 'McIntosh' (Block 13), 'Pacific Rose' (Block CC35/12), 'Pink Lady' (Block CC25/17) and 'Sir Prize' (Block 28). Tissue samples of 'Royal Gala' were obtained from the same orchard in a different season and were used to create EST libraries. 'Royal Gala' leaves infected with *Venturia inaequalis* were from seedlings grown in the laboratory. 'Akane', 'Jonagold' and 'Sir Prize' have 'Jonathan' in their parentage, 'Goldsmith' and 'Baujade' have 'Granny Smith' in their ancestry, and 'Royal Gala', 'Jonagold', 'Sir Prize', 'Goldsmith', 'Baujade' and 'Pink Lady' have 'Golden Delicious' in their ancestry (see Noiton and Alspach, 1996). *Malus* species were *M. baccata* L. Borkh. var *gracilis* (Siberian crab apple; Block 23/656), *M. hupehensis* (Pampan.) Rehder (Chinese crab apple; Block 30), *M. ioensis* (Wood) Britton (Prairie crab apple; Block 23), *M. niedzwetzkyana* Dieck**.** (Block 23/614), *M. sieversii* (Ledeb.) M. Roem. (ex-Kazakhstan; Block 2/17, Pop94) and *M. sylvestris* (L.) Mill. (European crab apple; Block 10/46).

Analysis of ethylene concentration and background colour

Excess numbers of unripe fruit from the 16 apple cultivars and the six other *Malus* species were harvested from February to mid-April 1999, and couriered overnight to the Mt Albert Research Centre, Auckland. Fruit were individually weighed, and for each fruit the concentration of ethylene was measured on 1 ml of gas from the internal cavity by gas chromatography (HP5890 Series II fitted with a flame ionization detector) and the background colour of the skin was measured as the a* value of the CIELAB L*, a*, b* scale using a colour reflectance meter (Minolta CR-300). For each cultivar and *Malus* species, three replicate sets of sliced whole fruit without the

core that had low (see Figure 2) ethylene concentrations (T_0) were frozen in liquid nitrogen and stored at −80 °C. The remaining fruit were then held at 20 °C. The ethylene concentration and background colour of the remaining fruit were measured weekly as the fruit ripened. Two further three replicate sets of fruit of each cultivar or species (T_1 and T_2) were frozen and stored as described above, based on changes in the internal ethylene concentration. Fruit comprising each replicate were sampled on the same date, i.e., same date and same ethylene concentration.

In another experiment, fruit from 'Royal Gala', transgenic 'Royal Gala' with reduced ethylene production due to down-regulation of ACC oxidase (J.-L. Yao, personal communication) and transgenic 'Royal Gala' with down-regulated ACC oxidase that had been treated with ethylene were compared. The fruit were harvested on 10 February and stored at 4 °C until 4 May (12 weeks). The fruit had a background colour between 3 and 5 (Ground Colour Charts, Gala /Royal Gala, New Zealand Apple and Pear Marketing Board January 1991) after storage. A subset of fruit was treated with either 100 μg l^{-1} ethylene in a closed chamber at a rate of 100 ml min^{-1} or air for 4 days. All fruit were then held at 20 °C for 8 days and samples were then frozen in liquid nitrogen and stored at −80 °C.

RNA extraction and northern analysis

Total RNA was extracted from fruit using either the method of Chang *et al.* (1993) or that of Langenkämper *et al.* (1998). To gain undegraded mRNA, SDS concentrations had to be increased to as much as 8% for *M. ioensis*. Northern hybridisation analysis was performed as per Gleave *et al.* (1998) using 20 μg total RNA per lane for each cultivar or species. [α-^{32}P]dCTP-labeled (RTS Radprime DNA Labeling System–Gibco BRL) AP15 was used as a probe (Atkinson *et al.*, 1996). The membrane was washed twice with 2x SSC at room temperature for 5 min and then twice with 2x SSC, 1% SDS at 65 °C for 30 min. Hybridisation with [α-^{32}P]dCTP-labeled crab apple 18S rRNA (Simon and Weeden, 1992) was used to check RNA loadings and efficiency of transfer. Hybridisation was visualised by scanning on a Storm 840 phospho-imaging system (Molecular Dynamics) and analysed using ImageQuaNT™ (Molecular Dynamics) software.

EST libraries were constructed from mRNA from a wide range of tissues (as indicated in the results), directionally cloned into either pBK-CMV or pBluescript (SK-) or pCMV.SPORT6 and sequenced from the 5'end in a high throughput approach. This resulted in over 160 000 ESTs (GenBank, unpublished data).

Protein extraction and western analysis

Fruit tissue (0.5 g) was ground in liquid N_2, added to 0.5 ml of preheated SDS loading buffer, and the hot SDS supernatant (Sonnewald *et al.*, 1993) loaded on pairs of 10% SDS PAGE gels. One gel of each pair was transferred to nitrocellulose with Tris/glycine transfer buffer (Towbin *et al.*, 1979) containing 20% methanol using a semi-dry transfer cell (Bio-Rad) (0.8 mA cm^{-2} for 2 h). To check protein loadings the second gel of each pair was stained with modified colloidal Coomassie G250 stain (Neuhoff *et al.*, 1988). The nitrocellulose blots were held overnight at 5 °C in blocking buffer (1x TBS, 5% dried milk, 0.1% Tween 20) and then protein bands immunolocalised with the 5H8 or 9C11 primary antibodies (kindly provided by R. van Ree, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam; Akkerdaas *et al.*, 1995), followed by an alkaline phosphatase conjugated goat anti-mouse secondary antibody (StressGen Biotechnology Corp.). The bound antibody was visualised with 1-step™ NBT/BCIP (Pierce). These two monoclonal antibodies have been shown to react with some patient sera and an allergenic birch pollen epitope (Akkerdaas *et al.,* 1995). In apple, 9C11 has a much lower avidity than 5H8 and both are believed to recognise overlapping epitopes, although they also show different cross reactivity to proteins from other species. The antibody 5H8 is known to bind at least two different Mal d 1-related isoforms, Mal d 1a and Mal d 1b (Helsper *et al.*, 2002).

Identification of Mal d 1-related ESTs

ESTs related to published Mal d 1-related sequences were identified by using keywords in a BLAST NRDB90 search (Altschul *et al.*, 1997), and by comparing known sequences (GenBank)

with those in the database. A cut off of e^{-10} was used. All full-length ESTs were then single-strand sequenced with overlapping regions. A 'virtual northern' of the 'Royal Gala' tissues was produced by identifying how many independent ESTs belonging to a fully sequenced EST were found in each of the cDNA libraries.

Semi-quantitative RT-PCR analysis

RT-PCR amplifications were performed on selected apple cultivars and species, according to the manufacturer's recommendations (Platinum® Quantitative RT-PCR Thermoscript One-Step System, Invitrogen). For each reaction 100 ng of total RNA, quantified using an Agilent Technologies 2100 Bioanalyzer, was used. cDNA synthesis was at 60 °C for 30 min, followed by denaturation at 96 °C for 5 min, then 40 cycles of amplification involving denaturation at 96 °C for 30 s, annealing at 55–62.5 °C (depending on the primer) for 40 s and extension at 72 °C for 60 s. For the final cycle extension at 72 °C was continued for a further 5 min. PCR primers (Table 1) were designed from regions that were differential between the ESTs. PCR amplifications using the plasmid cDNA for each EST as a template were used as both positive and negative controls, i.e., primers were used with the wrong plasmid cDNA as negative controls. Expected products using the correct cDNA template/primer combinations were: Mal d 1b (361 bp), Mal d 1d (367 bp), Mal d 1e (426 bp), Mal d 1g (445 bp). The resulting amplification products were analysed by electrophoresis through 1.5% agarose (AppliChem), followed by staining with ethidium bromide and visualisation on an UV transilluminator (UVP, Ultra-Violet Products) attached to a camera (UV tec, Total Lab Systems Ltd).

