



Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-S-methyl, a functional analogue of salicylic acid

SMAÏL ZIADI^{1,2}, PASCAL POUPARD^{1*}, MARIE-NOËLLE BRISSET²,
JEAN-PIERRE PAULIN² and PHILIPPE SIMONEAU¹

¹Unité Mixte de Recherches 'Pathologie végétale' 077, Laboratoire de Microbiologie Végétale, Université d'Angers, UFR Sciences, 2 boulevard Lavoisier, F-49045 Angers Cedex 01, France and ²Unité de Pathologie Végétale et Phytobactériologie, INRA, Centre d'Angers, 42 rue Georges Morel BP 57, F-49071 Beaucouzé Cedex, France

(Accepted for publication July 2001 and published electronically 1 October 2001)

This study is focusing on the identification of PR-10 genes expressed in leaves of *Malus domestica* L. cv. Golden Delicious treated with acibenzolar-S-methyl (ASM), a functional analogue of salicylic acid. The complete sequence of four cDNAs (named AP1 to AP4) was determined and the corresponding genes were grouped into two subclasses: APa including AP1 and AP4, and APb regrouping AP2 and AP3. The nucleotide sequence of AP2 plus upstream regulating sequences was found nearly identical to the previously described gene *Ypr10*Md.a*. By contrast, members of the APa subclass were considered as yet undescribed apple PR-10 genes. Results of gene expression show that the transcripts of the APa subclass strongly accumulated from 20 up to 120 h after ASM application in treated leaves and systemically in upper untreated leaves 120 h after ASM treatment. Significantly lower levels of induction were recorded with transcripts of the APb subclass. Using immunoblot analysis, two polypeptides of 17 and 18 kDa, respectively, were detected in leaves 48 h after ASM application. Immunodetection performed on leaf sections allowed the localization of proteins in the palisade parenchyma and the vascular tissues. The results suggest that the apple PR-10 genes, in particular those of the APa subclass, which are strongly inducible by ASM, could play a role in local and systemic defences. © 2001 Academic Press

Keywords: *Malus domestica* L. cv. Golden Delicious; PR-10 genes; acibenzolar-S-methyl; defence mechanisms.

INTRODUCTION

Pathogenesis-related (PR) proteins are part of the battery of plant inducible defences. They accumulate locally and systemically upon pathogen infection and the coordinated activity of several PR genes may be necessary for resistance against pathogens [17]. PR genes are mainly activated in a salicylic acid dependant manner [10], leading to the expression of systemic acquired resistance (SAR). However some PR genes are activated through a salicylic acid independent pathway involving jasmonic acid and ethylene [46]. PR proteins are currently

classified in 14 families. With the exception of the PR-10 family, they have known biological properties with mainly antimicrobial activities [38].

The PR-10 family whose type member is Parsley "PRI" [32] regroups acidic proteins with high similarity in protein size (16–19 kDa), amino acid sequence and gene organization. They are closely related to allergens, such as Bet v 1, the major birch pollen allergen [5] and Mal d 1, the major apple allergen [41]. The induction of PR-10 genes has been described in several plant species upon pathogen attack or pathogen derived-elicitor treatment [4, 21, 34]. In addition, the expression of these genes can be induced by various abiotic stimuli such as application of salicylic acid [39, 42], wounding [26, 44], ozone exposure or drought stress [23], and hormonal treatment [25]. Moreover some plant physiological stages such as leaf senescence [42] are also known as PR10-inducible factors.

These various studies suggest that PR-10 proteins are functionally involved in plant defence mechanisms as well as in plant development. However their biological function remains unclear. These proteins contain no signal

* To whom all correspondence should be addressed. E-mail: pascal.poupard@univ-angers.fr

Abbreviations used in text: ASM, acibenzolar-S-methyl; DEPC, diethylpyrocarbonate; NYS, *N*-glycosylation site; PCR, polymerase chain reaction; PR, pathogenesis-related; SAR, systemic acquired resistance; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

The CDNA sequences reported will appear in the GenBank data base under the following accession numbers: AY026911 (*Ypr10*Md.a*), AY026908 (*Ypr10*Md.b*), AY026910 (*Ypr10*Md.c*), AY026909 (*Ypr10*Md.d*).

peptide, suggesting that they are intracellular [37]. They have been functionally related to ribonuclease in bean [42], in birch [7] and more recently in lupin [3]. On the other hand, conserved motifs located on PR-10 sequences have been related to specific functions like phosphorylation sites characteristic for protein kinases [3, 18].

In apple, several studies have been reported on PR-10 members, most of them in relation to the allergic nature of these proteins [36, 41]. However, a cDNA named AP15, encoding a PR-10 protein and accumulating during fruit ripening was characterized [2], whereas the promoter of the gene encoding AP15, was demonstrated to be stress- and pathogen-inducible [27].

Recently, acibenzolar-S-methyl (ASM or 1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester), a functional analogue of salicylic acid described as a SAR inducer [14, 20], has been shown to protect apple seedlings and apple trees from fire blight, a disease caused by *Erwinia amylovora* [6]. This protection was related to the accumulation of two defence-related enzyme families, β -1,3-glucanases and peroxidases. In the present study, we used ASM as a potential inducer of PR-10 genes in leaves of apple seedlings. This allowed us to identify two subclasses of PR-10 genes and to analyse their pattern of expression at the transcriptional and translational levels.

MATERIALS AND METHODS

Plant material and ASM treatment

Apple seedlings from open-pollinated *Malus domestica* L. cv. Golden Delicious were grown in a greenhouse at temperatures between 15 and 22°C under natural photoperiod. Two days before ASM treatment, a supplementary artificial light was applied to maintain permanent light during the experiment. ASM (Novartis) was dissolved in distilled water at a concentration of 1 mM. Beyond this concentration some ominous physiological effects such as a plant growth reduction are observed as previously reported in cauliflower [16]. Apple seedlings (six to eight leaves, approx. 30 days old) were sprayed to run off with ASM (or water for the control). The two youngest leaves were collected at different times (8, 16, 20, 24, 32 and 48 h) after activator or water treatment and stored at -80°C until extraction of RNA or proteins. For some experiments, the two youngest leaves were protected from the application of ASM or water. Treated and untreated leaves were sampled 48 and 120 h after the treatment and stored as described above.

