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## Short communication

# A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also ripening-related

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**Abstract** The screening of a ripe apple (*Malus domestica* 'Golden Delicious') cDNA expression library with polyclonal antibodies to spinach sucrose-phosphate synthase resulted in the isolation of a cDNA clone pAP15. This clone encodes a homologue of a class of stress and pathogenesis-related clones, and is also highly homologous to a class of allergenic proteins from apple without known biological function. The pAP15 cDNA insert is 817 base pairs in length and codes for a protein of 17.5 kD. Genomic Southern analysis suggests that the gene belongs to a family of at least 15 members. Northern analysis indicates that pAP15 is ripening-related, with an accumulation of homologous mRNA coincident with ethylene production during apple fruit ripening.

**Keywords** allergen; apple; pathogenesis-related; ripening; stress

## INTRODUCTION

A large number of stress- and pathogenesis-related cDNA clones have been isolated from plant tissues. One class of these clones encode proteins (Group 1; Ohashi & Ohshima 1992) that range in size between 14 and 18 kD (e.g., Matton & Brisson 1989; Constabel & Brisson 1992; Warner et al. 1992) but have an unknown function, although it has recently been suggested that they may code for a ribonuclease (Moiseyev et al. 1994). Expression of mRNAs homologous to these cDNAs is induced by fungal elicitors (e.g., Walter et al. 1990), and by stress treatments such as high osmoticum, excess phosphate, addition of abscisic acid, ethylene and wounding (Crowell et al. 1992; Warner et al. 1992; Barratt & Clark 1993). These clones encode proteins which share homology with the major birch pollen allergen *Bet v I* (Breiteneder et al. 1989; Walter et al. 1990).

Patients suffering from Type I allergy caused by birch pollen can have their condition exacerbated if apples are consumed at the same time of the year as peak birch pollen production (Eriksson et al. 1982). This is thought to arise through cross-reaction of anti-birch allergen IgEs with a number of common antigenic epitopes on a 17–18 kD protein present in the fruit (Ebner et al. 1991; Vieths et al. 1994a). Northern analysis has indicated cross hybridisation between a birch *Bet v I* cDNA clone and a homologous mRNA in apple (Ebner et al. 1991), and recently a sequence coding for the apple allergen *Mal d I* was entered into the databases (Genbank accession no. X83672). Here we report the isolation and characterisation of an apple cDNA (pAP15, Genbank accession no. L42952) which codes for an additional member of this family of proteins. We show by Northern analysis that steady state mRNA levels are strongly up-regulated during the early stages of fruit ripening. This clone was isolated during an antibody screen of a ripe fruit cDNA library, using polyclonal antibodies to spinach sucrose-phosphate synthase (SPS).

