

MdAP, a novel protein in apple, is associated with the major allergen Mal d 1[☆]

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Abstract

Mal d 1, an 18-kDa intracellular pathogenesis-related protein (PR-10), has been known since long as the major apple allergen in Middle and Northern Europe. However, its biological function, as that of many other PR-10 proteins, is still unknown. In order to identify proteins putatively interacting with Mal d 1, an expression library of *Malus domestica* was screened using the yeast-two-hybrid (Y2H) system. A novel protein binding to two isoforms of Mal d 1 being used as 'bait' was isolated. The deduced amino acid sequence from the corresponding full-length cDNA of the predicted Mal d 1-Associated-Protein (MdAP) do not display any homology to known proteins, but shares 45% identity with a 'hypothetical protein' in *Arabidopsis thaliana*. Southern analysis of the apple genome indicated that MdAP, comprising 190 amino acids, is encoded by a single gene. The expression pattern of the 1-kb MdAP transcript resembled the expression profile of the different Mal d 1 isoforms in various apple organs, however at a much lower level. Furthermore, a huge variation in transcription levels of Mal d 1 isoforms was observed in apple tissue. For both, Mal d 1 and MdAP highest amounts of mRNAs were measured in ripe fruits and significantly lower amounts in vegetative tissue by quantitative reverse transcription–polymerase chain reaction (RT-PCR).

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1. Introduction

The expression and accumulation of a vast range of proteins, collectively termed pathogenesis-related (PR) proteins, is a general response of plants upon pathogen and stress attack. Due to their diverse structure, function and biological activity, meanwhile they were classified into

17 families (van Loon and van Strien, 1999; <http://www.bio.uu.nl/fytopath/PR-families.htm>). Amongst them, the pathogenesis-related protein 10 (PR-10) family comprises ubiquitous intracellular proteins with similar structure, size and conserved motifs. Interestingly, many PR proteins of various plant species have been identified as important allergens during the past years (Breiteneder and Ebner, 2000; Hoffmann-Sommergruber, 2000). A considerable number of pollen- and food-derived allergens are grouped to PR-10 or PR-10-like proteins. Bet v 1, the major birch pollen allergen, was the first being classified as one of the PR-10 family (Breiteneder et al., 1989), followed by many allergens identified in other tree pollen, in fruits, nuts and vegetables. Like Mal d 1, the most prominent allergen in apple fruit, a number of homologous proteins share common epitopes reacting with IgE antibodies of tree pollen-sensitized patients. The cross reactivity with a broad range of plant food often results in the so-called oral allergy syndrome, which displays IgE-mediated symptoms at the mucosa of lips, tongue and throat.

Abbreviations: AD, activation domain; Ade, adenine; BD, binding domain; DO, dropout medium; GD, Golden Delicious; His, histidine; Leu, leucine; MdAP, Mal d 1-associated-protein; MI, MacIntosh; PR-10, pathogenesis-related protein 10; RT-PCR, reverse transcription–polymerase chain reaction; T_{ann} , annealing temperature; TE, 10 mM Tris+1 mM EDTA; Trp, tryptophan; Y2H, yeast-two-hybrid; 3-AT, 3-amino-1,2,4-triazole.

[☆] The MdAP sequence is deposited in the EMBL, GenBank and DDBJ Database under the accession number AY262166.

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Based on sequence similarities with a ribonuclease isolated from *Panax ginseng* (Moiseyev et al., 1997) PR-10 proteins were designated as ‘ribonuclease-like’ proteins. However, only weak ribonuclease activity could be determined for the homologous proteins Bet v 1 from birch (Bufe et al., 1996; Swoboda et al., 1996) and LaPR-10 from white lupine (Bantignies et al., 2000), but not for any other member of this protein family. A role as an enzyme of the core phenylpropanoid pathway, which is involved in a general defense response, was suggested for AoPR1 in the monocot asparagus (Warner et al., 1994).

Despite the ubiquitous occurrence in the plant kingdom, the biological role of PR-10 proteins still remains to be elucidated. In many plant species, these proteins are encoded by multiple genes (*Ypr10*), which holds also true for the rosaceous genus *Malus*. The biological meaning of the high variation of PR-10s within one species, e.g. ranging from 69% to 99% identity in *Malus domestica*, still remains a challenging question. In all PR-10 proteins, there is a striking conserved glycine-rich motif GXGXXG at residues 46–51 (Hoffmann-Sommergruber et al., 1997). The three-dimensional structure of Bet v 1, investigated by X-ray crystallography and magnetic resonance spectroscopy (Gajhede et al., 1996), revealed that the glycine rich loop is involved in the formation of a large cavity in the protein. This finding already let suppose an involvement of this structure in a physiological function. Interestingly, an unexpected high structural similarity between the START domain of the human protein MLN64 involved in cholesterol transport and Bet v 1 was reported (Tsujishita and Hurley, 2000). Subsequently, phytosteroids were proposed as putative ligands for Pru av 1, the major cherry allergen (Neudecker et al., 2001). Recently, a Bet v 1 isoform was assumed to have a plant steroid binding activity due to its ability to strongly bind deoxycholate molecules in the deep hydrophobic cavity of the protein (Markovic-Housley et al., 2003). Not being plant metabolites these molecules are supposed to act as molecular mimic. Deoxycholate shares striking structural similarity with brassinosteroids suggesting that these are the natural ligands of PR-10 proteins. Commonly proteins interact in a network with other biomolecules to execute their function. In order to identify a potential protein ligand for Mal d 1, a cDNA expression library was screened for candidates. Here, we report for the first time the isolation and characterization of a novel protein as a putative binding partner for the apple PR-10 Mal d 1.

2. Material and methods

2.1. Plant material and treatment

Three-year-old apple plants (cultivar ‘Jonagold’) grown in the greenhouse were infected under controlled conditions by spraying 2.5×10^5 conidia/ml of *Venturia inaequalis* in

an aqueous solution on young apical leaves. Three weeks after infection, young leaves were harvested, shock frozen in liquid nitrogen and stored at -80°C until RNA extraction. For DNA and RNA extractions, healthy plant material from different cultivars (as indicated) was either derived from rooted tissue culture plants (Laimer da Camara Machado et al., 1991) or from greenhouse grown plants. In some cases (fruits, flowers) field derived material was used.

2.2. Nucleic acid extractions, DNA manipulations and sequence analysis

Total RNA was extracted following the method described by Chang et al. (1993). For constructing the expression library, total RNA was extracted by the GITC-method (Chirgwin et al., 1979). Poly(A)+mRNA was isolated from total RNA by two rounds of oligo-dT coupled magnetic particle selection as indicated by the manufacturer (Dyna). DNA was extracted from plant material according to Lodhi et al. (1994), except that the 5 M NaCl step was omitted and an additional chloroform-isoamylalcohol extraction was performed after RNase digestion.