RNA extraction

Total RNA was extracted from 0.2 g of leaves ground in liquid nitrogen then transferred to a microfuge tube

containing 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 5 % (w/v) SDS). The tube was mixed by inversion and incubated for 10 min at 60°C. Potassium acetate 3 M pH 5.0 (240 µl) was added and the mixture was incubated for 2 min at room temperature. After centrifugation (5 min, 8000 g), one volume of isopropanol was added to the supernatant, and the tube was incubated for 15 min in ice, followed by centrifugation for 15 min at 15 000 g. The pellet was dissolved in 100 µl H₂O treated with diethylpyrocarbonate (DEPC), supplemented by 20 µl of 100 % ethanol and incubated for 10 min in ice. After centrifugation (5 min, 8000 g), the supernatant was collected and total RNA was precipitated overnight at -20°C by adding 40 µl of 10 M LiCl. After centrifugation for 30 min at 15 000 g at 4°C, the pellet was washed first with 3 M LiCl and then with 80 % ethanol. The pellet was then dried at room temperature, dissolved in H₂O (DEPC), treated with RNase-free DNase and stored at -80°C.

cDNA cloning

Two degenerated primers, PR-10-1 (5' GGATCCTTGARGGARAYGGWGG 3') and PR-10-2 (5' CCHGGAACCATCAARAAGATC 3'), custom-designed from multiple sequence alignments, were successively used to amplify putative PR-10 transcripts from apple leaves collected 24 h after ASM treatment. The first strand of cDNA was synthesized with M-MLV reverse transcriptase (200 U) from 1 µg of total RNA primed by 100 ng of an oligo-d(TT) adapter primer (5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT 3'). A first round of amplification was performed with PR-10-1 and oligo-d(T) adapter primers (500 nM each) using 2 µl of the reverse transcription products in polymerase chain reaction (PCR) buffer [75 mM Tris-HCl, pH 9.0, 20 mM (NH₄)₂SO₄, 0.01 % (w/v) Tween 20, 3 mM MgCl₂] containing 0.2 mM dNTPs and 1 U of Taq DNA polymerase (Eurogentec, Liège, Belgium). PCR was performed using a thermojet equibio thermocycler (Eurogentec, Liège, Belgium) with the following parameters: one cycle of denaturation at 92°C for 5 min, annealing at 48°C for 3 min and extension at 72°C for 15 min; three cycles at 92°C for 1 min, 49°C for 1 min and 72°C for 2 min and 22 cycles at 92°C for 1 min, 50°C for 1 min 30 s and 72°C for 2 min. A final elongation was performed at 72°C for 15 min. A nested PCR amplification was then realized using 2 µl of diluted PCR products as template, and 500 nM of the PR-10-2 and adapter (5' GACTCGAGTCGACATCG 3')-primers. Thirty five cycles of denaturation at 95°C for 30 s, annealing at 50°C for 50 s and extension at 72°C for 2 min were performed with a final extension at 72°C for 15 min. Amplified cDNA fragments were isolated from a 1.2 % agarose gel in 1 × TAE buffer (40 mM Tris-acetate,

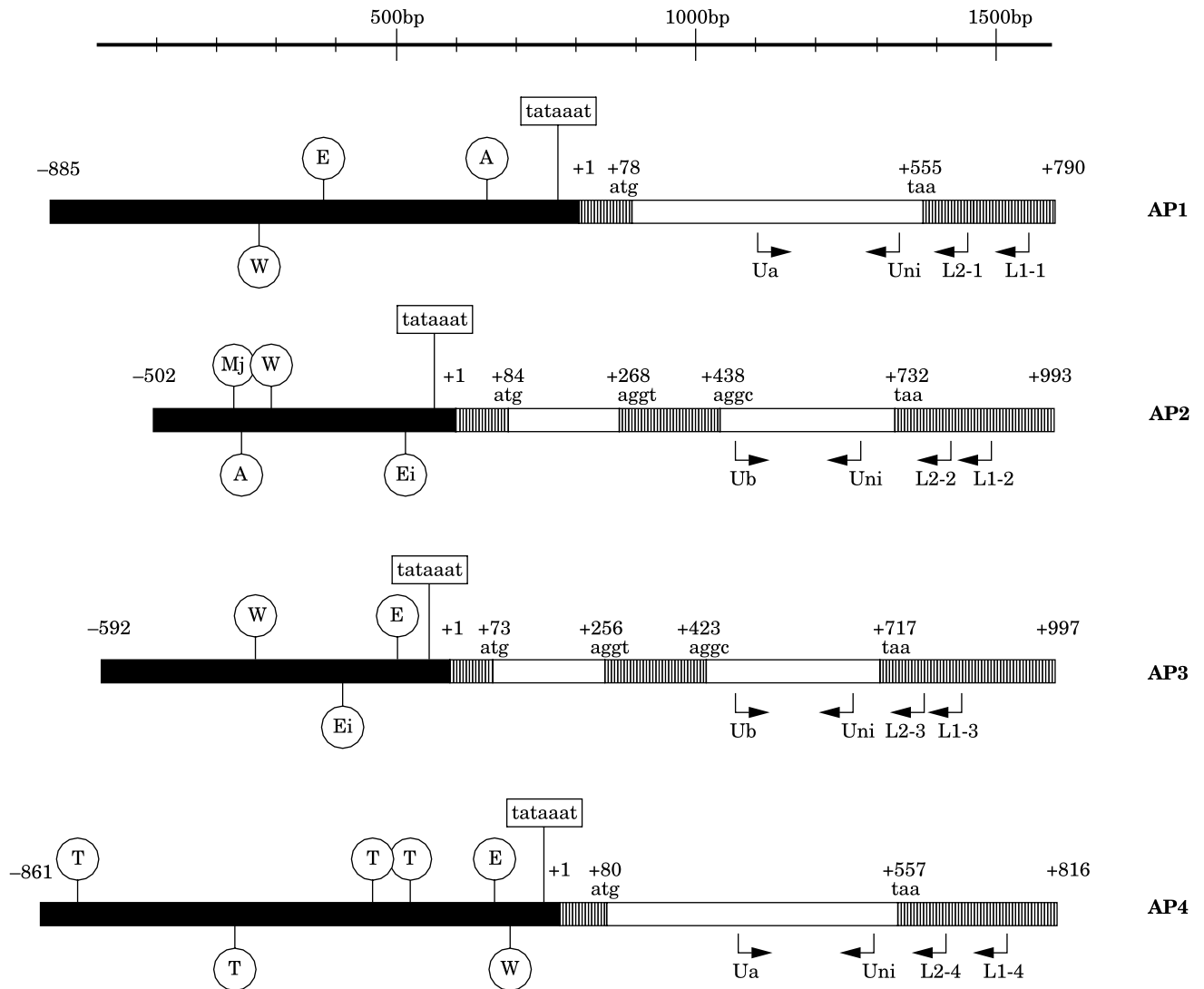


FIG. 1. Schematic representation of the four genomic sequences AP1, AP2, AP3, AP4 and their upstream promoter regions (represented by black boxes). Untranslated regions of the transcribed sequences (including the single intron for AP2 and AP3 sequences) are represented by grey boxes and translated ones by white boxes. Position of initiation and stop codons, potential transcription start and TATA box, PCR primers and putative binding sites for transcription factors (A: AUXRR-core, E: ERE-element, Ei: EIRE-motif, Mj: CGTCA-motif, T: TCA-element, W: box-W motif) are indicated.

pH 8.0, 1 mM EDTA) using a GeneClean kit (BIO 101, Carlsbad, CA, U.S.A.). Purified DNA fragments were ligated into pGEM-T vector (Promega, Madison, WI, U.S.A.) and transformed into the *DH5a* F'IQ strain of *Escherichia coli*. The cDNA inserts were sequenced by Genome Express (Grenoble, France).