For each primer combination, efficiency of amplification was calculated with Quantitative Real-Time PCR of the plasmid cDNA for each EST (Kühne and Oschmann, 2002). The LightCycler 2.0 System (Roche) was used for amplification and data collection (Software Version LCS4 4.0.0.23, Roche). Amplification reactions were performed using the Fast-Start Real-Time Mastermix (Roche) according to the manufacturer's recommendations. The amplification efficiencies calculated were as follows: Mal d 1b, 1.640 (error 0.0397), Mal d 1d, 1.659 (error 0.00827), Mal d 1e, 1.635 (error 0.0150) and Mal d 1g, 1.799 (error 0.0291). Therefore, amplification efficiencies of the four primer pairs were similar.

Table 1. Oligonucleotide primers used for gene-specific semi-quantitative RT-PCR.

Name	Oligonucleotide primer
Mal d 1b -A1	5′-CTTTTGGTGAAGGTAGCCAA-3′
Mal d 1b -A3	5′-CACACAACCTTCGACTGAAG-3′
Mal d 1d-A1	5′-CTTTTGGTGAAGGCAGCCAG-3′
Mal d 1d-A3	5′-CCTTCGACTGACCATATAGT-3′
Mal d 1e-A2	5-TTGTTGCCAGATGGATGGTC-3′
Mal d 1e-A3	5′-TTGATGCTGACAATCTCATT-3′
Mal d 1g-A2	5′-AAGATTGTTGCCATATGGAA-3′
Mal d 1g-A3	5′-ACAATGCCCTTGTCCTTGAC-3′

2-D gel electrophoresis and protein sequence analysis

'Royal Gala' fruit skin was ground in liquid nitrogen and extracted at room temperature with a buffer containing multiple chaotropes (Barraclough *et al.,* 2004). The acetone-precipitated protein was resolubilised and isoelectrically focussed on a pH 3-10 non-linear IPG strip followed by SDS-PAGE electrophoresis according to Barraclough *et al.* (2004). The gels were stained with Sypro Ruby (Molecular Probes) for 3 h or overnight. The Sypro stained gels were digitally imaged (GelDoc, BioRad) under UV excitation, and the image analysed on ImageMaster 2-D Elite software (Amersham BioSciences). Western analysis was performed as described above but using only the 5H8 antibody and ECL visualisation (Amersham Biosciences).

Protein spots selected for sequence analysis were removed and digested with trypsin (0.5 μg trypsin (modified sequencing grade from Roche) per sample in 0.2 M NH_4HCO_3 with 0.5 mM $CaCl_2$). The resultant peptide fragments were then analysed by LC-MS, employing an LCQ Deca ion trap mass spectrometer fitted with a nanospray ESI interface (ThermoQuest, Finnigan, San Jose, CA, USA) coupled to a Surveyor™ HPLC. Each digested protein sample (20 μl) was injected on to a reversed phase column (Inertsil ODS-3, C18, 300 mm ID × 15 cm, 3 mm, LC Packings, San Francisco, CA, USA). Tryptic peptides were separated at a flow rate of 4 ml min^{-1} with a linear gradient from 2 to 80% Solvent B (acetonitrile +

0.1% formic acid) over 50 min. Solvent A was 0.1% aqueous formic acid. The column flow rate was produced by splitting the primary flow rate of 40 ml min^{-1} from the Surveyor^{TM} HPLC system via an Acurate^{TM} flow splitter (LC Packings, San Francisco, CA, USA). The nanospray interface was used with a 30 mm ID fused-silica standard coated PicoTip^{TM} (New Objective, Woburn, MA, USA) and the spray voltage was supplied directly to the coated needle tip at 2.2 kV. The mass spectrometer was operated in the positive ion mode and the mass range acquired was m/z 300–2000. The heated capillary temperature was set at 210 °C. Data was acquired using a triple play experiment in data-dependent mode with dynamic exclusion enabled.

Results

The Mal d 1/PR-10 family of 'Royal Gala' contains at least 12 expressed members

We have identified ESTs (GenBank Accession AY428578) that are identical in nucleotide sequence to the previously characterised Mal d 1b (Atkinson *et al*., 1996), but not Mal d 1a or c (Son *et al.,* 1999). Further ESTs encoding members of the Mal d 1 family were also found. We have named these new sequences Mal d 1d–Mal d 1n (GenBank Accession numbers AY428579–AY428589) following the convention set by Son *et al*. (1999) (Figure 1). Mal d 1a (Schöning *et al*., 1995; Son *et al*., 1999) is most similar to Mal d 1d, differing by only 11 nucleotides in the cDNA sequence (data not shown) and two amino acids in the predicted protein sequence (Y4 to C4 and V135 to A135). The predicted amino acid sequences for the new Mal d 1 family members identified from 'Royal Gala' cover a small range in predicted pI values of 4.89–6.02 and predicted molecular masses of 17 454–18 433 Daltons (Table 2).

A 'virtual northern' analysis shows that eight of the 12 family members were expressed in either the skin or the cortex of tree-ripened fruit (150 DAFB) (Table 3). Seven members, some of them different to those found in ripe fruit, were expressed in young (10 DAFB) fruit. Seedling leaves that had been challenged with the pathogen *V. inaequalis* and partially senescing leaves also showed expression of a range of family members.

Mal d 1b was the most highly expressed Mal d 1-related isoform, in both the skin and the cortex of ripe fruit (Table 3). Mal d 1d was the second most highly expressed family member in both the skin and cortex of ripe fruit. Together the expression of Mal d 1b and Mal d 1d comprised 74% and 91% of Mal d 1-related expression in ripe fruit skin and cortex, respectively. ESTs for these two gene products were 0.7% of the ESTs sequenced from ripe apple skin and 0.3% of the ESTs from the cortex. Mal d 1b was also the most dominant isoform expressed in 'Royal Gala' apples following different storage conditions including altered atmospheres and short- and long-term storage (data not shown). In addition, Mal d 1d transcripts were always detected. Both isoforms comprised approximately 50% of the Mal d 1-related ESTs from young fruit undergoing active cell division (10 DAFB - Table 3). Mal d 1b was detected also in low abundance (1 of 4558 - 0.02%) in fruit cortex 126 DAFB. In leaf tissues, Mal d 1d was more frequent than Mal d 1b, but both were present in leaves challenged with a pathogen, as well as several other isoforms. Only Mal d 1d was found in early floral meristems (next year's flowers).

Mal d 1e–Mal d 1i, and Mal d 1n were also found in tree-ripened fruit skin or cortex, but at a lower frequency than Mal d 1b or d (Table 3). Mal d 1m and n were expressed in young fruit, together accounting for 45% of the Mal d 1-related isoforms in this tissue. Mal d 1k was found in only developing seeds. Mal d 1j and Mal d 1l were expressed in non-fruit tissues. Several other 'Royal Gala' cDNA libraries had no Mal d 1-related ESTs, including flowers, conducting tissues and young expanding leaves (data not shown).