Unless otherwise stated all DNA manipulations and molecular cloning techniques were carried out according to standard procedures (Sambrook et al., 1989).

Sequencing reactions were performed with the DNA sequencer ABI 377 using the ‘ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit’.

Protein and DNA similarity searches were performed using the NCBI BLAST server and TAIR (The Arabidopsis Information Resource) database. Sequence analysis was performed by Lasergene99 software (DNASTAR, Madison).

2.3. Construction of an expression library

For first strand synthesis, two set-ups each with 3 μg mRNA derived from *V. inaequalis* infected leaves were primed either with random hexanucleotide primers or oligo-d(T)₁₈VN anchor primers to ensure a high representation of domains from the amino-to the carboxy-terminus of the fusion proteins in the library. cDNA synthesis and EcoRI-adaptor ligation was carried out using the TimeSaver cDNA synthesis kit (Amersham) following the supplier’s instruction. Pooled cDNA was purified and size-fractionated (cut off 350 bp) over a 1.2% agarose gel and subsequently ligated into the EcoRI-linearized and dephosphorylated pGADT7 (Clontech) harboring the GAL4-activation domain (AD) and LEU2 reporter gene. cDNA constructs were transformed into *Escherichia coli* DH5 α and approximately 10^6 independent clones were selected on ampicillin containing medium.

2.4. Construction of ‘bait’ and ‘prey’ vectors used in Y2H screening

The complete coding region of two cloned Mal d 1 isoforms, derived from cultivar ‘Golden Delicious’ (GD)

and ‘McIntosh’ (MI), were polymerase chain reaction (PCR) amplified with the degenerate primers *EcoRI*-Md1F 5' NNN GAATTC ATG GGT GTS TWC ACA TWY GAA WMC G 3' and *BamHI*-Md1R 5' NNN GGATCC TTA GTT GTA GGC RTC CKG GTG 3' ($T_{\text{ann}} = 56^{\circ}\text{C}$) under standard conditions. *EcoRI/BamHI* digested and purified PCR products were fused in frame as ‘bait’ to the GAL4-binding domain (BD) in the *EcoRI/BamHI* cut pGBKT7 carrying the *TRP1* reporter gene (Clontech). Additionally, GD and MI Mal d 1 cDNAs were ligated in frame to the GAL4-AD of the *EcoRI/BamHI* cut pGADT7 and the *EcoRI* excised MdAP cDNA from pGADT7 was religated into the *EcoRI*-linearized and dephosphorylated pGBKT7. Correct orientations and in frame fusions were checked by sequencing with vector specific primers. pGBKT7-53 (murine p53 fused to GAL4-BD) and pGADT7-T (SV40 large T-antigen fused to GAL4-AD) were used as a positive control for the Y2H screening (supplied by Clontech).

2.5. Y2H screening

The MATCHMAKER Two-Hybrid-System3 (Clontech) was used for the identification of protein-protein interactions. All manipulations, media and strains were as described in the supplied manual (PT3247-1). 240 μg ‘bait’ vector carrying GD Mal d 1 cDNA were simultaneously co-transformed with the purified expression library plasmids in a molar ratio of 2:1 into the *Saccharomyces cerevisiae* strain AH109 carrying two nutritional marker (*His3*–/*Ade2*–) and two enzymatic reporter (*lacZ* and *Mel1*) genes driven by different GAL4-responsive promoters. Transformants were plated on minimal synthetic dropout medium (DO) lacking tryptophan (Trp), leucine (Leu), histidine (His) and adenine (Ade) (in the following termed $4 \times \text{DO}$). Growing colonies were restreaked on $4 \times \text{DO}$ supplemented with 2.5 mM 3-amino-1,2,4-triazole (3-AT) and 100 μl 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -gal, 2 mg/ml in dimethylformamide) to eliminate background growth and to screen for positive clones showing a blue phenotype, respectively. β -Galactosidase filter assay was performed as described in the ‘Yeast Protocols Handbook’ PT3024-1 (Clontech). Plasmids of positive Y2H-clones were purified and retransformed into *E. coli* DH5 α . Library plasmids carrying an Amp^R marker gene were rescued on ampicillin containing medium. Inserts from the isolated library plasmids were sequenced using vector specific primers.

2.6. Generation of full-length MdAP cDNA

The 5' end of MdAP cDNA was completed by 5' Rapid Amplification of cDNA Ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech) and the gene specific primer 5' GTC TTC TCG CAC TTC CTG ACG AAC TTT CC 3'. The same RNA pool as for the construction of the expression library was used as template and the amplification reaction was carried out as described in the

manufacturer's protocol. The resulting 260-bp fragment was ligated into the TA cloning vector pCRII (Invitrogen) and transformed in *E. coli* INV α F'. Inserts from purified plasmids of two independent clones were sequenced using vector-specific primers.

2.7. Single stranded cDNA synthesis

Total RNA was treated with RNase free DNase I (Roche) and recovered by ethanol precipitation after phenol/chloroform extraction. 2.5 μg total RNA and 2 μM oligo d(T)₁₈VN anchor primer were denatured at 75°C for 5 min and immediately chilled on ice. cDNA was synthesized in a 20 μl reaction by adding 50 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 0.5 mM dNTPs, 20 U RNase Inhibitor (MBI) and 200 U M-MuLV-RT (MBI) at 37°C for 1 h. Reaction was stopped at 95°C for 5 min and chilled on ice.

2.8. Standard and RealTime PCR

Standard PCR was carried out in 25 μl volume containing 50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X-100, 2.5 mM MgCl₂, 200 μM dNTPs, 200 nM primers each and 0.5 U Taq polymerase (Promega) under the following cycling conditions: initial denaturation at 94°C 3 min, 35 cycles of 94°C 45 s, T_{ann} 45 s, 72°C 45 s and a final extension at 72°C for 5 min; T_{ann} is as indicated in brackets for each primer pair. For the amplification of the MdAP gene primer pair MdAP-fwd 5' AAG ATG CAG TGC AAG ACT GAG G 3' and MdAP-rev 5' GCT ACC GCT TGC TTT AGG AAA A 3' was used ($T_{\text{ann}} = 60^{\circ}\text{C}$).