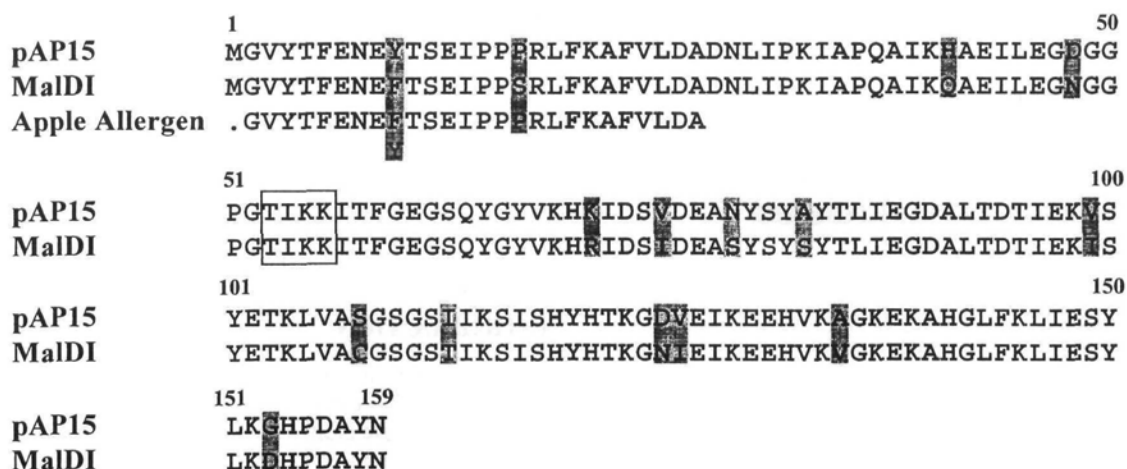


**Fig. 1** Sequence of the polypeptide encoded by the pAP15 cDNA insert (Genbank accession no. L42952) lined up against the MalDI polypeptide (Genbank accession no. X83672) and the N-terminal sequence (Schoning et al. 1995) of the allergenic protein from 'Golden Delicious' apples (*Malus domestica*). Differences between the sequences are shaded. The boxed region (residues 53–56) encodes a putative protein kinase C phosphorylation site.

## MATERIALS AND METHODS

A ripe Golden Delicious apple cDNA expression library (Ross et al. 1992) was plated at a density of c. 400 colonies/plate, and duplicate filter lifts were made from seven master plates. Immunoscreening of these filters was carried out according to Sambrook et al. (1989), using polyclonal antibodies raised against spinach SPS (Sonnewald et al. 1993) which had been purified by passage through an *Escherichia coli* protein-linked affinity column (Sambrook et al. 1989). The purified antibody was used at a 1:500 dilution, and positive antibody reactions were visualised with horseradish peroxidase. A third control filter was screened with pre-immune serum. A single positive colony came through three rounds of screening. The cDNA insert from this colony was designated pAP15.

Apple DNA for Southern analysis was prepared by the technique of Dellaporta et al. (1983). DNA (20 µg/lane) was fractionated on a 1% agarose gel and transferred to a Hybond N<sup>+</sup> membrane (Amersham) by capillary transfer in 0.4M NaOH, 0.6M NaCl. Membranes were hybridised with <sup>32</sup>P-labelled pAP15 insert for 16 h at 65°C, in 1M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. The membrane was washed at a stringency allowing c. 15% mismatch (300 mM NaCl, 30 mM tri-sodium citrate, 1% SDS, 75°C), and exposed to Kodak XAR film with intensifying screens at

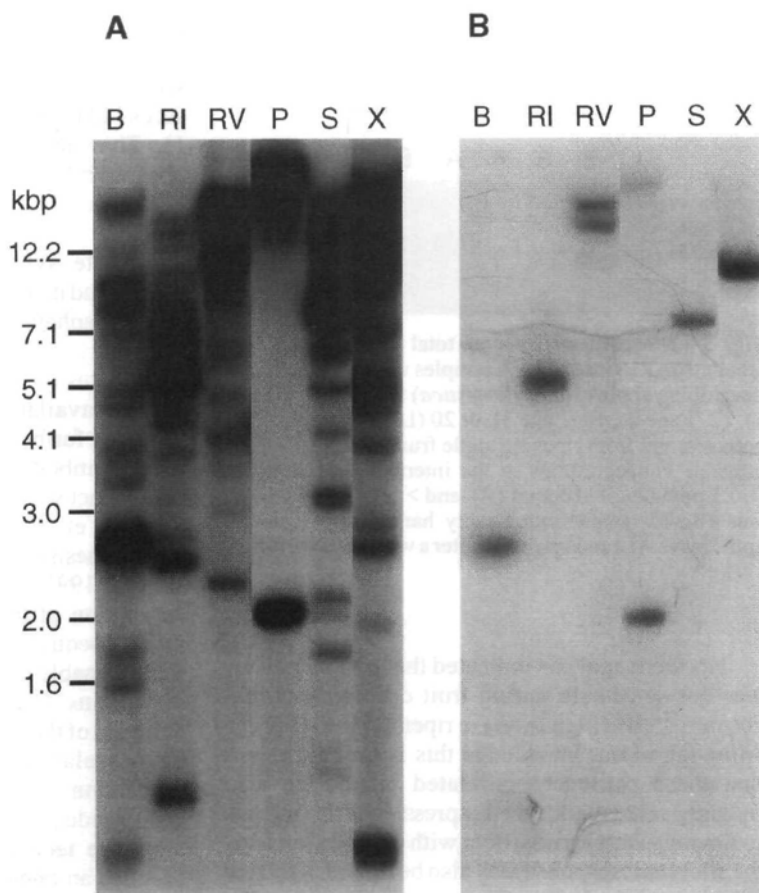
–80°C. This membrane was re-washed at a stringency allowing 5% mismatch (75 mM NaCl, 7.5 mM tri-sodium citrate, 1% SDS, 75°C), and exposed to film again.