Mal d 1-related ESTs are found in cDNA libraries from other apple cultivars

ESTs very similar but not identical (2 or 3 conservative base changes) to Mal d 1d were highly represented in a cDNA library constructed from the roots of a dwarfing rootstock 'M9' (4857 ESTs, data not shown), suggesting allelic differences between apple cultivars. Mal d 1-related ESTs were present in 'Braeburn' cultured cells (4805 ESTs), 'M9' phloem (4898 ESTs), 'Pacific Rose' vegetative buds and the expanding leaves of several cultivars selected for their pest or disease resistance status ('Pinkie', 'Northern Spy' and 'Aotea'),

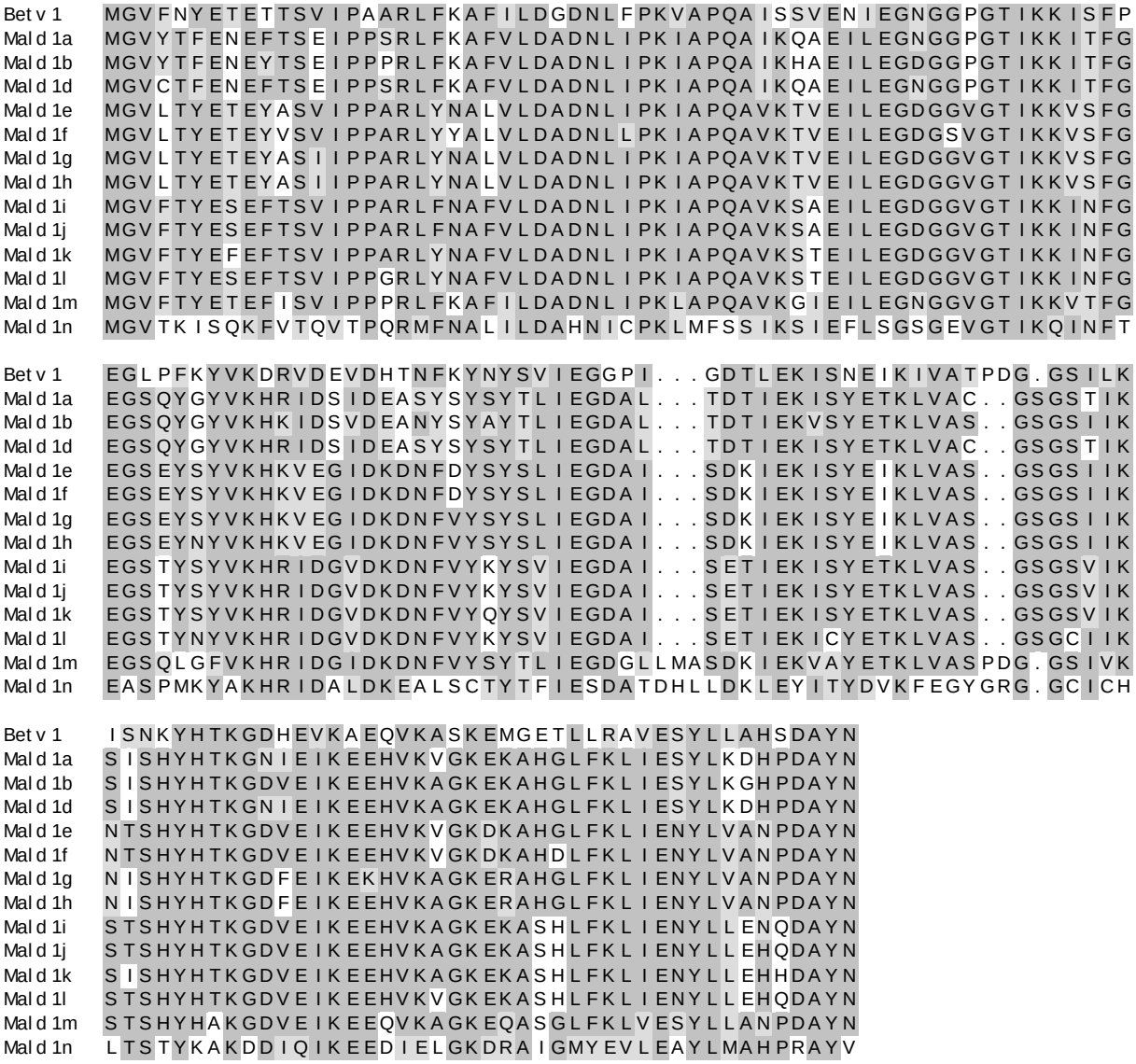

Figure 1. An amino acid comparison of the predicted translations for the Mal d 1-related ESTs from 'Royal Gala' that were identified in the HortResearch EST Database. Bet v 1 (GenBank Accession X15877) and Mal d 1a (GenBank Accession X83672) are included for comparison.

indicating that this gene family has high representation in apple and across a wide range of tissues.

All cultivars and species of apple express Mal d 1-related genes during fruit ripening

Genotypes did not ripen in a similar manner (Figure 2). 'Akane' and 'Pacific Rose' showed little change in ethylene concentration and background colour with time, suggesting that fruit from these cultivars did not ripen over the experimental period. Most of the apple species also maintained low ethylene levels and showed a limited increase in background colour. It was not possible to measure background colour on three of the species (*M. baccata*, *M. hupehensis* and *M. niedzwetzkyana*) because the skins were fully red.

M. sieversii and the cultivar 'Goldsmith' both had elevated ethylene levels at harvest (T_0) (8.47 and 2.57 $\mu l\ l^{-1}$, respectively) when compared to the other fruit which were typically 0.3 $\mu l\ l^{-1}$ or

Table 2. Predicted isoelectric point (pI) and molecular mass (MM) of each Mal d 1 isoform.

Isoform	pI	MM (Da)
Mal d 1b	5.88	17 538
Mal d 1d	5.94	17 562
Mal d 1e	5.05	17 454
Mal d 1f	4.89	17 619
Mal d 1g	6.02	17 525
Mal d 1h	5.29	17 553
Mal d 1i	5.29	17 555
Mal d 1j	5.59	17 558
Mal d 1k	5.56	17 705
Mal d 1l	5.59	17 711
Mal d 1m	5.03	17 711
Mal d 1n	6.01	18 433

less. However these concentrations were still low compared to concentrations found in ripe fruit. 'McIntosh', 'Jonagold', 'Braeburn', 'Sir Prize' and 'Golden Delicious' all showed significant increases in ethylene production as the fruit ripened (Figure 2A). *M. sieversii* and *M. ioensis* were the only species showing a significant ripening-related increase in ethylene.

In most instances the decreasing 'a' value (Figure 2B) indicated increased yellowing in background colour. However, the cultivars 'Baujade', 'Granny Smith' and the species *M. ioensis* showed little or no change in background colour during the ripening period although ethylene levels had increased significantly. Large changes in background colour did not correlate with large changes in ethylene production in most instances.

Unripe, freshly harvested fruit (Figure 3, T_0 N) showed different concentrations of normalised steady state mRNA levels of Mal d 1 homologues. Based on the hybridisation conditions (sequences showing 82% identity over a region of 100 bp or more – found between bases 370 and 470 in the Mal d 1b cDNA sequence – cross hybridise to the full length Mal d 1b sequence when used as a probe), probes were able to detect Mal d 1a–i isoforms. 'Braeburn' was highest, followed by 'Akane' and three of the apple species (*M. ioensis*, *M. niedzwetzkyana* and *M. sieversii*). 'Granny Smith', 'Jonagold', 'Golden Delicious', 'Goldsmith', 'Pacific Rose', 'McIntosh', 'Sir Prize' and the two species *M. baccata* and *M. hupehensis* were all low.

Most cultivars showed a marked increase in mRNA levels in response to ripening (Figure 3, T_2 N). In particular, with the exception of 'Baujade', all cultivars with 'Golden Delicious', 'Granny Smith' or 'Jonathan' in their ancestry had higher levels of Mal d 1-related steady state mRNA in ripe fruit (T_2) than in unripe fruit (T_0). Steady state Mal d 1 mRNA showed little change during ripening in 'Baujade', 'Akane', 'Cortland', 'Cox's Orange Pippin', 'Pacific Rose' and three of the *Malus* species (*M. baccata*, *M. hupehensis* and *M. sylvestris*).

mRNA encoding Mal d 1b and Mal d 1d are the most highly expressed of the Mal d 1 isoforms in ripe apple fruit from different cultivars and species

To test whether hybridising mRNA in northern analysis was likely to be similar in composition across species and cultivars, primers differentiating mRNA transcripts (Table 1) corresponding to the Mal d 1-related ESTs identified in 'Royal Gala' were used to examine the composition of mRNA species during ripening across a subset of species and cultivars. Mal d 1d primers would also amplify Mal d 1a alleles (Schöning *et al*., 1996), if present. The primers were first tested on skin and cortex mRNA extracted from tree-ripened 'Royal Gala' fruit (Figure 4a) after 25 and 40 cycles of amplification. As expected, Mal d 1b was the most highly expressed of the two isoforms in the cortex. Mal d 1e and g were not detected. In contrast Mal d 1b and d/a isoforms were expressed at similar levels in the skin. Mal d 1e and g were also expressed in the skin, with Mal d 1e present in higher levels. These results confirmed indications from the virtual northern and showed that Mal d 1b and d/a are likely to be the dominant expressing mRNA in ripe 'Royal Gala' fruit.