Templates for standards used in RealTime PCR were amplified from cloned cDNAs of MdAP and Mal d 1. The MdAP cDNA insert was amplified with MdAP-fwd and MdAP-rev primer resulting in a 420-bp product. Mal d 1 cDNAs, representing the four different isoform clusters, were amplified with the degenerate primers *Ypr10*-Fdeg 5' ATG GGT GTB TWC AMW TWY GAR AMY G 3' and *Ypr10*-Rdeg 5' TTA GTT GTA GGC ATC SGR GTG 3' ($T_{\text{ann}} = 60^{\circ}\text{C}$), spanning the entire coding region of 480 bp. Amplicons were purified with the QIAquick PCR purification kit (Qiagen) and an aliquot was quantified on a 2% agarose gel by comparing the intensity of the ethidium-bromide (EtdBr) stained fragments with a 100-bp DNA marker of known concentration using the UVP® Image Store7500 system (Ultra-violet Products). Standard concentrations were converted and adjusted to 10^9 molecules/ μl in TE buffer (pH 7.5). Freshly prepared 10-fold dilutions ranging from 10^6 to 10^2 molecules/per reaction for MdAP and 10^8 to 10^3 molecules/per reaction for Mal d 1 were used as standard templates. The RealTime PCR mix was as described for standard PCR, except for 3 mM MgCl₂, 160 nM primer each, 0.75 U Taq polymerase (QBiogene) and 0.2 mg/ml BSA. SYBR®Green I dye (Roche) in a final dilution of 1:20,000 and 4 μl cDNA (diluted 1:20 in

distilled water) were added to a total volume of 24 μ l. The reaction mix was amplified in a thin wall 0.2 ml PCR tube (greiner bio-one) in the RotorGene 2000 thermocycler (Corbett Research) under the following conditions: initial denaturation at 94 °C 90 s and 35 cycles of annealing at 57 °C 5 s, extension at 72 °C 12 s and denaturation at 94 °C 10 s. The optimum temperature for data acquisition has been determined by melting point analysis. Fluorescent signals were recorded at 84 °C, which gave the maximum reading for all specific amplification products when non-specific products could no longer be detected. Specific primers allowing the discrimination of the different Mal d 1 isoform clusters (see Fig. 7) were designed based on the alignment of database stored sequences from highly conserved regions within one cluster and highly divergent regions between different clusters. Primers used for Mal d 1 isoform and MdAP cDNA quantification by RealTime PCR are listed in Table 1.

2.9. Nucleic acid blot analysis

For Southern blots 15 μ g DNA was fully digested with 25 U *Bam*HI and 25 U *Eco*RI, respectively and separated on a 1% agarose gel. DNA was depurinated 10 min in 0.25 M HCl, denatured 2 $\times$ 15 min in 0.5 M NaOH+1.5 M NaCl, neutralized 2 $\times$ 15 min in 1 M Tris–HCl (pH 7.5)+1.5 M NaCl and equilibrated 10 min in 20 $\times$ SSC prior to capillary transfer with the same buffer over night. DNA immobilized on Hybond N+Nylon membrane (Amersham) was fixed by baking 2 h at 80 °C. For Northern blots, total RNA was separated and transferred to the nylon membrane as described by Fourny et al. (1988). Equal loading of the gel was checked by EtdBr-stained rRNA. For preparation of an anti-sense RNA probe the MdAP cDNA was excised with *Eco*RI from pGADT7 and subcloned into the dephosphorylated *Eco*RI site of pBluescript-KS in antisense orientation. The *Eco*RV linearized and purified plasmid was transcribed *in vitro* by the T7 RNA polymerase using the DIG RNA labeling mix

(Roche). The DIG-labeled probe (corresponding to nt 83–216 and 1244–1806 of the *MdAP* gene; Fig. 3) was quantified according to the ‘DIG System and Application Manual’ (Roche). Nucleic acid blots were prehybridized for 2 h and hybridized over night at 65 °C in Church buffer containing 10 ng/ml MdAP-antisense-RNA. Buffer compositions, post hybridization washes and detection were as described previously (Pühringer et al., 2000). Chemiluminescent signals were recorded on X-ray films (Kodak X-omat AR) over night.

3. Results

3.1. Identification of MdAP by Y2H screening

In order to identify a protein in the apple cytosol, capable of interacting with Mal d 1, the GAL4-based two-hybrid system was used. Transcriptional upregulation of *Ypr10* genes upon fungal elicitation has been demonstrated in various plant species (Somssich et al., 1986; Walter et al., 1990; Pühringer et al., 2000). Hence, an expression library of *M. domestica*, containing random cDNAs from *V. inaequalis* infected young leaves fused to the GAL4 AD, was constructed as a source of putative binding partners for the apple PR-10 protein. Two Mal d 1 isoforms derived from cultivar ‘Golden Delicious’ and ‘McIntosh’ and representing rather distantly related isoforms (see also Fig. 7; GD=AJ417551, MI=AY186248), were used as fusions to the GAL4 DNA-BD. Due to an internal *Eco*RI site the Mal d 1 of MI is an 8 amino acid (aa) N-terminal truncated isoform differing in 33 aa from the one isolated from GD. Both constructs were separately transformed into the yeast reporter strain AH109 and none of the constructs activated the expression of the reporter or the nutritional marker genes by itself (data not shown).

The fusion of BD with GD Mal d 1 cDNA was used as ‘bait’ for the initial screening of $\sim 1.1 \times 10^6$ transformants in the library. Nine colonies growing on 4 $\times$ DO were isolated and retested on the same medium+X- α -gal for true protein–protein interaction. Seven colonies showed a blue phenotype expressing Ade, His and α -galactosidase (α -Gal), whereas two colonies displayed a white phenotype lacking α -Gal expression (Fig. 1A). Sequence analysis of library plasmids from positive Y2H clones revealed identical inserts in four out of seven clones coding for a 169 aa peptide of MdAP (Fig. 1A, cl2, 6, 7 and 9). Two clones clearly turned out to be false positive as they contained only the poly(A)-tail with a short 3’ untranslated region (UTR) in the wrong orientation and an untranslatable insert, respectively (Fig. 1A, cl3 and cl4). For the verification of a true protein–protein interaction purified library plasmids of the remaining two positive clones with different inserts (cl5 and MdAP), a false positive clone carrying an untranslatable insert (cl4) and a control vector bearing an unrelated protein (T) were retransformed into AH109 together with

Table 1
Isoform specific Mal d 1 and MdAP primers used in RealTime PCR

cDNA	Primer	Sequence 5'–3' ^a	Product size (bp)
Mal d 1.01	Md1-1F	AAGCTGAAATCCTTGAAGGAA	275
	Md1-1R	GTGCTCTTCCTTGATTCAATG	
Mal d 1.02	Md1-2F	ACACCTCTGAGATTCCACCAC	287
	Md1-2R	CAACTTGGTYTCGTAAGAGAC	
Mal d 1.03	Md1-3F	ACCTCCGTCATCCCCCTG	265
	Md1-3R	TCTTCTCAATTGTCTCAGAGAT	
Mal d 1.04	Md1-4F	CATCGAAGGCGATGGAGGT	241
	Md1-4R	CCTTAGCAYGGTAGTGGCTA	
MdAP	MdAP-F	GTCACCTGACGAGGTGCTCAA	266
	MdAP-R	GGAAAAGCTTCCTGCTGATG	

^a Minimum mismatches in sequences of different isoform clusters are bold; maximum mismatches in sequences belonging to the same isoform cluster are in italics.