A rapid amplification of cDNA ends (RACE) method [15] was then used to determine the unknown 5'-end sequence of the cDNAs. First, cDNAs were synthesized from 4 µg of total RNA primed with 100 pmol of an antisense specific primer (L₁-1 for the AP1 gene, L₁-2 for the AP2 gene, L₁-3 for the AP3 gene and L₁-4 for the AP4 gene, see Fig. 1). Then cDNAs were purified with a GLASSMAXDNA isolation kit (GIBCO BRL, Rockville, MD, U.S.A.) following the manufacturer's protocol.

A polyA tail was incorporated at the 5'-end of the cDNAs using terminal transferase and the tailed cDNAs were used as template for PCR amplification (first round PCR) using the oligo-d(T) adapter primer (see above) and a specific reverse primer (L₂-1 for AP1, L₂-2 for AP2, L₂-3 for AP3 and L₂-4 for AP4, see Fig. 1). For second round amplification, the diluted first round PCR products were amplified using the adapter primer (see sequence above) and the Uni reverse primer (Fig. 1). The PCR parameters were as described above.

Sequence analysis

Alignment of amino acid and nucleic sequences was performed with the CLUSTALW program on line.

Phylogenetic analysis was achieved according to the neighbour joining method using the phylogeny inference package (PHYLIP, version 3.5). Homology search was done with the BLAST program [1]. The sequences were screened for putative transcription factor binding sites (nucleic sequence) and specific amino acid patterns (proteic sequence) by scanning the Plantcare and Prosite databases on line respectively.

PCR walking in genomic DNA

Genomic sequences corresponding to AP1–AP4 cDNAs plus upstream promoter regions were obtained using a PCR-based approach [31] as adapted by Rosati *et al.* [28]. Genomic DNA was extracted from apple leaves according to Doyle and Doyle [11] and digested with either *Eco* RV or *Pvu* II restriction enzymes (Eurogentec, Liège, Belgium) according to supplier's instructions. Specific reverse primers were as for the 5' RACE experiments.

RT-PCR analysis

Expression of target genes was studied by reverse transcription-polymerase chain reaction (RT-PCR) as described in Poupard *et al.* [26], except that 29 cycles were used in standard conditions. Amplifications were performed using either the primer pair Ua-Uni or Ub-Uni. The positions of Ua and Ub primers are indicated in Fig. 1. A constitutive control was carried out using the elongation factor 1 alpha gene (*Ef 1α*) which was amplified using the following degenerate primer pair: 5' CTTTCAGGATGTBTACAAGATTGG 3' and 5' GCAGCCTTGGTVACCTTGGC 3' [29].

Northern blot hybridization

Total RNA (40 µg per sample) was separated by electrophoresis in 1% agarose gel using the NorthernMax kit (Ambion, Austin, TX, U.S.A.). Staining of the gel with ethidium bromide was performed to ensure that equivalent RNA quantities were loaded in each lane. RNA was transferred by capillary blotting to nylon membrane (Hybond-N, Amersham, Buckinghamshire, U.K.). After prehybridization for 2 h at 65°C, hybridization was performed with a [α -³²P]-dUTP labelled antisense RNA probe overnight at 65°C. The [α -³²P]-dUTP labelled RNA probe was prepared using the Lig'nScrib and the Maxiscript kits according to the manufacturer's instructions (Ambion, Austin, TX, U.S.A.). This probe corresponds to a fragment of 221 bp complementary to the region of the AP1 or AP2 transcripts located between primers Ua or Ub and Uni (Fig. 1). Washing steps were performed using the low stringency and high stringency solutions provided by Ambion (2 × 5 min at room temperature and 2 × 15 min

at 65°C, respectively). The membrane was autoradiographed using Kodak AR X-Omart films.

Protein extraction and immunoblotting

Apple leaves (0.2 g per sample) were homogenized in 2 ml extraction buffer [50 mM NaH₂PO₄, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarkosyl and 15 mM β -mercaptoethanol] according to Constabel and Brisson [9], supplemented with 0.2% (w/v) insoluble polyvinylpyrrolidone and 15 mg of Fontainebleau sand. The extracts were centrifuged for 30 min at 15 000 g and the proteins were precipitated by the addition of 5 volumes of cold acetone to the supernatant for 2 h at -20°C. After centrifugation for 30 min at 16 000 g at 4°C, proteins were solubilized in a suitable volume of Laemmli [19] sample buffer [50 mM Tris-HCl, pH 6.8, 2.0% (w/v) SDS, 15% (v/v) glycerol, 5% (v/v) β -mercaptoethanol]. The concentration of proteins was determined using the Micro BCA protein assay reagent kit (Pierce, Rockford, MI, U.S.A.). Total proteins (250 µg per sample) were boiled at 95°C for 5 min and were separated by polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [19] using a 5% polyacrylamide (w/v) stacking gel and 12% polyacrylamide (w/v) resolving gel. Western blotting and immunodetection with a rabbit anti-Bet v 1 polyclonal antibody kindly supplied by Allergopharma (Dr B. Weber, Reinbek, Germany), were performed in the conditions previously described by Poupard *et al.* [26].

Immunodetection of proteins on leaf sections

Leaf pieces (approx. 4 × 1 mm) were fixed and embedded in paraffin as previously described [25]. Serial sections (10 µm thick) were cut with a rotary microtome, placed on microscope slides (Superfrost plus, Fischer Scientific, Elancourt, France) and baked overnight at 45°C. Paraffin was removed in HistoClear II (National Diagnostics, Atlanta, GA, U.S.A.) for 10 min twice and sections were rehydrated through a graded ethanol series for 1 min each [from 100 to 30% in the presence of 0.85% (w/v) NaCl in the last three baths]. After two washes in TBS 1 × (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 min and then in blocking solution [TBS 1 ×, 1% (w/v) BSA, 0.3% (w/v) Triton X-100] for 1 h twice, leaf sections were incubated for 2 h at room temperature with the anti-Bet v 1 polyclonal antibody supplied by Allergopharma (500 µl per slide, dilution 1/500 in blocking buffer). After five washes in TBS 1 ×, slides were incubated for 1 h at room temperature in the blocking solution containing the secondary alkaline phosphatase antibody (dilution 1/5000) supplied by Boehringer (Mannheim, Germany). Slides were washed five times in TBS 1 × and the colour reaction was performed in the conditions mentioned in

Poupard *et al.* [25]. Sections were photographed using a Leitz Dialux 20EB microscope.