The PCR primers were then used in an analysis of five of the cultivars ('Braeburn', 'Fuji', 'Golden Delicious', 'Granny Smith' and 'Pacific Rose') and the crab apple *M. sieversii* (Figure 4b) using RNA from the northern experiment. The cultivars were chosen on the basis of the following: low ('Pacific Rose') or high ('Braeburn', 'Fuji') Mal d 1-related mRNA levels, and reported previous data ('Golden Delicious' and 'Granny Smith') (Son *et al*., 1999). *M. sieversii* was included as a species representative as it is from Kazakhstan, the proposed origin of modern apple.

Both Mal d 1b and Mal d 1d/a were the most highly expressed Mal d 1-related isoforms in all five cultivars and the species. However, there was

Table 3. A virtual northern of 'Royal Gala' apple cDNA libraries containing Mal d 1-related ESTs. Each Mal d 1-related EST has been given a letter because of differences in sequence compared to Mal d 1a and Mal d 1c (Son *et al.*, 1999). Total ESTs indicates the total number of independent ESTs sequenced from that library, DAFB indicates fruit that were sampled a number of days after full bloom, seedling leaves refer to leaves that were challenged with *V. inaequalis*. The numbers in each row represent the total number of ESTs in each library that were identical to the Mal d 1-related EST in column one.

cDNA library	Young vegetative shoots	Early floral bud	10 DAFB fruit	59 DAFB seeds	126 DAFB cortex	126 DAFB core	150 DAFB skin	150 DAFB cortex	Seedling leaves	Partially senescing leaves
Total ESTs in library	23 378	1031	9468	5449	4558	5202	8050	6947	5049	11 024
Mal d 1b			10		1		31	17	4	4
Mal d 1d		6	7				23	4	6	7
Mal d 1e							10		3	
Mal d 1f							2		2	
Mal d 1g			1				3			1
Mal d 1h			1				1			
Mal d 1i							3		1	
Mal d 1j									1	
Mal d 1k				1						
Mal d 1l										1
Mal d 1m			5							
Mal d 1n			4					2		2

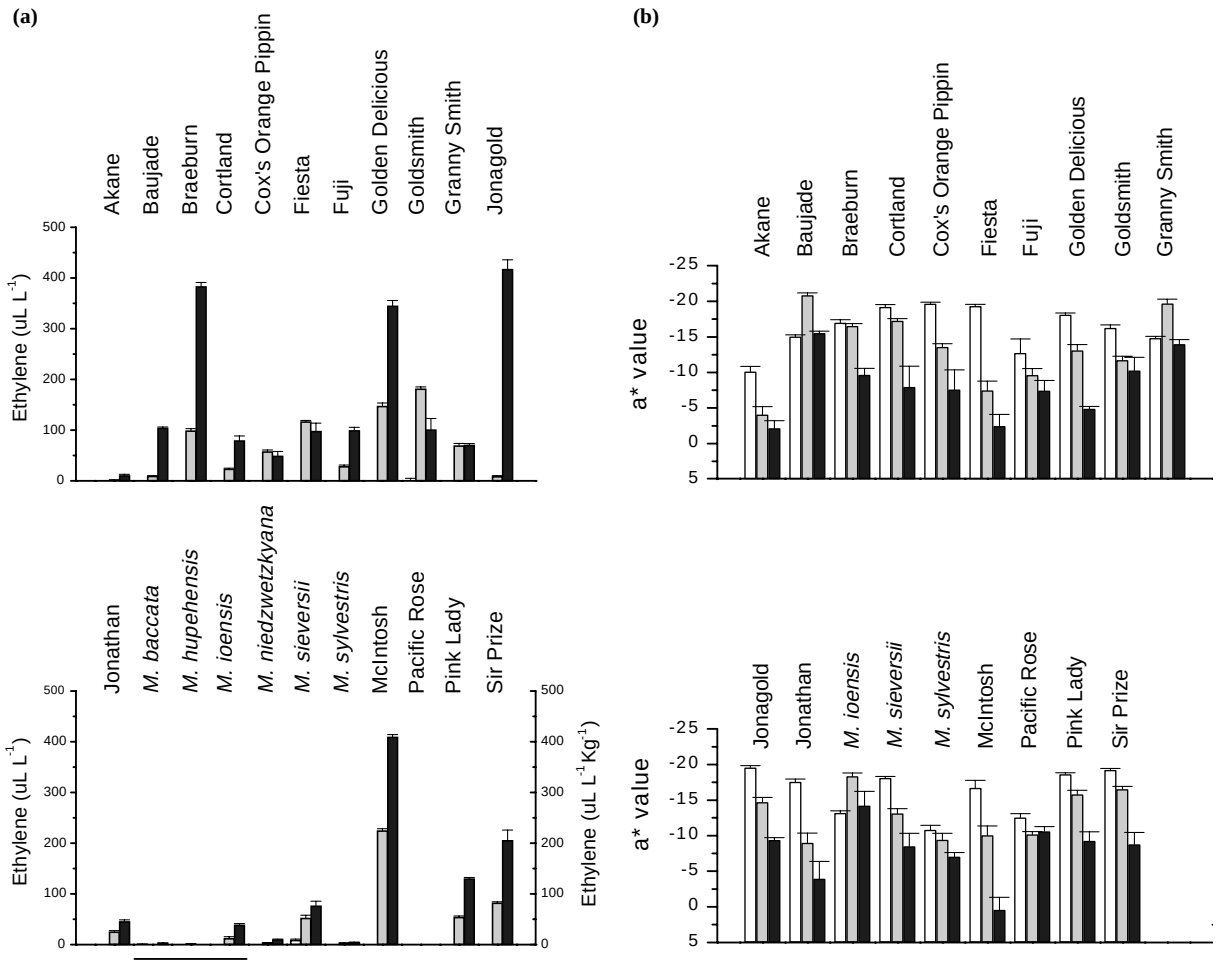

Figure 2. Comparisons of ethylene evolution (a) and background colour (b) between apple cultivars and some crab apple species. Fruit were sampled directly after harvest (T_0-white bars), during postharvest ripening (T_1-grey bars) and when ripe (T_2-black bars). Values are means of 20 fruit with SEM. Ethylene is reported as internal ethylene measurements sampled from the core of the fruit, except for some of the species that had no core cavity. These fruit were sampled after leaving batches of 5 fruit for 1 h in a closed container of known volume, and are denoted by an underline in the figure. Increases in the yellowness of background colour are indicated by falling 'a' values. Some species were completely red and background colour was unable to be measured.

also variation between the expression of Mal d 1b and the expression of Mal d 1d depending on genotype. Mal d 1b expression dominated in 'Braeburn', 'Granny Smith', and *M. sieversii* and the expression levels were relatively similar across all genotypes. The level of expression of Mal d 1d/a was similar to Mal d 1b in 'Golden Delicious', 'Fuji' and 'Pacific Rose', although overall expression was low in 'Pacific Rose' as expected from the northern data (Figure 3). Mal d 1e showed that a faint amplification product of the predicted size was detected in 'Fuji', 'Golden Delicious', 'Granny Smith' and *M. sieversii* after 25 cycles, and in 'Braeburn' and 'Pacific Rose' after 40 cycles. However, in the case of Mal d 1g, amplification products were detected in 'Braeburn', 'Fuji', 'Golden Delicious' and 'Granny Smith' after 40 cycles, but not in 'Pacific Rose' or *M. sieversii*.