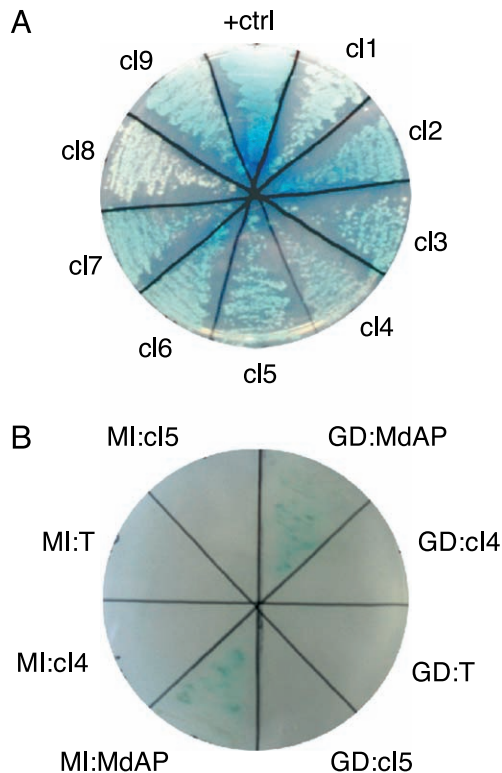


Fig. 1. Verification of initial Y2H colonies obtained by screening an apple library using Mal d 1 as bait. (A) Nine clones (cl) co-expressing fusions of BD-Mal d 1 (GD) and AD-library protein (indicated by numbers) were tested on $4 \times$ DO + 2.5 mM 3-AT + X- α -gal to screen for blue phenotypes showing α -Gal expression: +ctrl=positive control for Y2H screening (as described in Section 2.4); cl1 and cl8 showed a white phenotype lacking α -Gal expression; cl2, cl6, cl7 and cl9 displayed identical library inserts = 169 aa MdAP; cl3 and cl4 were false positives containing poly(A) + 3' UTR and an untranslatable insert, respectively; cl5 harbored a 141 aa peptide. (B) Y2H analysis for confirmation of true positive interactions; fusions of BD (before colon) with MI-Mal d 1 at the left side and with GD-Mal d 1 at the right side were co-expressed with the respective protein fused with AD (after colon): cl4 and cl5 as described in (A), T = SV40 large T-antigen was used as negative control.

the bait vector carrying either the full-length (GD) or the 5' truncated (MI) Mal d 1 cDNA, respectively. Only clone MdAP remained positive with both bait vectors when plated on high stringent medium ($4 \times$ DO supplemented with 2.5 mM 3-AT) + X- α -gal, thus strongly indicating an interaction of the expressed protein with Mal d 1 resulting in Ade, His and α -Gal expression (Fig. 1B). The 169 aa library insert of MdAP was transferred from the AD to the DNA-BD vector and the 'baits' GD and MI vice versa. In order to confirm the specificity of the interaction, the Y2H analysis was repeated with the interchanged 'bait' and 'prey' vectors. The interaction of the cDNA-encoded library protein MdAP with Mal d 1 was still detectable and specific as proven in a β -galactosidase filter assay. Only colonies co-expressing Mal d 1 and the MdAP peptide displayed a blue phenotype in the assay for β -galactosidase activity (Fig. 2A and B).

3.2. MdAP is a novel protein showing homology to a 'hypothetical' protein in *A. thaliana*

The insert of the MdAP clone comprised 169 aa followed by a stop codon and an 187 bp 3' UTR. The 5' end of MdAP cDNA was completed by 5' RACE. The sequence of the amplified fragment entirely matched to 104 overlapping bp at the 5' end of the initial clone. Thus, the open reading frame could be elongated by 21 aa starting with a methionine and additional 19 bp of 5' UTR (Fig. 3). The deduced protein of the full length MdAP cDNA comprising 190 aa has a predicted molecular mass of 21 kDa and a pI of 4.67. The nucleotide and protein sequence did not show any significant similarity to any gene or protein with known function present in databases. The homology search revealed 45% identity to a deduced protein sequence from *A. thaliana* (acc. no. AAD21467) with a similar size of 192 aa (Fig. 4). However, no function has been assigned to this *Arabidopsis* protein, which is annotated in the database as 'hypothetical' protein due to gene prediction programs.

Preliminary experiments using a crude yeast extract containing the expressed MdAP and purified native Mal d 1 to confirm the interaction in a co-immunoprecipitation assay were not successful, possibly due to weak binding and not yet optimized assay conditions. Therefore, the expressed protein was named tentatively *Mal d 1-Associated-Protein* (MdAP) as the interaction has not been proven in an

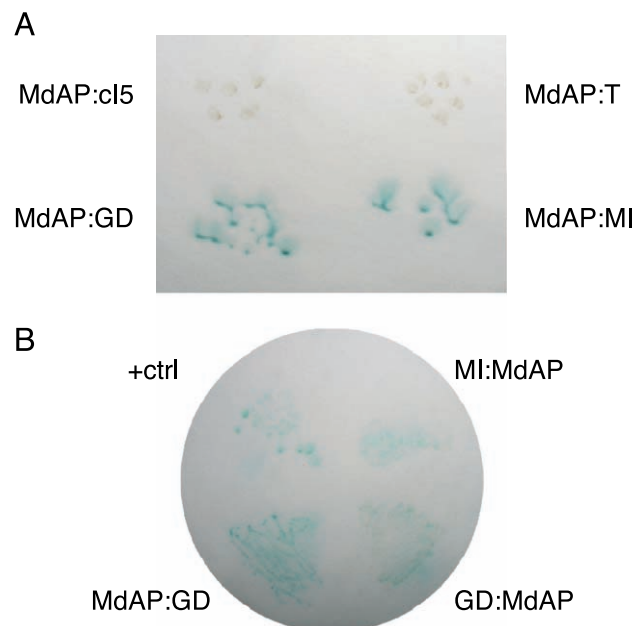


Fig. 2. Confirmation of true protein-protein interactions by β -Galactosidase filter assay. Yeast strain AH109 expressing fusion proteins was grown on selection medium for 3 days, subsequently lifted on filter paper and assayed for β -galactosidase activity at 30 °C for 24 h. Proteins before colon indicate fusions with BD; proteins after colon indicate fusions with AD. (A) Clones were grown on $2 \times$ DO (lacking Trp and Leu); cl5 and T (described in Fig. 1) were used as negative controls. (B) Clones were grown on $4 \times$ DO; +ctrl=positive control (as described in Section 2.4).