RESULTS

Isolation and sequence analysis of two subclasses of apple PR-10 genes

In the purpose of identifying PR10 genes in apple seedlings, first-strand cDNAs were synthesized from total RNA isolated from young leaves collected 24 h after ASM treatment. A 3' RACE strategy with sense degenerated oligonucleotides complementary to a conserved sequence in several PR-10 genes was used and the amplified cDNA fragments were then cloned. Restriction analysis of several resulting recombinant plasmids allowed identification of four different classes of cloned sequences, which were named AP1 to AP4. Complete sequences of the full-length cDNAs were obtained after 5' RACE using reverse primers specific for each class (Fig. 1).

The deduced amino-acid sequences were found highly similar to other PR-10 with, as expected, highest degrees of sequence identity to Mal d 1 isoforms. In particular, AP2 was found nearly identical to AP15 (accession No: L42952), a Mal d 1 isoform from *M. domestica* cv. Golden Delicious induced during fruit ripening [2], and AP3 nearly identical to a Mal d 1 isoform expressed in mature fruit from the same cv. (accession No: AF126402). The amino-acid sequences of AP1 and AP4 share 94 and 93 % identities, respectively, with that of a Mal d 1 isoform (accession No Z724251) from *M. domestica* cv. Gala [18], but only 76–77 % identities with the above-mentioned isoforms from the Golden Delicious cv.

The tree [Fig. 2(a)] resulting from the phylogenetic analysis of the deduced amino-acid sequences of AP1 to AP4 and those of Mal d 1 isoforms from *M. domestica* cv. Golden Delicious found in databanks reveals that the Mal d 1 isoforms can be grouped into two subclasses named APa and APb: APa comprising AP1 and AP4, and APb including AP2 and AP3. Amino acid residues that are unique to all members of either the APa or the APb subclass are indicated in Fig. 2(b). Despite these amino-acid changes, the P-loop motif conserved throughout the PR-10 protein family [18] and the serine residue in position 112 essential for IgE binding [33] are present in all members of the APa and APb subclasses. Similarly, the amino-acid sequences of AP1 to AP4 located between positions 89 and 120 share 88 % similarity with the PR-10 family signature pattern (Prosite accession number PS00451). The amino acid changes between APa and APb mainly result in the loss of two potential phosphorylation sites in members of the former subclass.

Information on the structure of the nuclear genes encoding AP1 to AP4 was obtained after cloning the

corresponding genomic sequences. The analysis of their nucleotide sequences revealed the presence of a single intron positioned at codon 62 inserted between nucleotides 1 and 2 only in clones belonging to the APb subclass (see Fig. 1). The intron found in the gene for AP2 is 171 bp in length and shares 98 % and 75 % identities to the corresponding non-translated sequences in AP15 (171 bp) and AP3 (168 bp), respectively.

The PCR-walking strategy used to obtain the genomic sequences also allowed us to clone up to 963 bp of the 5' regulating regions upstream of the start codons of AP1 to AP4 genes. The AP2 gene and the previously reported pMal2 genomic clone encoding pAP15 [27] showed 98 % identity in this region. The AP2 sequence was therefore considered as corresponding to the registered *Ypr10**Md.a gene. By contrast, alignment of the 5' regulating sequences of AP1, AP3 and AP4 with that of other PR10-genes revealed no significant long stretches of homology. Accordingly, the genomic clones for AP1, AP3 and AP4 were designated as *Ypr10**Md.b, *Ypr10**Md.c and *Ypr10**Md.d, respectively. Scanning these sequences for putative binding sites for transcription factors revealed the presence in all 5' regulating regions of a motif called Box-W originally identified in the promoter of the parsley *pcpr1-1* gene, the type member of the PR-10 family [22]. Other consensus patterns similar to ERE-element, TCA-element, AuxRR-core, CGTCA-motif, and EIRE-motif (involved in ethylene, salicylic acid, auxin, methyl jasmonate and biotic elicitor responses, respectively) also occurred in some of the 5' regulating regions without any apparent correspondence with their subclass origins (Fig. 1).

Induction of apple PR-10 genes by ASM

The kinetic of expression of the apple PR-10 genes was first studied by RT-PCR between 8 and 48 h after treatment with ASM or water. The amplification of the cDNAs belonging either to the APa or to the APb subclass was performed using primer pairs Uni/Ua and Uni/Ub respectively (as indicated in Fig. 1). Typical results (Fig. 3) showed that the constitutive control *Ef 1α* exhibited a comparable level at the different times studied while the expression of the APa and APb transcripts was enhanced after ASM application in comparison with the water treatment: transcripts of the APa or the APb subclass strongly accumulated 20 h after ASM treatment and remained at a high level up to 48 h. However, the transcripts of the APb subclass were induced by ASM treatment to a lower extent than the APa transcripts.

To confirm the results obtained by the RT-PCR experiments, the expression of the PR-10 genes was also investigated by Northern blot analysis. Experiments were carried out with total RNA isolated from leaves of apple seedlings sampled 8, 24 and 48 h after ASM or water