Different amounts of 5H8-antigenic protein were present in apple genotypes

Protein loadings for all fruit were equal as determined by Coomassie staining of replicate

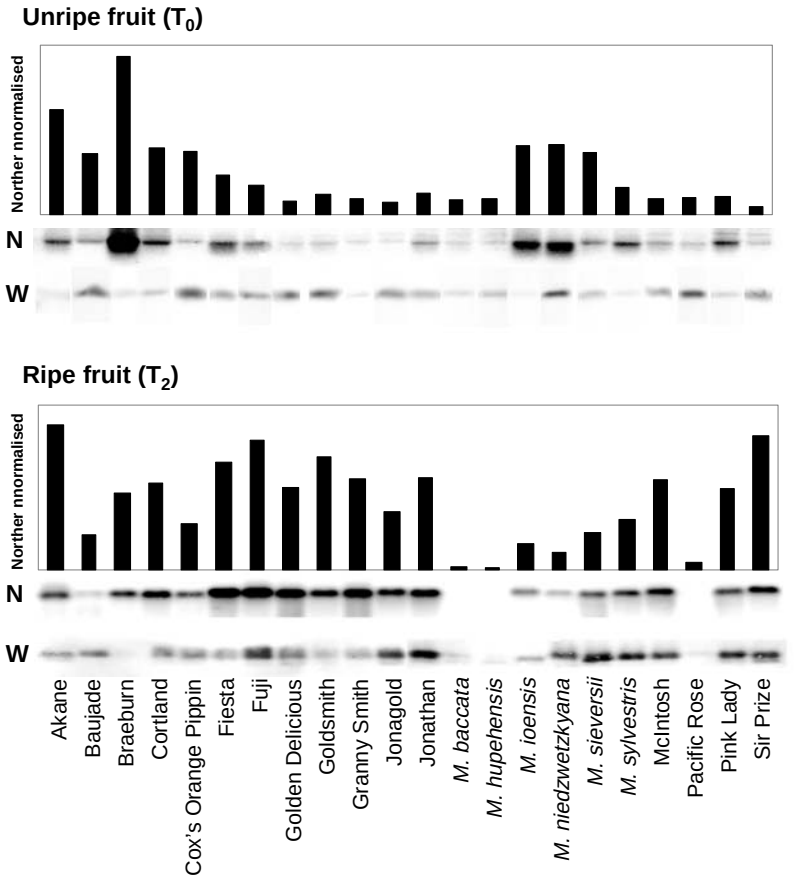

Figure 3. Northern and western analyses of unripe freshly harvested (T_0) and ripe (T_2) fruit from 16 apple cultivars and six *Malus* species. (N). Northern hybridisation analysis was carried out on blots loaded with 20μg RNA using full-length AP15 cDNA (Mal d 1b) as a hybridisation probe. For each cultivar or species the level of Mal d 1-related mRNA was adjusted for loading differences by hybridisation with crab apple 18S rRNA. A histogram of Mal d 1-related mRNA levels normalised to the loadings assessed with the ribosomal probe is plotted above the hybridisation bands. (W). Western analysis was carried out using the monoclonal 5H8 as the primary antibody (Akkerdaas *et al*., 1995). Gels were loaded with equal volumes of hot-SDS extracted protein from equal fresh weights (0.5 g) of apple fruit tissue. Matching protein gels stained with Coomassie Blue indicated similar concentrations of protein. Bands on blots were visualised with alkaline phosphatase secondary antibodies.

protein gels (data not shown). Higher levels of 5H8-antigenic protein were present in unripe fruit of 'Baujade', 'Cox's Orange Pippin', 'Goldsmith', and *M. niedzwetzkyana* than in the other genotypes (Figure 3, T_0 W). In particular, 'Cortland', 'Granny Smith', 'Pink Lady' and the species *M. baccata*, *M. ioensis*, *M. sieversii*, and *M. sylvestris* had little or no 5H8 antigenic protein. In ripe fruit of 'Fuji', 'Jonagold', 'Jonathan', and *M. sieversii* and *M. sylvestris* 5H8-binding was higher than in other genotypes (Figure 3, T_2 W). Large changes in 5H8-binding protein occurred with ripening in all cultivars and species tested except 'Baujade', 'Braeburn', 'Fiesta', 'Golden Delicious', *M. baccata*, and *M. niedzwetzkyana*. In 'Goldsmith', 'Pacific Rose', 'Cox's Orange Pippin', and *M. hupehensis* antibody binding decreased in ripe fruit. A small subset of the samples were reanalysed comparing all three ripeness stages. 'Braeburn' had low levels of antigenic protein through ripening as expected, and 'Pink Lady' and *M. sylvestris* showed increases during ripening, also as expected (data not shown).

In a further experiment, 'Royal Gala' fruit, where ethylene production had been reduced due to the presence of antisense ACC oxidase, had a similar presence of 5H8-antigenic protein to that in control fruit. Treating these fruit with ethylene made little difference (Figure 5), although ethylene-

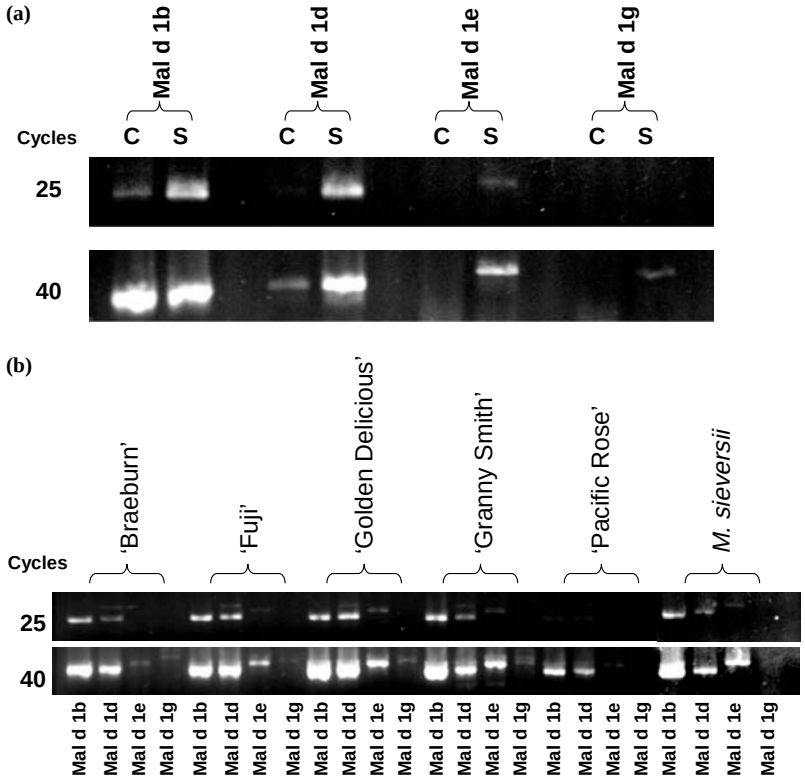

Figure 4. RT-PCR analysis of Mal d 1-related ESTs expressed in mRNA (100 ng RNA loadings) isolated from (a) 'Royal Gala' cortex and skin and (b) whole fruit RNA used in Figure 3 from five apple cultivars ('Braeburn', 'Fuji', 'Golden Delicious', 'Granny Smith' and 'Pacific Rose') and *M. sieversii*. C, cortex; S, skin from tree-ripened fruit (150 DAFB); 25, 25 amplification cycles; 40, 40 amplification cycles, conditions as in Methods. Mal d 1-related identities as in Figure 1.

treated fruit and non-treated fruit were of very different ripenesses (data not shown). In our experiments, antibody 9C11 did not bind any proteins in a subset of the apple cultivars and species ('Golden Delicious', 'Jonagold', 'Granny Smith', 'Baujade', 'Pacific Rose', 'Braeburn', *M. sylvestris*, *M. sieversii and M. niedzwetzkyana*) tested in a duplicate blot in parallel with antibody 5H8 (data not shown).