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gacagaagggaaagagaaagATGGGGTGGGTGTGGAGGACGACGAGGATGATGACAACAA 60
TTCAACGGCGATTGACATAAAATCCTAGATCGGACGGCGATCGATGCTTGACGAGGAAGGT 120
GGTGAAGATGCAGTGCAAGACTGAGGAGGTGGAGCCTGGAAAGTTCGTCAGGAAGTGCGA 180
      MdAP-fwd      ← 5' RACE primer
GAAGACCGAGCAGCTTCTCAGGGACTGCGCCGGCCGgtaaattcccaatctctctcattct 240
ctctatcgatttctccatttccctaagacccaattggcaaatgggaatatcttatctagg 300
gtttccgtgaattgttggaattgcctgttggtgtcactttgattttgtcatatctatct 360
taattttgggatttttttttaaaattttatgttgggggtgttattattgggcttatggc 420
ttacgatagaaattgacgagaatcataacggttatgatatttgatcgattgttgtaggaaa 480
tattggtttttctgtaattttcattagaacacgattcgacattggatccaaatttgtagag 540
tggttaaattagaagaaatatatatggaataacgagacaagagagatgagaatatgccgc 600
gtctttaatctttatgcattcgtttataaattgttcgagtgacggttggttacagttcag 660
tgaagtatatatagcgtgtggttaataagagcgtgttaataagagcgcacgcataagacgt 720
ctgattagattgtattgattctgcatacagaaagagtaggggctttgatttgattacat 780
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tgtctttcgagtcctttgggacgtaattggagctatctagtgcgtgcagtgcataaaaaag 1240
aaccttttcgctttcttccttgtttgagtatgggcggtttccatgttgcgataacattg 1200
accaatgtagagccaattgctgagcactcttgctttttgttagGCCTGTAGAAGTGGTGC 1260

AATCCAACAAAGAGTACACTGAACATGACGTCAGGAGGTGCTCAAAGGGTCTGCCA 1320
      MdAP-F      →
CCTTTGGGTCATCACAGCACGGAGCATTGGACTTCCCAGGGCTACGCGGTGACATTGAAG 1380
ACATTGAACGCAACTTTATGGGTGGCCTCAGCCGATTCTTTGAGGCTGCTGAGGAGTTGA 1440
AGAGCGGCTTCTTCAGTGCTTTTGGGAACATCTTTGATGAGGGACCTTCTCCCCACTAC 1500
CTCCTAGGAGGCGCGAGGTACCCATAGAAGGCCATCATACAGCAGGAAGCTTTTCCTAAAG 1560
      MdAP-R      ←
CAAGCGGTAGCGACGACTCAGGACACGTTGATCTCTCTGGCTTGGCAAGAGAAGTTtgaa 1620
MdAP-rev
gcagagttgtccattgcccgtgataagttaaaagaatgaccacctcggtttacctgtag 1680

tatcgatatcttcccttgtacttaatatagagtacaacatgaaatttctcgcgtagagta 1740
gagcagttcctatgaaggaatttagcatgaatccatcatcctgccgtacgatgtgaatg 1800
cgctg 1806

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Fig. 3. DNA sequence of *MdAP* gene. Coding region is represented by capital letters; lower case letters indicate 5' and 3' UTR; intron sequence is indicated in italics. Start and stop codon and *Bam*HI site (in intron) are given in bold; 5' RACE primer and RealTime PCR primer sequences are displayed by arrows; primers used for gene amplification are boxed in grey.

independent assay. Besides several putative phosphorylation sites, the tripeptide motif RGD, known as cell attachment sequence, was found at aa position 107–109. However, the deduced protein sequence does not contain a signal peptide nor any known organelle-targeting or transmembrane motifs and therefore points to a cytosolic protein rather than a cellular matrix or transmembrane protein.

3.3. The single *MdAP* gene consists of two exons and an intron

Southern analysis of genomic DNA was conducted in order to investigate the gene copy number. Isolated DNA from two randomly chosen apple cultivars was digested with *Eco*RI and *Bam*HI, both enzymes were selected as

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M.d.  MGWVWRDDEDDNNSTAIDINPRSD----GDRCLTRKVVKMQCKTEEEVEP 46
A.th.  MGWIWIDDSSASKAGADLTDKPLGDSAAAENCSTTTTVVRSQCKTEEEVEP 50

M.d.  GKfVRKCEKTEQLLRDCAGRPVEVVQSNKEYTEHDVTDEVLKGSATFGSS 96
A.th.  GKfVRKCDKTEEILRHCFGKPSSEVVQSNTEETEDVTDQMVGRSALPNQF 100

M.d.  Q-HGALDFPGLRGDIEDIERNFMGGLSRFFEAABEELKSGFFSAFENIFDE 145
A.th.  EDKNPLNFPGLRRDVAIERHFLSGMKSFDDAAEEMTSSLFDIMG---DH 147

M.d.  GPSSPLPPRRREVPIEGHHQOEAFP----KASGSDDSGHVDLSGLAREV 190
A.th.  HPST----RRGGIPIENHPKVEEHRNDETAPTRPHSSGEIDLSGLAKDV 192

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Fig. 4. Amino acid alignment of the deduced sequence of MdAP in *M. domestica* (M.d.) and the homologous protein in *A. thaliana* (A.th.). Alignment was performed using ClustalW (<http://www.ebi.ac.uk/clustalw>); identical residues are boxed in black; conservative substitutions are in grey boxes; intron position is indicated by arrows.