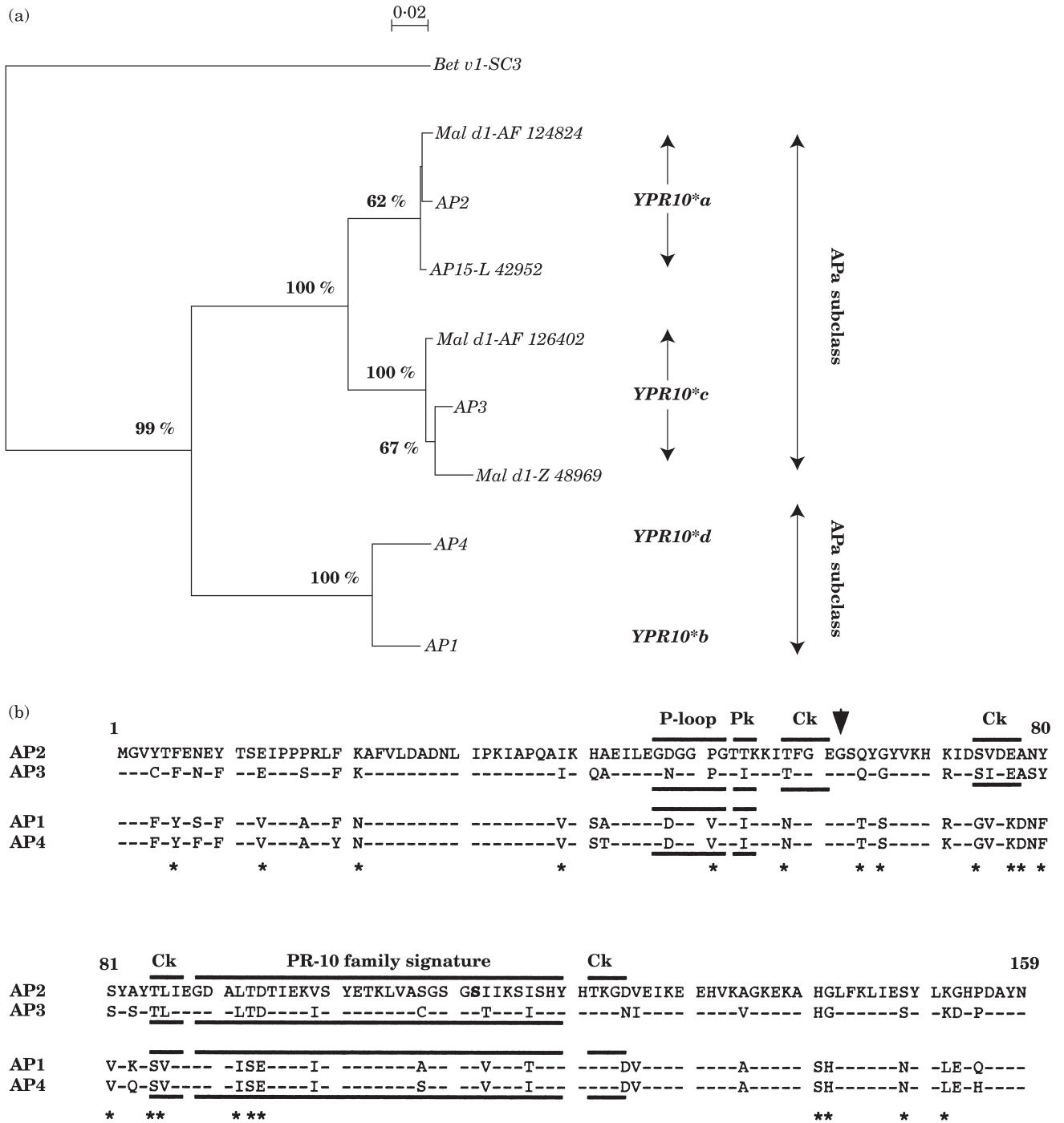


FIG. 2. (a) Phylogenetic tree obtained from the alignment of the deduced amino acid sequences of AP1, AP2, AP3 and AP4 with other PR-10 amino acid sequences from *M. domestica* cv. Golden Delicious: Mal d 1—AF124824 and Mal d 1—AF126402 [32], pAP15—L42952 [2], Mal d 1—Z48969 [36] and from *Betula verrucosa* Bet v 1-SC3—X77601 [34]. The tree was constructed by neighbour-joining method using programs from PHYLIP package. The PR-10 sequences of apple are grouped into two subclasses, APa and APb. Bootstrap values from 500 bootstrap replications are indicated as bold numbers. (b) Alignment of the deduced amino acid sequences of AP1 to AP4. Amino acid residues unique to members of either the APa or the APb subclass are marked by asterisks. The conserved motifs characteristic for phosphate binding loop (P-loop), protein kinase C (Pk) and casein kinase (Ck) phosphorylation sites are indicated. Intron position is marked as an arrow above the AP2 and AP3 sequences. The serine residue in position 112 essential for IgE binding is indicated as a bold letter.

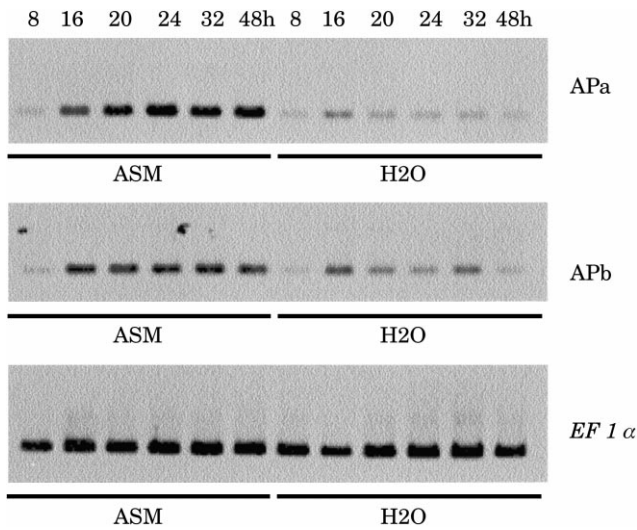


FIG. 3. Kinetics of expression of APa and APb mRNAs analysed by RT-PCR in apple leaves at different times (indicated in h above the gel) after treatment with ASM or water. RT-PCR was performed with primer pairs specific to each gene subclass, Ua-Uni for the APa subclass and Ub-Uni for the APb subclass. A constitutive control was carried out using the elongation factor 1 alpha gene (*EF 1α*).

treatments. Hybridization was performed using a probe complementary to a sequence fragment of AP1 located between the Ua and Uni primers (as indicated in Fig. 1). The nucleotide sequence of AP1 in this region shares 91 % identity with the corresponding region in AP4 but lower degrees of identity with the same regions in AP2 and AP3 (79 and 75 % identity, respectively): in the hybridization conditions, this probe was specific for the APa subclass [Fig. 4(c)]. As shown in Fig. 4(a), hybridization with apple leaves RNA revealed a very weak signal at 8 h after water or ASM treatment. Conversely, the amount of APa mRNA increased dramatically in leaves at 24 and 48 h after ASM application. In a following step, the expression of APa genes at a systemic level was investigated [Fig. 4(b)]. Apple seedlings were treated by ASM or water except the two youngest leaves, which were protected from the activator or water

application. At 48 or 120 h after the treatment, total RNA was extracted from treated or untreated leaves and hybridization carried out with the RNA probe specific for the APa subclass. Results indicate that the APa transcripts in the leaves treated with ASM strongly accumulated at 48 h (as expected) and even at 120 h after activator application. In the upper untreated leaves, the APa transcripts were also induced but only at 120 h after application of ASM. However this systemic induction was weak by comparison with the induction of the APa genes observed in ASM-treated leaves. Similar results were obtained when the RNA membranes presented in Fig. 3 were hybridized with a probe specific for the APb subclass, although significantly weaker hybridization signals were observed (data not shown).