Mal d 1 isoforms in 'Royal Gala' fruit skin that bind antibody 5H8

Binding of the antibody 5H8 is dependent on the presence of an antigenic epitope(s) in a Mal d 1-related isoform. To determine whether only Mal d 1b and Mal d 1d/a bind the protein, 2D-PAGE followed by western analysis was performed on the extracted proteins in tree-ripe 'Royal Gala' skin. A large protein spot of pI 5.9 was visualised at a position corresponding to 17–18 kDa (Figure 6a). Other minor protein spots at more acidic pIs were also found in this molecular mass range. The 5H8 antibody bound at a position (Figure 6b, a) corresponding to the large protein spot and to two other smaller protein spots at more acidic pIs (Figure 6B, b and c).

Based on the predicted pI and molecular mass for Mal d 1b and Mal d 1d (Table 2), the two isoforms are likely to position closely together in 2D-PAGE. Of the other isoforms expressed in skin, only Mal d 1g would migrate to a position close to Mal d 1b and Mal d 1d. Protein sequence analysis of the large spot (Figure 6b, a) produced amino acid sequences from regions that were common between Mal d 1b and Mal d 1d. However, sequencing of the equivalent immunoresponsive protein spot in the cultivar 'Fuji' revealed that both Mal d 1b and Mal d 1d were present (data not shown).

In 'Royal Gala' the smaller and more acidic protein (Figure 6b, a) migrates to a mass equiva-

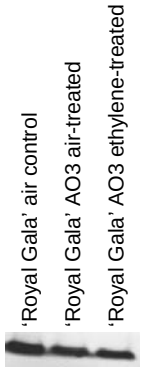

Figure 5. Western analysis of protein hot-SDS extracted from apple fruit (0.5 g FW). Blots were probed with 5H8 monoclonal antibody as in Figure 3. Control and transgenic (AO3) 'Royal Gala' were compared after 12 weeks storage and 12 days shelf life. Transgenic apples had been down-regulated for endogenous ethylene production and half were held in air and half in ethylene for 4 days after storage. Matching protein gels indicated that the apple lanes had similar concentrations of protein.

lent that may correspond to isoforms Mal d 1e, f, g, h or i. However, sequencing of an equivalent antigenic protein from 'Fuji' shows that an isoform in this position is similar to only Mal d 1b or Mal d 1d and not to any of the other Mal d 1-related isoforms identified thus far in 'Royal Gala' (data not shown). Protein sequence obtained from the 'Royal Gala' equivalent also could not be distinguished from either Mal d 1b or Mal d 1d, i.e. the protein fragments sequenced were the same in both proteins, but could be distinguished from the other identified isoforms. Sequencing of the third 5H8 antigenic protein (Figure 6b, c) produced sequences that were consistent with isoforms Mal d 1e, g and h. However, based on predicted pI and molecular mass we conclude that the isoform corresponding to this antigenic protein is Mal d 1e.

Discussion

We have shown that one cultivar of apple, 'Royal Gala', expresses many Mal d 1-like gene products and have demonstrated that the postulated large family (Atkinson *et al.,* 1996) of genes exists and that most, if not all genes, are actively transcribed. This diversity is similar to birch (Swoboda *et al*., 1995b) and may be the same for PR10-related proteins in other species. The Mal d 1 genes are expressed not only in different tissues, but a large number of them are also expressed in the same tissue, namely in ripe fruit skins (Mal d 1b, d–h and i) or in leaves from disease-challenged seedlings (Mal d 1b, d–f, i and j). Variations in sequence are not attributable to PCR error as suggested for a cause of sequence variation within other cultivars studied (e.g. Son *et al.,* 1999). We have demonstrated that knowledge of the degree of ripeness as measured by standard parameters will not predict the amount of allergenic protein present, and that cultivars differ widely. Variation in ethylene production does not explain the differences, confirming the conclusions reached by Pühringer *et al*. (2000) in examining the expression of the reporter gene GUS fused to promoter regions of Mal d 1b. Finally, we have shown that the predominantly expressed genes in ripe apple fruit are translated *in vivo* into proteins. These proteins bind to a monoclonal antibody that has been shown to bind to an allergenic epitope found in apples (Akkerdaas *et al*., 1995), and proteins antigenic to the antibody were found in all cultivars and species examined.

Mal d 1-like proteins form a large family in apples

At least 15 individual family members were predicted for the Mal d 1 family (Atkinson *et al*., 1996) in 'Golden Delicious', based on southern analysis. Their hybridisation conditions would have identified the equivalent of all the 'Royal Gala' genes up to and including Mal d 1l, indicating that we have isolated cDNAs coding for 10 of the putative 15 members. Mal d 1m and n cannot be included as they fall outside the hybridisation conditions. Apple is a cryptic diploid (Guilford *et al*., 1997; Maliepaard *et al*., 1998) and one hypothesis is that it arose from two genomes of nine chromosomes with one chromosome lost after fusion to give 17 chromosomes in all. So depending on the content of the lost chromosome, theoretically there should be a further four or five genes to identify, or else these have become pseudogenes.

A recent analysis of Mal d 1 genes available in the public databases identified four groups of homologues (Pühringer *et al.,* 2003). We carried out a neighbour joining phylogenetic analysis of the 'Royal Gala' genes (Figure 7) including the key sequences from Pühringer *et al*. (2003). The results