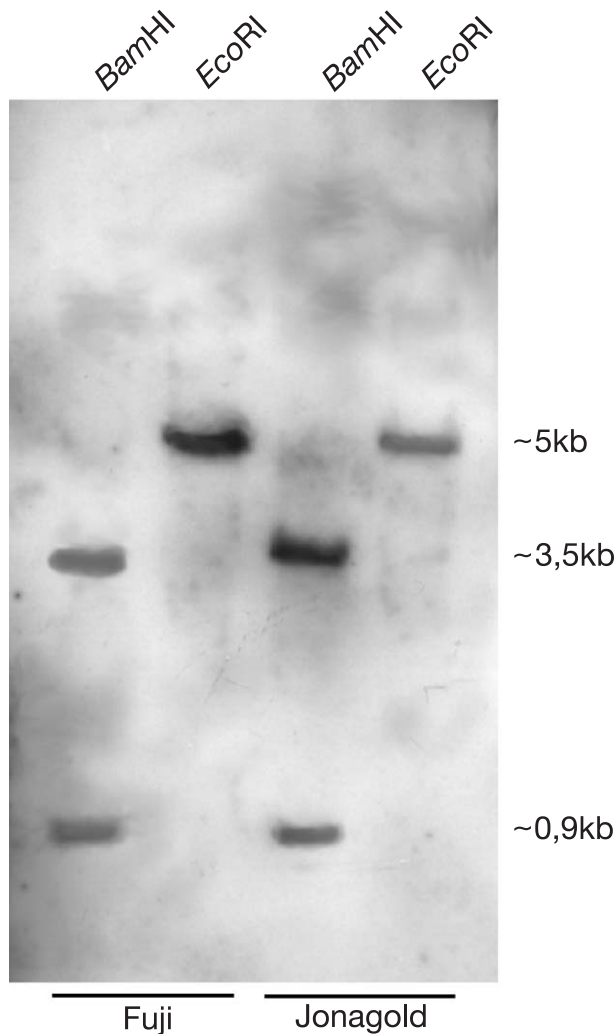


Fig. 5. Southern hybridization for detection of *MdAP* gene copy number. A total of 15 µg genomic DNA isolated from apple cultivars Jonagold and Fuji was restricted as indicated and hybridized to the DIG-labeled *MdAP* antisense RNA probe under high stringent conditions. Approximate fragment size is indicated in kb.

non-cutting in *MdAP* cDNA. Separated fragments were probed under high stringent conditions with a DIG-labeled *MdAP*-antisense RNA. Both cultivars displayed an identical pattern with a single band in the *EcoRI* digest and surprisingly two bands in the *BamHI* digest (Fig. 5). Sequence analysis of the *MdAP* gene amplified from genomic DNA revealed an A/T rich (65%) intron sequence with an internal *BamHI* site. Hence, *MdAP* is encoded by a single gene, consisting of two exons and an intron showing the conserved splice sites GT and AG at the 5' and 3' end, respectively (Fig. 3). The 1027 bp intron interrupted the coding region within codon 66 at the 2nd nucleotide position. A similar structure displayed the gene encoding the homologous protein in *Arabidopsis*, where the coding region is interrupted by a single 759-bp intron at codon 69 (see Fig. 4).

3.4. In apple organs *Mal d 1* isoforms and *MdAP* are transcriptionally expressed in a similar pattern but at different levels

Mal d 1 mRNA was demonstrated to be highly expressed in ripe fruits and old leaves, whereas the expression in young leaves was reported to be at a low level, but could be upregulated upon challenge with various abiotic and biotic stress factors (Atkinson et al., 1996; Puehringer et al., 2000). In view of a putative interaction of *Mal d 1* and *MdAP*, the question whether both transcripts are co-regulated or rather differentially expressed in defined plant organs is of great interest.

By Northern blotting a single transcript approximately 1 kb in size hybridizing to the *MdAP* antisense RNA probe could be detected in 10 µg total RNA of apple fruits, but was not detectable in mature leaves (Fig. 6). A weak hybridization signal was recordable after over night exposure that in relation to the intense signal previously obtained with a similar labeled *Mal d 1* probe on half amount of RNA after 30 min exposure (Puehringer et al., 2000) pointed to a much lower expression of *MdAP*. A quantitative PCR based on the monitoring of intercalated SYBR®Green I fluorescence was applied for relative quantification of *MdAP* and *Mal d 1* transcript expression. As *Mal d 1* is encoded by a multigene family, the expression profile of different isoforms was investigated by RealTime PCR using isoform-specific primers. Recently cDNA sequences derived from apple fruits were summarized in two major groups, *Mal d 1a* and *Mal d 1b* (Son et al., 1999). But many sequences mainly identified from vegetative tissue are clearly diverging from these two groups. Therefore, the numerous sequences stored in the database were divided into four clusters based on DNA sequence similarities of their coding regions. *Mal d 1* isoform primers, designed to discriminate between the four clusters, were tested for their specificity on recombinant DNA representative for each cluster (Fig. 7A). A phylogenetic tree was drawn to give a

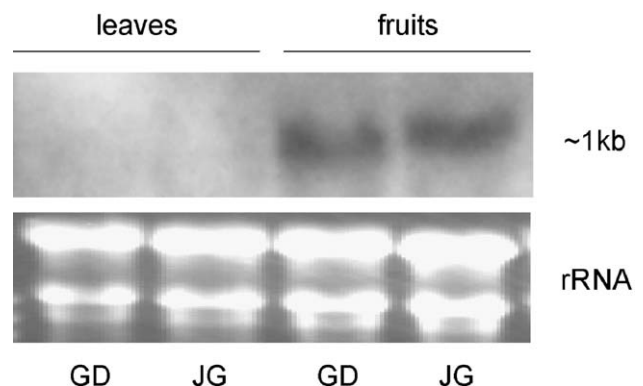


Fig. 6. Northern analysis of *MdAP* transcript expression in non-infected apple fruits and leaves. 10 µg total RNA was analyzed by gel blotting using the same probe and conditions as described in Fig. 5. Equal loading of the gel was checked by EtBr stained rRNA. GD=Golden Delicious, JG=Jonagold.