The presence of the proteins encoded by the AP transcripts in leaves of apple seedlings was investigated at 8, 24 and 48 h after treatment with ASM or water. The analysis was carried out by SDS-PAGE followed by immunoblotting using a heterologous polyclonal antibody raised against Bet v 1, a PR-10 protein from birch pollen. As shown in Fig. 5, no protein serologically-related to Bet v 1 could be detected in the extracts corresponding to the leaves treated with water at the three times studied or to the leaves sampled at 8 and 24 h after application of ASM. At 48 h following the activator treatment, two polypeptides migrating at 17 and 18 kDa, respectively, were revealed. The proteins encoded by the AP transcripts were localized in cross sections of apple leaves using the same anti-Bet v 1 polyclonal antibody. Sections were realized in apple leaves collected 48 h after ASM or water application (Fig. 6). In sections treated with ASM, the immunostaining reaction was mainly associated with the palisade parenchyma as indicated in Fig. 6(a). Moreover, in Fig. 6(b) showing a vein cross-section, the staining was peculiarly intense in some sites of the vascular bundles, which correspond to cells of the phloem tissue. In control sections treated with water [Fig. 6(c)], although the intensity of the signal is much lower than in Fig. 6(b), the staining seems also to be associated with the phloem cells. By contrast, other parts

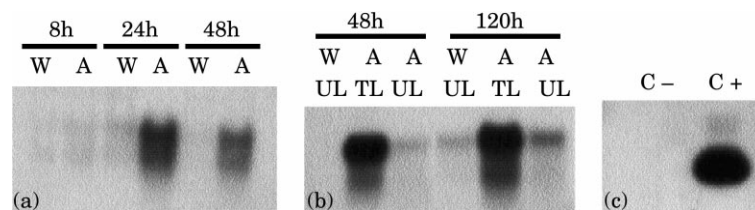


FIG. 4. Northern blot analysis of APa mRNAs expression in apple leaves after treatment with ASM or water. For all blots, hybridization was carried out with a [α^{32} -P]-labelled antisense RNA probe specific for the APa subclass. (a) Total RNA (40 μ g per sample) was prepared from apple leaves sampled 8, 24 and 48 h after treatment with ASM (A) or water (W). (b) Apple leaves were treated with ASM (A) or water (W) and total RNA (40 μ g per sample) was extracted from treated (TL) and untreated leaves (UL) collected on the same plant 48 or 120 h after treatment. (c) Positive (C+) and negative (C-) controls of the above experiment corresponding to 2 μ l of a diluted PCR product (dilution 1 : 50) from apple leaves cDNAs amplified with primer pairs Ua/Uni and Ub/Uni, respectively.

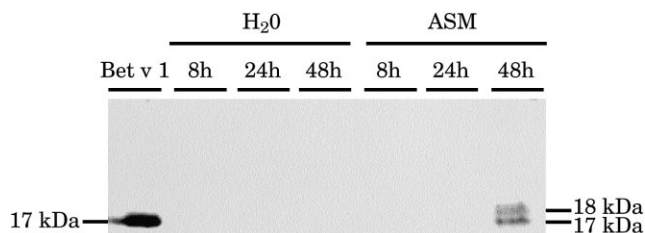


FIG. 5. SDS-PAGE and immunoblot analysis of protein extracts from apple leaves 8, 24 and 48 h after treatment with ASM or water. A positive control (first lane on the blot) was performed using the recombinant protein Bet v 1 (major allergen of birch pollen) produced in *Escherichia coli*.

of the leaf sections (including the palisade parenchyma) are apparently free of protein signal.

DISCUSSION

Apple is one of the most important models for studying PR-10 proteins at the immunological level [40], as well as at the physiological level particularly in relation to fruit maturation [2]. Aspects of the role of PR-10 in the defence response of apple have also been recently considered by Pühlinger *et al.* [27]. However, with the exception of this last work, little is known concerning the expression of PR-10 genes in apple under biotic or abiotic stresses. In the present study, a functional analogue of salicylic acid, ASM has been used, which is commercialized as a plant health promoter compound, in Germany under the trade name Bion-50 WG, Switzerland as Unix Bion-63 WG and in the U.S.A. as Actigard. ASM is a potent systemic acquired resistance inducer providing protection against a wide spectrum of diseases in various plant species such as tobacco [14], Arabidopsis [20] or apple [6].

In a first step, PR-10 transcripts accumulating in apple leaves were characterized. The results indicate that at least four different PR-10 genes could be expressed in this organ following ASM application. This finding is not surprising since it has been previously reported in numerous plant species that PR-10 are encoded by a family of multiple genes [45]. In particular, Atkinson *et al.* [2] reported that nearly 15 PR-10 gene members could exist in apple. On the basis of their nucleotide and deduced amino acid sequences, the genes encoding the transcripts accumulating in ASM-treated leaves could be separated into two subclasses that were named APa and APb. These two subclasses were easily differentiated both at the protein level by numerous amino acid changes and at the nucleotide level by the presence of an intron in members of the APb subclass only. PR-10 genes nearly identical to the members of the APb subclass described here, have already been identified in previous studies

[2, 33] and were shown to be expressed in apple fruit during maturation. By contrast, members of the APa subclass could correspond to genes encoding yet undescribed apple PR-10 isoforms. Characterization of two different subclasses of PR-10 transcripts was also recently reported in *Lilium longiflorum* [43]. In that study, the authors have shown that members of the two subclasses were all inducible by abscisic acid and methyl jasmonate. While both the genes of the APa and APb subclasses were found to be inducible by ASM with similar kinetics, transcripts corresponding to the APa subclass accumulated at a significantly higher level in leaves after induction. This may be related to the presence of four putative TCA-elements involved in salicylic acid response in the promoter sequence of AP4, a member of the APa subclass. Scanning the 5' regulating regions revealed the presence in all four *Ypr10* genes described here of a W box motif similar to the fungal elicitor responsive element originally described in the promoters of parsley PR-10 genes [22] and also recently reported in the *Ypr10***Md.a* gene [27]. The distribution of other putative binding motifs in the different regulating regions of the *Ypr10* genes suggests a complex regulation of individual genes without correlation with their subclass origin.

The present study indicates that PR-10 proteins accumulate in apple leaves 48 h after treatment with ASM and two polypeptides at 17 and 18 kDa were detected with the anti-Bet v 1 monospecific polyclonal antiserum. Binding of these antibodies or IgE from patients suffering allergic reactions to a 17–18 kDa double band in apple extract has already been observed [12, 18, 30, 36, 41]. Similarly, immunodetection also revealed microheterogeneities in the population of PR-10 proteins in other plant species such as birch [35] and potato [9]. As it has been shown, in the case of apple, that the two polypeptides did not react with the same monoclonal anti-Bet v 1 antibody [30], it may be suspected that they represent different PR-10 isoforms. The molecular weights of AP1–AP4 encoded proteins, calculated from the deduced amino acid sequences, are nearly identical (17.5 kDa). The observed variations in electrophoretic mobility may thus reflect different post-translational modifications. In line with this hypothesis, a potential *N*-glycosylation site (NYS) was found only in the protein encoded by AP2 and two putative phosphorylation sites are lacking in proteins encoded by genes of the APa subclass when compared with those of the APb subclass [see Fig. 2(b)]. However, it should be noticed that such post-translational modifications have not yet been found in PR-10 proteins when investigated [8, 34, 35].