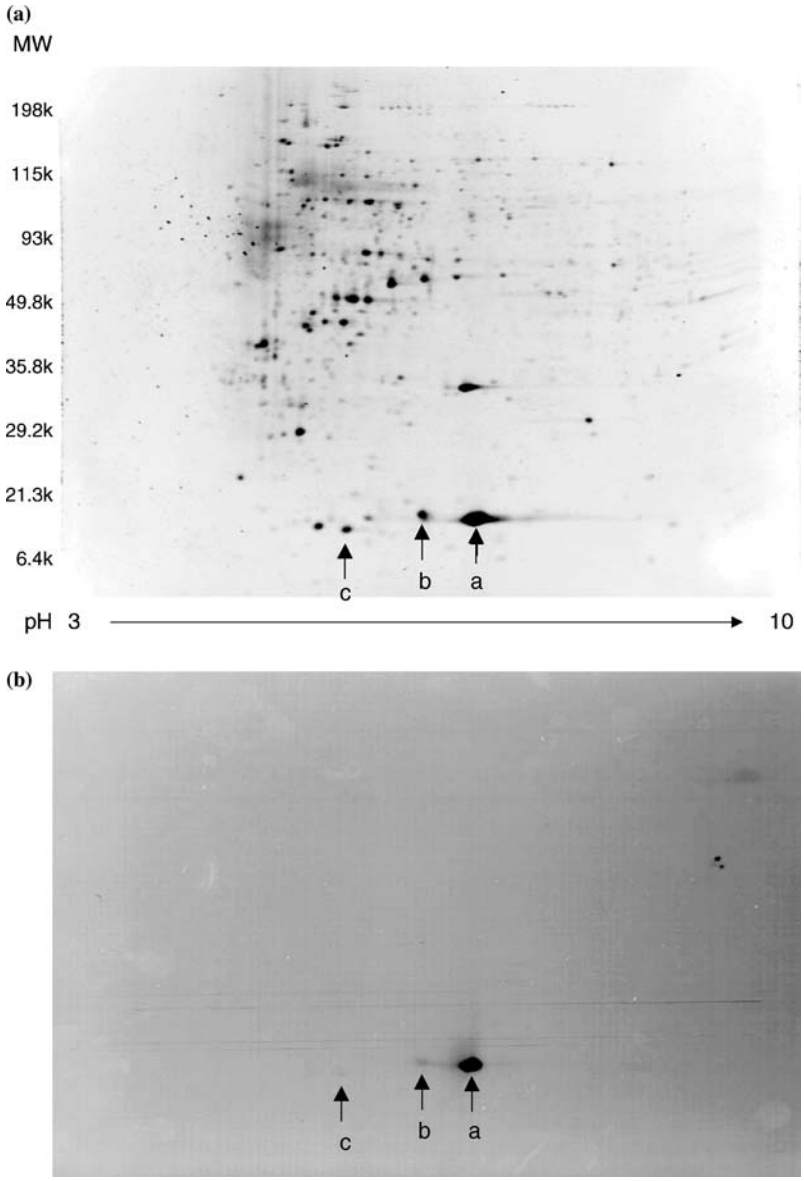

Figure 6. 2-D SDS-PAGE of the proteins extracted from 'Royal Gala' apple skin (a). First dimension (pH 3–10), second dimension molecular weight sizing with standard molecular mass markers annotated on the *y* axis. (b) Western analysis of a matching gel with arrows identifying the three protein spots (a, b and c) that bound the antibody 5H8.

showed that only Mal d 1b belonged to their Mal d 1.02 family, and that Mal d 1d must be an allelic variant of Mal d 1a, belonging to the Mal d 1.01 family. The Mal d 1b (Mal d 1.02) family was concluded by Pühringer *et al*. (2003) and Helsper *et al*. (2002) to be the dominantly expressed gene in ripe apple fruit and our data confirms these conclusions, while also showing that in some cultivars and possibly species, the Mal d 1.01 family can also be expressed at similar levels (see Figure 4). Ripe fruit of 'Granny Smith' was previously suggested to express Mal d 1a (Mal d 1.01) at very low levels ($<2\%$, Helsper *et al.,* 2002) and ripe fruit of 'Golden Delicious' and 'Jonagold' at levels of $\sim$12% (Pühringer *et al.,* 2003). Our data shows that while 'Granny Smith' fruit have considerably lower expression of Mal d 1.01 family genes, 'Golden Delicious', 'Royal Gala', 'Fuji' and 'Pacific Rose' fruit have similar levels of expression of genes from the two families. Hence there are

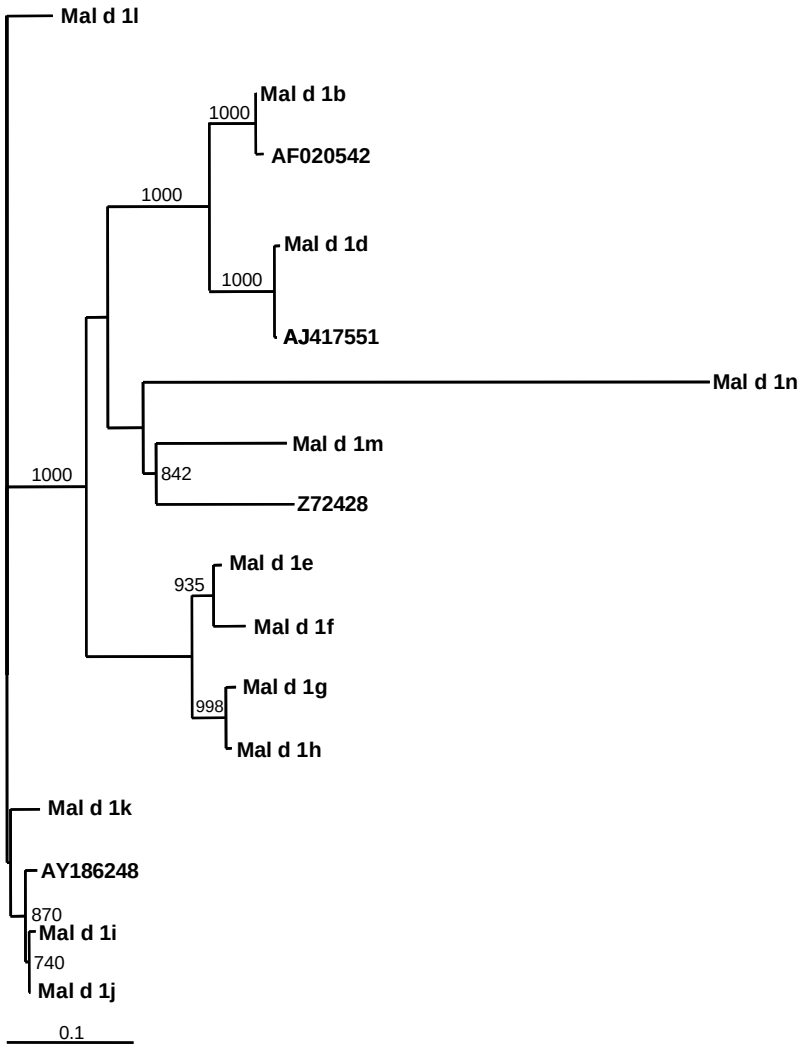

Figure 7. Phylogenetic analysis of full length 'Royal Gala' Mal d 1 translated proteins. An unrooted neighbour joining tree with boot straps (1000 replicates) was used. Separation of sequences with values greater than 700 are indicated on the figure. Key sequences (GenBank accession numbers) from Pühringer *et al*. (2003) were included for comparison and identification of defined Mal d 1 subfamilies.

major differences between the two European studies and ours, either due to analysis methods or due to fruit with a very different metabolic background.

We have also confirmed that at least one member of the Pühringer *et al*. (2003) Mal d 1.03 family – Mal d 1i – is expressed in fruit at much lower levels, but do not confirm that members of the Mal d 1.04 family – Mal d 1m and n – are expressed in only mature leaves. We have evidence for their expression in young fruit, ripe fruit and senescing leaves, although not demonstrating their presence in pathogen-challenged leaves as suggested by the experiments of Ziadi *et al*. (2001). In addition we have now demonstrated the presence of at least two more Mal d 1 families (Figure 7). Mal d 1l is unique and was found in only senescing leaves; Mal d 1e–g and h together form a further one or possibly two families and are also important contributors to Mal d 1 gene expression in ripening fruit. Based on the 'virtual northern' of ripe 'Royal Gala' fruit five isoforms (Mal d 1e–i) together would amount to only 13% of the total Mal d 1-related expression. Therefore, most of the

expression detected by our northern analysis of ripening 'Royal Gala' fruit would be of mRNA corresponding to Mal d 1b and Mal d 1d, and based on RT-PCR results (Figure 4) this appears to be likely for other apple cultivars. We have sound evidence that protein of Mal d 1e is present in ripe fruit and that it is recognised by the 5H8 monoclonal antibody, suggesting that it is likely that human sera will also recognise this protein. Screening a cDNA library database in which thousands of ESTs have been identified by random sequencing has avoided any bias caused by PCR isolation of clones, and this is the first report of a large collection of paralogues from one apple cultivar, 'Royal Gala', where PCR error or bias cannot be a factor. It would be valuable to test all the Mal d 1 variants for their reactivity to IgE from allergic patients, and gain an understanding of whether low allergen apples could be produced by changing the balance of isoforms expressed in ripe fruit, or if low levels of all isoforms are necessary.

The many 'Golden Delicious' isoforms isolated by PCR (GenBank Accessions AAK13027.1, AAK13028.1, AAK13029.1, AAK13030.1, CAA 88833.1 and CAD10375.1) as well as the identification in our study of variants from other apple cultivars that were not found in 'Royal Gala' suggest that all apple cultivars are going to contain a range of Mal d 1-like proteins. Some of these may vary in only one or two amino acids from the equivalent isoform in another cultivar, suggesting an allelic variant rather than a different locus, if the differences are not due to PCR error. A key question will be whether the variants are allelic or duplications in alternative chromosomal positions. For example, an analysis of GenBank sequences has shown that in 'Golden Delicious' there are two isoforms that differ from Mal d 1b by only one amino acid. In the case of Mal d 1d, in 'Golden Delicious' there are two isoforms that differ by one amino acid and one that differs by three amino acids. Determining which isoform can be assigned to alleles and which to different genes for at least one cultivar of apple will assist in attempts to breed for low allergenic apples.