Total RNA for Northern analysis was extracted from apple cortical and leaf tissues as described by Ross et al. (1994). RNA (30 µg/lane) was denatured and fractionated on 1% agarose gels containing 0.66M formaldehyde. RNA was transferred to Hybond N<sup>+</sup> membrane by capillary transfer in 0.05M NaOH for at least 15 h. Membranes were hybridised with <sup>32</sup>P-labelled pAP15 insert for 16 h at 65°C, in 750 mM NaCl, 75 mM tri-sodium citrate, 5 × Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. The membrane was then washed for successive 15 min periods in 300 mM NaCl, 30 mM tri-sodium citrate, 0.1% SDS (65°C, twice) and 150 mM NaCl, 15 mM tri-sodium citrate, 0.1% SDS (65°C). The membranes were exposed to Kodak XAR film with intensifying screens at –80°C.

## RESULTS AND DISCUSSION

The pAP15 cDNA insert was fully sequenced (Genbank accession no. L42952) and shown to be 817 base pairs (bp) in length. The sequence contained a single open reading frame of 480 bp encoding a protein of 160 amino acids with a predicted molecular mass of 17.5 kD. The protein encoded by pAP15

**Fig. 2** Southern analysis of apple (*Malus domestica*) genomic DNA probed with  $^{32}$ P-labelled pAP15 insert. DNA was digested with either *Bam*HI (B), *Eco*RI (RI), *Eco*RV (RV), *Pst*I (P), *Sph*I (S), or *Xba*I (X). The membrane was first washed at a stringency allowing c. 15% mismatch (Panel A), then at a stringency allowing 5% mismatch (Panel B).



shares high homology (90%, see Fig. 1) to the protein encoded by *Mal d I* (Genbank accession no. X83672). Both clones code for proteins estimated at the same size. Over the full polypeptide, there are 15 amino acid differences between pAP15, isolated from 'Golden Delicious', and *Mal d I* which was isolated from 'Granny Smith' apple. This suggests the existence of a family of closely related proteins in apple with at least two members.

When the N-terminal amino acid sequence of the allergen protein in the cultivar 'Golden Delicious' was reported (Schoning et al. 1995), two possibilities were given for the amino acid at Position 10 (phenylalanine or tyrosine; Fig. 1). pAP15 codes for the phenylalanine at this position, whereas *Mal d I* codes for the tyrosine (Fig. 1). The sequence of the polypeptide coded by *Mal d I* also differs from the N-terminal sequence of the allergen at Position 17. Southern analysis provided further

evidence for a pAP15-related gene family in apples. When a genomic Southern blot was washed at a stringency that allowed for 15% mismatch, up to 15 hybridising bands could be observed, but after a high stringency wash (5% mismatch) only one or two fragments hybridised (Fig. 2).

The estimated pI of the polypeptide encoded by pAP15 is 5.9. This compares with pIs of 5.3–5.5 from two dimensional SDS-PAGE analysis of IgE responsive proteins in apple extracts (Schoning et al. 1995), a pI of 5.5 for the 18 kD apple allergen (Vieths et al. 1994b), 4.9–5.5 for several other apple isoproteins of 16 kD (Vieths et al. 1994b), and 5.96 for the putative protein encoded by the *Mal d I* clone. The size and pI of the pAP15 polypeptide is also very similar to one of the up-regulated ripening-related proteins from apple which were observed in a previous study in this laboratory (Lay-Yee et al. 1990).