In situ localization of PR-10 proteins in apple leaf sections at 48 h following gene induction by ASM, allowed the demonstration of the protein expression mainly in the palisade parenchyma and the vascular

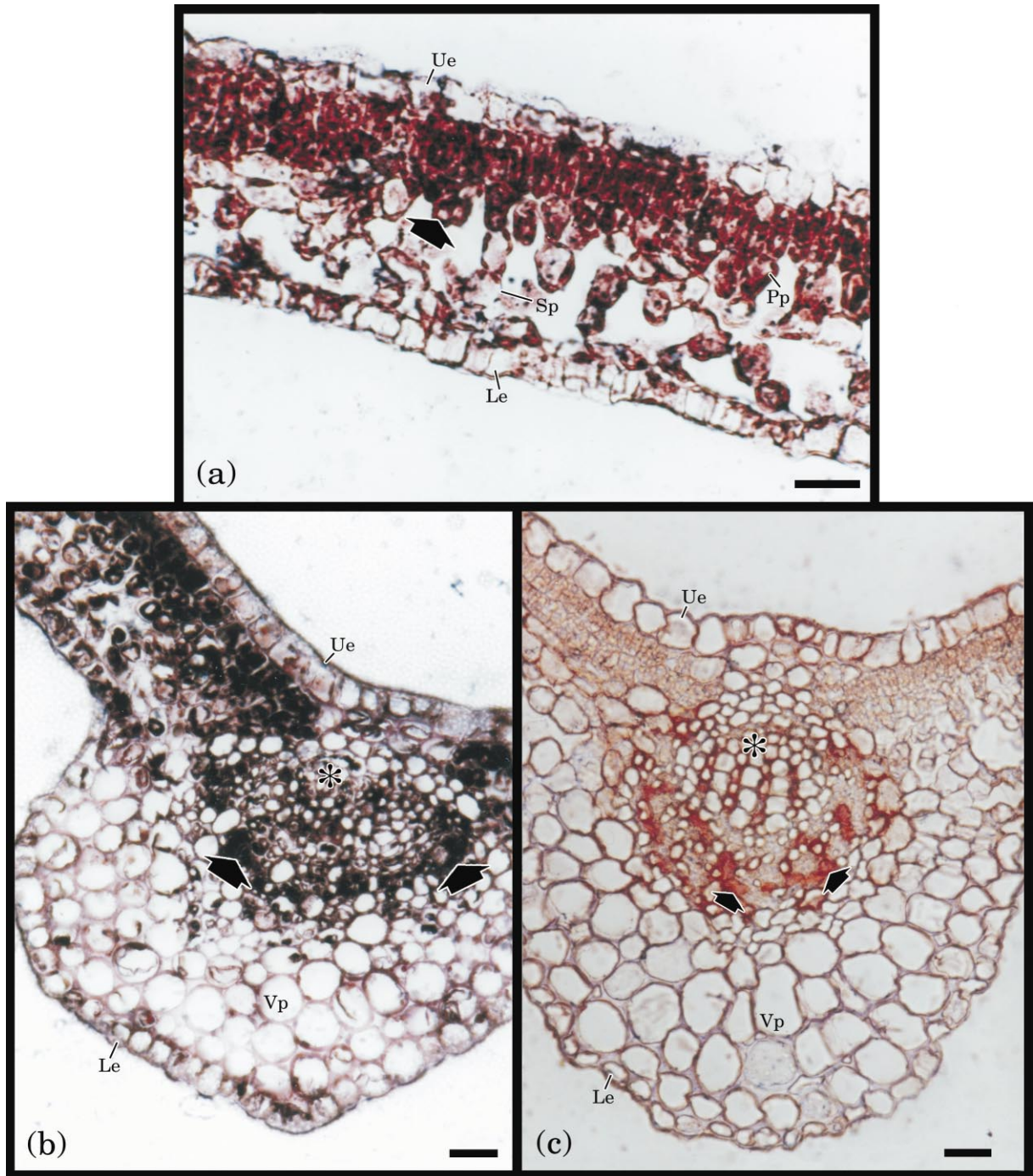


FIG. 6. *In situ* localization of PR-10 proteins in apple leaves. Cross sections (10 μm) were achieved using apple leaves collected 48 h after treatment with ASM (a and b) or water (c). Proteins are visualized by violet staining (indicated by arrows) using the immunodetection system described in “Materials and Methods”. In (a) and (b), showing a vein cross-section, an asterisk marks the site of the vascular bundles. Le: lower epidermis; Pp: palisade parenchyma; Sp: spongy parenchyma; Ue: upper epidermis, Vp: vein parenchyma. Bars = 25 μm .

tissues. In leaf mesophyll, palisade and spongy parenchyma basically differ in cell organization, which is known to be related to specific tissue function. Indeed, high photosynthetic activity is associated with the palisade tissue [13]. Different functional specialization of

the mesophyll tissues could explain why PR-10 proteins accumulate preferentially in the palisade layer. The anatomic resolution of the technique used in this study has led to the localization of the protein signal in specific areas of the vascular bundles, corresponding to phloem.

The expression in the phloem may be constitutive as a weak protein signal was observed in control leaves. According to the results, previous studies of spatial expression of PR-10 gene products in leaves indicated also their localization in vascular tissues [4, 24, 44]. Moreover, a putative function of some PR-10 members in the differentiation of vascular bundles has been hypothesized in bean [42] or birch [25]. In apple, the present results suggest that further work is needed to elucidate the role of PR-10 proteins in the palisade parenchyma and the phloem of leaves after the application of a salicylic acid analogue. Experiments are underway to examine more precisely the *in situ* localization of PR-10 proteins of apple leaves at the cellular level using immunogold transmission electron microscopy.

In the present study, the authors demonstrate the systemic expression of apple PR-10 transcripts after local application of ASM. This confirms the systemic nature of ASM action in a perennial crop, previously demonstrated with the accumulation of β -1,3 glucanases and peroxidases in apple untreated tissues [6]. In other plant models than apple, many studies indicate that PR-10 transcripts accumulate locally, i.e. near or at the sites of wounding or pathogen infection [9, 21, 44]. Nevertheless, in alfalfa, the systemic character of the expression of *MsPR10-1* was reported by Breda *et al.* [4]; indeed, accumulation of this PR-10 transcript was observed in vascular tissues adjacent to and distant from the site of leaf infiltration by the pathogenic bacterium *Pseudomonas syringae* pv. *pisi*. The present results also suggest that PR-10 proteins could not only play a defensive role locally but may be involved in mechanisms of systemic resistance. As ASM treatment is known to lead to the same spectrum of pathogen resistance and gene expression as does pathogen infection [14, 20], it would be of special interest to analyse the spatio-temporal pattern of PR-10 expression in apple leaves after a pathogen challenge, such as infection by fungi or bacteria (e.g. *Venturia inaequalis* or *Erwinia amylovora*).