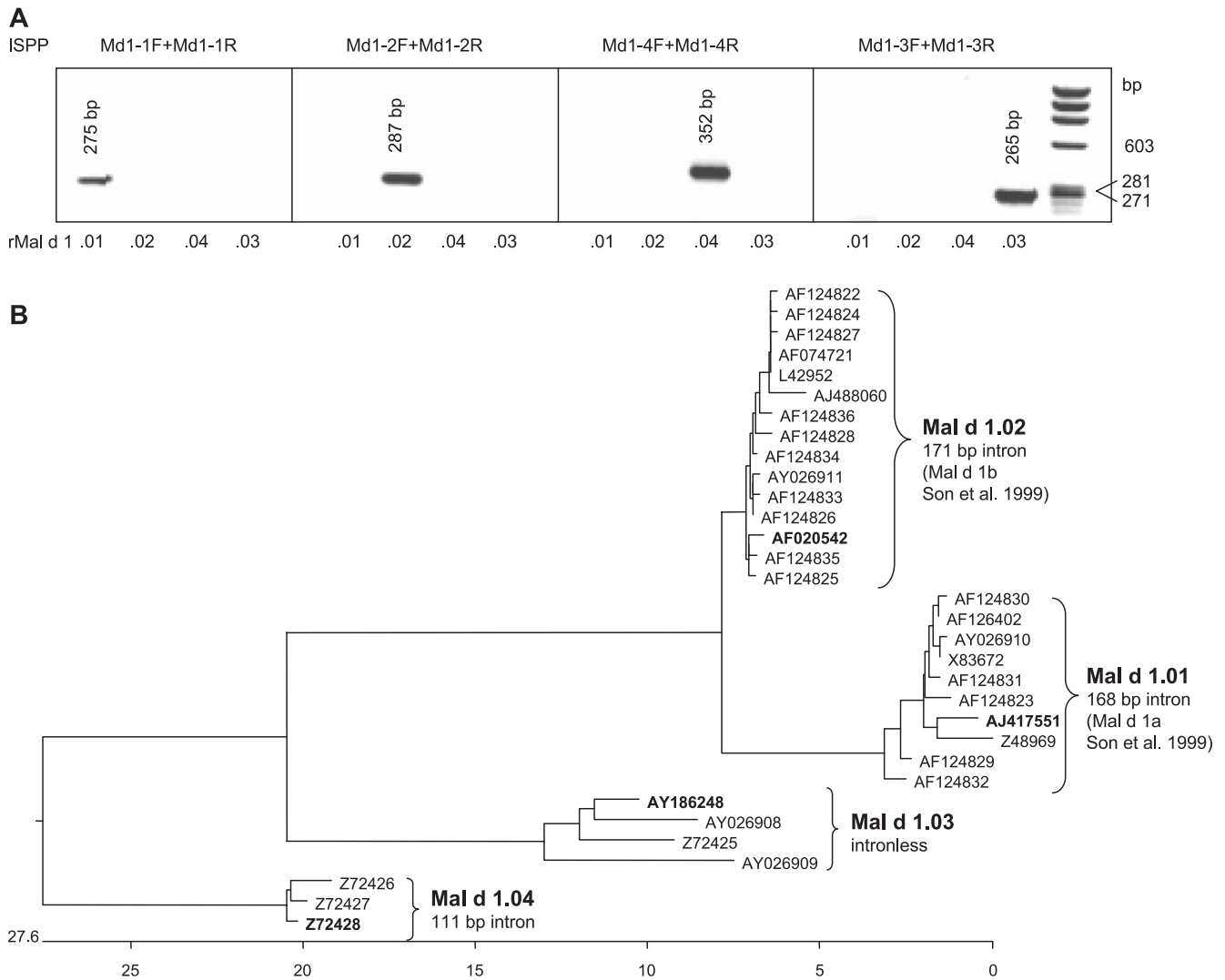


Fig. 7. Mal d 1 isoform clusters. (A) Isoform specificity of Mal d 1 primers was verified by amplification of cloned DNA (highlighted in panel B) representing the different clusters. Amplified fragments were separated on a 2% agarose gel. ISPP=isoform specific primer pair; rMal d 1=recombinant Mal d 1 cDNA [Mal d 1.01 and 1.02] and recombinant Mal d 1 gene [Mal d 1.03 and Mal d 1.04 (comprises 111bp intron)]. (B) Phylogenetic relationship of sequences encoding Mal d 1 isoforms. DNA sequences were retrieved from GenBank and coding regions were aligned and displayed in a cladogram using Megalign from Lasergene99 software. The length of each pair of branches represents the distance between sequence pairs, while units at the bottom of the tree indicate the number of substitution events. Mal d 1 sequences used as 'baits' in Y2H screening or as templates in RealTime PCR are in bold.

first estimate of the relationship between homologous sequences (Fig. 7B). In addition to isoform clusters Mal d 1.01 and Mal d 1.02 corresponding to Mal d 1a and Mal d 1b, clusters Mal d 1.03 and Mal d 1.04 were introduced. Subsequently all Mal d 1 isoform clusters were investigated in terms of their mRNA transcription in apple tissue. Expression of MdAP and Mal d 1 isoform transcripts in relation to each other was measured in the same cDNA pool transcribed from 25 ng total RNA. A huge variation in transcript levels between Mal d 1 isoforms and MdAP was observed in all investigated apple organs (Fig. 8). The differences in expression varied from >6 million cDNA copies of Mal d 1.02 in ripe fruits to values slightly above the detection limit of 1000 copies for MdAP in roots (note the different scaling in Fig. 8). Mal d 1.02 was by far the

most abundantly expressed isoform in apple fruits, followed by Mal d 1.01 and Mal d 1.03. A similar profile of isoform transcript distribution was observed in flowers, however at a several magnitudes lower expression level, much like that observed in young leaves. In contrast, Mal d 1.01 was predominantly expressed in vegetative tissues with the highest accumulation in mature leaves, an intermediate expression in roots and a low expression in young leaves. A comparable pattern, but with decreased transcription levels was observed for Mal d 1.02 and Mal d 1.03, except for the latter almost similar values were measured for mature leaves and roots. Surprisingly, only in mature leaves a considerable transcript expression of Mal d 1.04 (>5.317 cDNA copies) was measured, while the expression of this isoform cluster was <1000 cDNA copies in all other apple

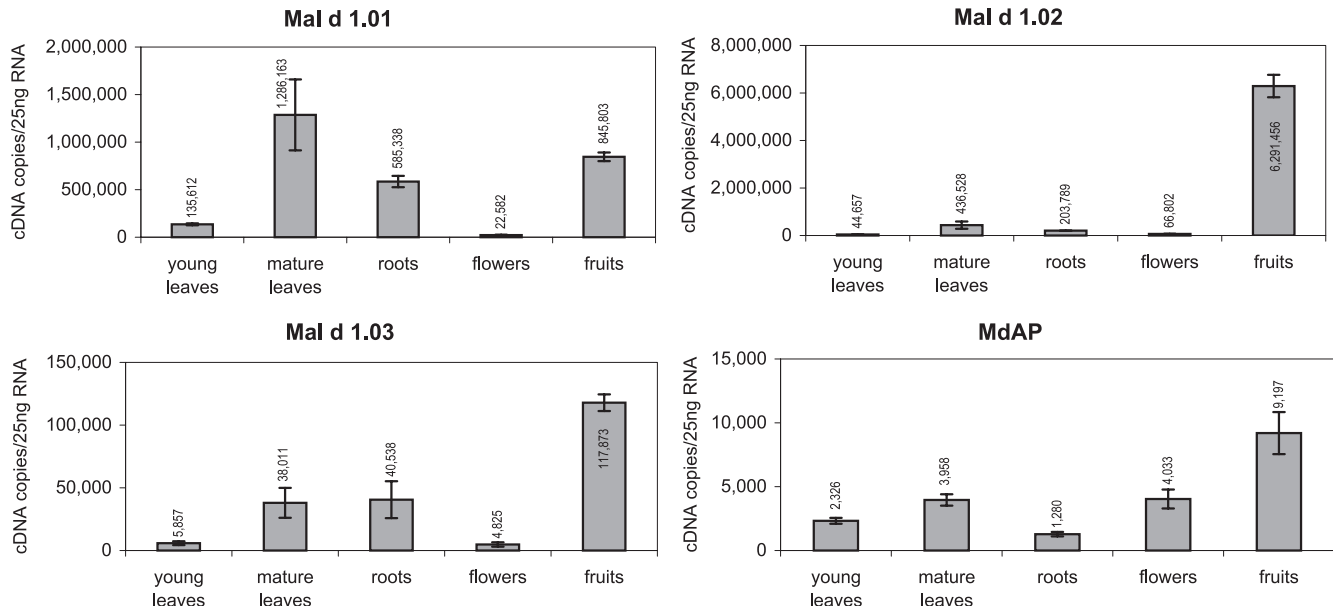


Fig. 8. Quantification and comparison of MdAP and Mal d 1 isoform transcript expression in healthy apple organs by RealTime PCR. Values represent the mean $\pm$ S.D. of at least two independent measurements with duplicates each from two different cultivars (Golden Delicious and Jonagold).

organs (data not shown) and thus below the reliable detection limit of our SYBR[®]Green I based quantitative PCR system.