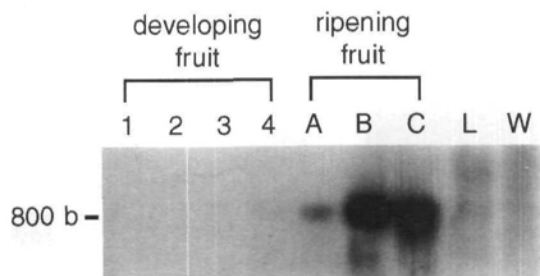


**Fig. 3** Northern analysis of total probed with  $^{32}$ P-labelled pAP15 insert. RNA samples were obtained from developing apple (*Malus domestica*) fruit either 8 (Lane 1), 12 (Lane 2), 16 (Lane 3), or 20 (Lane 4) weeks after anthesis, and from ripening apple fruit, with postharvest ethylene concentrations in the internal core cavity of 0–0.1 ppm (A), 1–10 ppm (B), and > 50 ppm (C). RNA was also extracted from freshly harvested, expanding apple leaves (L) and leaves 8 h after a wounding treatment (W).

Northern analysis indicated that pAP15 mRNA was not detectable during fruit development, but accumulated to high levels in ripening fruit (Fig. 3). Although to our knowledge this is the first report that these pathogenesis-related clones are also ripening-related, enhanced expression of this mRNA in ripening fruit is consistent with observations that the allergenic response may also be ripening-related (Vieths et al. 1993). There was no detectable hybridisation of the pAP15 insert with mRNA from freshly harvested expanding apple leaves, nor with leaf mRNA up to 8 h after a wounding treatment (Fig. 3).

The isolation of a false positive clone using the spinach SPS antibodies suggests the presence of common epitopes between SPS and the polypeptide encoded by pAP15. However, this same antibody has been used previously to screen spinach and potato leaf cDNA libraries, and has resulted in the isolation of only SPS clones (Sonnewald et al. 1993; Sonnewald pers. comm.). Also, Western analysis of apple fruit and apple leaves using the same spinach antibodies, results in only one positive band corresponding to the 120 kD expected size of SPS (Wegrzyn, Reimholz, & MacRae unpubl. data), and no band in the size range of the polypeptide encoded by pAP15. One explanation for this inconsistency is that the pAP15 gene product expressed in *E. coli* is folded differently to the apple protein in vivo, and different antigenic regions of the polypeptide that are displayed externally for

antibody cross-reaction. A comparison of the pathogenesis-, stress-, or allergen-related protein sequences indicates there is a region that is conserved across all the homologues (amino acids 53–56; Fig. 1). This encodes a putative protein kinase C phosphorylation site, with enhanced  $V_{max}$  and  $K_m$  because of the two basic lysine residues following the threonine (Woodgett et al. 1986). Sucrose phosphate synthase is a multi-phosphorylated protein and its activity is regulated by protein kinases and phosphatases (Huber & Huber 1991). One possibility is that a common phosphorylation epitope contributes to the antigenic cross-reaction.

The availability of the clone reported here provides further opportunities in several research areas. Antibodies to the birch pollen allergen *Bet v I* cross-react with an 18 kD protein in apple extracts (Vieths et al. 1994b), but antibodies to the pathogenesis-related protein *PR-10a* (Constabel & Brisson 1992) do not (data not shown). Therefore expression of the pAP15 gene product in culture and subsequent production of specific antibodies should enable better characterisation of this family of proteins. Observations across a range of apple cultivars of the allergenic response in patients could be correlated with analysis of pAP15 gene expression, to provide further evidence that this gene product is a causative agent. Finally, use of antisense technology involving this cDNA may produce an apple which elicits reduced allergenic response. Study of such transgenic apples should also help establish the significance of the production of this class of pathogenic-related proteins.

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