Are some isoforms hypoallergenic variants?

Research with the birch pollen orthologues has demonstrated that very small changes in amino acid sequence can change a protein from being hyperallergenic to hypoallergenic (Spangfort *et al.,* 1996; Ferreira *et al.*, 1996, 1998). Bet v 1l has been shown to be a hypoallergenic isoform of the Bet v 1 protein family (Ferreira *et al.*, 1996), and point mutations in six amino acids were sufficient to change Bet v 1a from an allergenic protein to a hypoallergenic protein (Ferreira *et al.*, 1998). Point mutations in both the cherry allergen Pru av 1 and Mal d 1 where serine 112 was altered to proline showed changed structure and loss of allergenicity (Scheurer *et al.*, 1999; Son *et al.*, 1999). A recent publication on the cytokinin-inducible Bet v 1 orthologue protein T1 from periwinkle (Laffer *et al.*, 2003) has also shown that this isoform too is likely to be hypoallergenic, and the protein has the variant amino acids 10, 57, 113 and 125. Examining the isoforms isolated from apple it is apparent that at least some forms could be predicted to be less allergenic than others. Mal d 1b, e–h and m always have amino acids similar to Bet v 1a at amino acids 57, 112, 113 and 125, although there are various substitutions at amino acids 10 and 30. Hence these isoforms would be predicted to be IgE responsive to some patient sera, and we have shown that at least Mal d 1b (a proven allergen) and Mal d 1e are present as proteins in ripe apple.

Three-dimensional structure is also important in determining immune responses. Immunological studies using monoclonal antibodies and IgE antibodies have indicated that at least two loop regions in the 3-D structure of Bet v 1 are important to the allergic response—amino acids 9–22 and 104–113 (Bet mim E) and amino acids 58–67 (Bet mim 1) (Ganglberger *et al.*, 2000). These correspond to loop regions on the surface of the 3-D models for both Bet v 1a and Bet v 1l (PDB accession codes 1bv1_ and 1fm4). Neudecker *et al.* (2003) have also demonstrated that a change in charge associated with the P-loop in Pru av 1 (~amino acids 44–54) and celery homologues can also affect patient IgE reactivity. 3-D modelling of variations between different Mal d 1 isoforms predicts changes in the structure of least two surface loop regions—Mal d 1b amino acids 88–98 and 103–112. Mal d 1f-k all superimpose on to Mal d 1b exactly or sufficiently closely to not differ when viewed from all angles (unpublished data). Mal d 1e superimposes onto Mal d 1a and d. All variation is in the loop associated with amino acids 103–112. Mal d 1 l–n differ more widely.

Reports of variations in quantities of Mal d 1-like proteins between fruit genotypes need to include clear physiological descriptions

Mal d 1-like isoforms seem certain to arise in fruits due to their plant function. As PR10-like proteins, at least some isoforms must express with disease challenge (Pühringer *et al*., 2000; Ziadi *et al.,* 2001; Hoffmann-Sommergruber, 2002), and stress responsiveness has also been reported (Pühringer *et al*., 2000). Both such challenges can normally be expected to have already occurred in some fruit when harvested, or to occur after harvest. We have demonstrated that many of the same isoforms are present both in ripening fruit and in leaves challenged with a pathogen, suggesting that processes involving this class of protein are not different between ripening and disease response. By comparing transgenic apple fruits with and without down-regulated ethylene production, as well as comparing ethylene production and fruit background colour across a wide range of cultivars and species, we have also demonstrated that it is unlikely that expression of Mal d 1-like genes and proteins respond to ethylene as a hormone but rather to other senescence processes. This is in accordance with promoter studies for Mal d 1b where senescing leaves gave higher GUS expression (Pühringer *et al*., 2000), and the reported abundance of Mal d 1b transcripts in senescing leaves in this work. However, there were still anomalies. For example, 'Pacific Rose' appeared not to ripen or senesce during the time period of our experiments, and Mal d 1 transcripts were low. This difference will also be true in fruit when purchased from the market and consumed by an allergic consumer. Our data shows clearly that levels of Mal d 1 expression and probably protein can differ between apple cultivars, but that physiological state is a major factor as well. Production of low-allergenic cultivars will require careful checking to ensure a genuine reduction in key Mal d 1-like proteins, that physiologically defined comparisons are made, and that such a reduction does not affect critical plant functions.

What is the function of the Mal d 1 family of proteins?

These proteins have now been implicated in a wide range of processes. We have demonstrated gene expression of this class of protein in a wide range of tissues in one genotype – 'Royal Gala'. Several of these isoforms have structural alterations as well as changes in charge or functional amino acids. The putative cytosolic localisation for this class of protein as well as the presence of diverse members is reminiscent of proteins involved in signalling pathways. Several functions have already been suggested which do not conflict with a role in signalling.

The 3-D structure of Bet v 1 (Gajhede *et al*., 1996) and Pru av 1 (Neudecker *et al.,* 2001) shows a P-loop motif (GXGGXGXXK; amino acids 46–54, Bet v 1a) that is found in many DNA- and ATP/GTP-binding proteins (Saraste *et al*., 1990). It is also conserved in the hypoallergenic orthologue from periwinkle (Laffer *et al*., 2003), and the cytokinin-binding homologue in mung bean (Fujimoto *et al.,* 1998). The importance of this loop may also be seen in its conservation across the isoforms of 'Royal Gala', except Mal d 1f and Mal d 1n where in each there is one mutation (S49G and E49G, respectively), and its involvement in at least one IgE antigenic site (Vieths *et al*., 2002; Neudecker *et al*., 2003). A leucine (L44) that may be responsible for the binding of the phosphoryl group of the nucleotide is also conserved across all the 'Royal Gala' isoforms. These data indicate a potential role in signalling associated with cytokinin challenge, which remains to be proven.

Compelling evidence has been produced for Bet v 1 having a role in the binding and transport of plant steroids or signalling associated with them. Bet v 1 can bind physiological ligands such as fatty acids, flavonoids and cytokinins (Mogensen *et al*., 2002); Pru av 1 can bind the phytosteroid homocastasterone (Neudecker *et al*., 2001) and the hypoallergenic isoform, Bet v 1l, can bind deoxycholate, a steroid molecule very similar to brassinosteroids, and the brassinosteroids brassinolide and 24-epicastasterone (Marković-Housley *et al*., 2003). Brassinolide was shown to bind in both 2:1 and 1:1 stoichiometry with Bet v 1l, whereas 24-epicastasterone bound more weakly with a 1:1 stoichiometry. These data suggest that there might be different affinities among isoforms in binding plant steroid ligands. Comparing apple isoforms and amino acid substitutions, there is sufficient conservation to suggest that all of these too could bind plant steroids with at least a 1:1 stoichiometry.

Most recently Pühringer *et al.* (2003) isolated a small protein that interacts with Mal d 1 – MdAP. Two isoforms were used to show binding – Mal d 1.01 family (Mal d 1a/d) and Mal d 1.03 family (Mal d 1i–k). The authors suggest this protein could act to stabilise a transport complex within the plant, and binding to divergent isoforms and the presence of only one gene in apple would be consistent with a signalling role where the Mal d 1 isoforms may define what binds to the complex, what receptor might interact and which other interacting proteins or factors define the pathway in response to different challenges such as brassinosteroids, cytokinins, a ripening/senescence related signal and so on. All these data suggest that the Mal d 1 family of proteins have a range of important functions in apples, and that variant isoforms may associate with different *in vivo* functions. It is time to attribute real functions to each of these.

Acknowledgments

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