Compared to the relatively high abundance of Mal d 1 transcripts MdAP mRNA turned out to be rather rare with values even below the lowest expressed Mal d 1.03 isoform. However, in apple organs with very high Mal d 1 transcript expression like fruits and mature leaves, MdAP mRNA levels were found to be also highest. Besides the expression in flowers where in relation to Mal d 1 MdAP showed a significantly higher expression, the MdAP mRNA pattern in apple organs resembled that of Mal d 1 at a much lower level.

4. Discussion

For the first time, a protein associated with a PR-10 is now described in apple. We have identified a novel protein that interacts with two rather divergent Mal d 1 isoforms in a Y2H system. Its novelty concerns both the protein as it does not resemble previously identified proteins, and the fact that a protein exists which is likely to be an interactor of Mal d 1. Besides the mere sequence of a 'hypothetical' protein in *Arabidopsis* up to now there is no report about a MdAP related protein in other plants. The RGD tripeptide, described as a cell attachment sequence, is present in MdAP but not in the homologous protein in *Arabidopsis*. This motif has recently been demonstrated to play a role in cell wall-associated defense responses to fungal pathogens (Mellersh and Heath, 2001). However, in regard of the most probably cytosolic nature of MdAP, the biological meaning of this motif remains speculative.

Although currently a biological significant interaction between Mal d 1 and MdAP has still to be proven by an independent method, the fact that both proteins interact in both configurations (as bait and as prey) in the Y2H system is usually considered as a 'true positive' interaction. Furthermore, based on the fact that in yeast MdAP interacts equally well with two rather distantly related Mal d 1 isoforms we conclude that the binding is likely to be isoform-independent. Nevertheless, a further assay (like an optimized co-immunoprecipitation assay, a far-Western analysis or a Fluorescence Resonance Energy Transfer test) aiming at the confirmation of the interaction between Mal d 1 isoforms and MdAP will have to be conducted.

Compared to the generally high expression levels of Mal d 1 in apple fruits and mature leaves MdAP showed a dramatically (100–1000-fold) lower transcript expression. Despite the low values a significantly enhanced MdAP transcription in ripe fruits as well as a clear tendency for transcript accumulation in mature leaves was observed and reflected the Mal d 1 pattern at a much lower level. Albeit the physiological relevance for both proteins is still not known, their mRNA accumulation in the same tissue might also point to a common rather than a diverse function.

Investigations on distinct Mal d 1 mRNAs revealed a high polymorphism in the expression. Our findings that Mal d 1.02 and Mal d 1.01 are by far the most abundantly expressed isoforms in ripe apple fruits underline the allergological relevance demonstrated for the corresponding groups Mal d 1b and Mal d 1a (Son et al., 1999). The tremendous accumulation of Mal d 1.02 transcripts is in accordance with the data obtained at the protein level by quadrupole time-of-flight mass spectrometry (Helsper et al., 2002). Only Mal d 1b-like isoforms could be detected in

affinity-purified Mal d 1 derived from ‘Granny Smith’ apples and the authors suggested that other isoforms most likely represent less than 2% of the total Mal d 1. However, in this work, we demonstrated that at least at the transcriptional level Mal d 1.01 represents approximately 12% of all Mal d 1 transcripts in fruits.

Furthermore, the results of the Mal d 1 isoform expression studies clearly pointed out that it is Mal d 1.01, which is contributing most to the high constitutive expression of Mal d 1 in mature leaves as reported previously (Atkinson et al., 1996; Pühringer et al., 2000). Although we cannot exclude an individual expression of single genes deviating from other members within the clusters, the RealTime PCR data obviously demonstrate that the four major Mal d 1 isoform clusters are represented differentially in apple organs concerning their mRNA levels.

Recently, the local and systemic upregulation of APa and to a lower extent of APb transcripts upon treatment of young apple leaves with acibenzolar-*S*-methyl, a functional analogue of salicylic acid, was reported (Ziadi et al., 2001). In our terminology, APa transcripts (acc. no. AY026908, AY026909) belong to cluster Mal d 1.03 and APb (acc. no. AY026910, AY026911) to cluster 1.01 and 1.02, respectively (see Fig. 7B). These data are in line with our previous finding that the promoter of a Mal d 1.02-like isoform is stress and pathogen-inducible (Pühringer et al., 2000) and further indicate that genes of all isoform clusters can be upregulated in young leaves upon stress attack. The differential expression of Mal d 1 isoforms in apple tissue might point to a specific function of each isoform.

Although the identification of MdAP with yet unknown function did not lead to the revelation of the meaning of Mal d 1 in apple plants, the existence of a novel protein associated with Mal d 1 is a further step in the complex puzzle of elucidating the biological role of PR-10 proteins. It is conceivable that these proteins might be associated in a multimeric complex to exert their biological function. However, the question what this complex is doing in the cell still has to be clarified. First indirect hints for a biological function of PR10s come from recent publications about the binding of steroid molecules by some members of this family. For Pru av 1 and Bet v 1 a binding activity for phytosteroids has been suggested (Neudecker et al., 2001; Markovic-Housley et al., 2003). Hydrophobic molecules, like brassinosteroids, need to be sequestered to overcome their low solubility in the cytosol and consequently to be transported to their site of action. This role is supposed to be taken over by PR10s that could exert a function as a transport protein.

As many cellular proteins function as components of larger multimeric complexes it is also conceivable that distinct components are involved in such a carrier function. In case of Mal d 1 we could hypothesize that the binding of MdAP for instance could lead to a stabilization of the complex, thus facilitating the transport of the target molecule. Mal d 1-MdAP interaction might provide a stronger

interface that enables the complex to fulfill its biological role. This hypothesis is therefore not in contradiction to the recent suggestion that the natural ligand of the PR-10 protein might be a phytosteroid. Considering the fact that there is no coordinate expression of PR-10s in plants it is difficult to assign a unique function to all members of this protein family.

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