In conclusion, these findings demonstrate the presence of two subclasses of PR-10 transcripts in apple leaves and their local and systemic induction by ASM. Therefore, the allergenic character of some PR-10 protein members should be considered before using ASM as a disease control molecule, since it has been suggested by Son *et al.* [33] that variation of allergenicity exhibited by apple cultivars could be correlated to the expression levels of the major allergen Mal d 1.

REFERENCES

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.
2. Atkinson RG, Perry J, Matsui T, Ross GS, Macrae EA. 1996. A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also ripening-related. *New Zealand Journal of Crop and Horticultural Science* **24**: 103–107.
3. Bantignies B, Séguin J, Muzac I, Dédaldéchamp E, Gulick P, Ibrahim R. 2000. Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. *Plant Molecular Biology* **42**: 871–881.
4. Breda C, Sallaud C, El-Turk J, Buffard D, de Kozak I, Esnault R, Kondorosi A. 1996. Defense reaction in *Medicago sativa*: a gene encoding a class PR-10 protein is expressed in vascular bundles. *Molecular Plant-Microbe Interactions* **9**: 713–719.
5. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M. 1989. The gene coding for the major birch pollen allergen *Bet v I*, is highly homologous to a pea disease resistance response gene. *The EMBO Journal* **8**: 1935–1938.
6. Briset M-N, Cesbron S, Thomson SV, Paulin J-P. 2000. Acibenzolar-S-methyl induces the accumulation of defense-related enzymes in apple and protects from fire blight. *European Journal of Plant Pathology* **106**: 529–536.
7. Bufe A, Spangfort MD, Kahlert H, Schlaak M, Becker W-M. 1996. The major birch pollen allergen. *Bet v I*, shows ribonuclease activity. *Planta* **199**: 413–415.
8. Constabel CP, Brisson N. 1992. The defense-related *STH-2* gene product of potato shows race-specific accumulation after inoculation with low concentrations of *Phytophthora infestans* and Potato Virus X. *Plant Molecular Biology* **188**: 289–295.
9. Constabel CP, Brisson N. 1995. Stigma- and vascular-specific expression of the *PR-10a* gene of potato: a novel pattern of expression of a pathogenesis-related gene. *Molecular Plant-Microbe Interactions* **8**: 104–113.
10. Dempsey D'MA, Shah J, Klessig DE. 1999. Salicylic acid and disease resistance in plants. *Critical Reviews in Plant Sciences* **18**: 547–575.
11. Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissues. *Focus* **12**: 13–15.
12. Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, Kraft D. 1991. Common epitopes of birch pollen and apple. Studies by Western and Northern blot. *Journal of Allergy and Clinical Immunology* **88**: 588–594.
13. Esau K. 1965. *Plant Anatomy*. New York, U.S.A.: J. Wiley and Sons.
14. Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincer S, Staub T, Uknes S, Métraux J-P, Kessmann H, Ryals J. 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *The Plant Journal* **10**: 61–70.
15. Frohman MA, Dush MK, Martin GR. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proceedings of the National Academy of Sciences, USA* **85**: 8998–9002.
16. Godard J-F, Ziadi S, Monot C, Le Corre D, Silué D. 1999. Benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* var *Botrytis*) to downy mildew of crucifers caused by *Peronospora parasitica*. *Crop Protection* **18**: 397–405.
17. Hammond-Kosack KE, Jones JDG. 1996. Resistance gene-dependent plant defense responses. *The Plant Cell* **8**: 1773–1791.
18. Hoffmann-Sommergruber K, Vanek-Krebitz M, Radauer C, Wen J, Ferreira F, Scheiner O, Breiteneder H. 1997. Genomic characterization of members of the

- Bet v 1* family: genes coding for allergens and pathogenesis-related proteins share intron positions. *Gene* **197**: 91–100.
19. **Laemmli UK**. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
 20. **Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J**. 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal* **10**: 71–82.
 21. **Lo S-CC, Hipskind JD, Nicholson RL**. 1999. cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. *Molecular Plant-Microbe Interactions* **12**: 479–489.
 22. **Meier I, Hahlbrock K, Somssich IE**. 1991. Elicitor-inducible and constitutive *in vivo* DNA footprints indicate novel cis-acting elements in the promoter of parsley gene encoding pathogenesis-related protein 1. *The Plant Cell* **3**: 309–315.
 23. **Pääkkönen E, Seppänen S, Holopainen T, Kokko H, Kärenlampi S, Kärenlampi L, Kangasjärvi J**. 1998. Induction of genes for the stress proteins PR-10 and PAL in relation to growth, visible injuries and stomatal conductance in birch (*Betula pendula*) clones exposed to ozone and/or drought. *The New Phytologist* **138**: 295–305.
 24. **Pinto MP, Ricardo CPP**. 1995. *Lupinus albus* L. pathogenesis-related proteins that show similarity to PR-10 proteins. *Plant Physiology* **109**: 1345–1351.
 25. **Poupard P, Brunel N, Leduc N, Strullu D-G, Viéumont J-D, Simoneau P**. 2001. Expression of a *Bet v 1* homologue gene encoding a PR 10 protein in birch roots: induction by auxin and localization of the transcripts by *in situ* hybridization. *Australian Journal of Plant Physiology* **28**: 57–63.
 26. **Poupard P, Strullu D-G, Simoneau P**. 1998. Two members of the *Bet v 1* family encoding birch pathogenesis-related proteins display different patterns of root expression and wound-inducibility. *Australian Journal of Plant Physiology* **25**: 459–464.
 27. **Pühringer H, Moll D, Hoffmann-Sommergruber K, Watillon B, Katinger H, Laimer da Câmara Machado M**. 2000. The promoter of an apple *Ypr10* gene, encoding the major allergen *Mal d 1*, is stress- and pathogen-inducible. *Plant Science* **152**: 35–50.
 28. **Rosati C, Cadic A, Duron M, Ingouff M, Simoneau P**. 1999. Molecular characterization of the anthocyanidin synthase gene in *Forsythia × intermedia* reveals organ-specific expression during flower development. *Plant Science* **149**: 73–79.
 29. **Rosati C, Cadic A, Duron M, Renou J-P, Simoneau P**. 1997. Molecular cloning and expression analysis of dihydroflavonol 4-reductase gene in flower organs of *Forsythia × intermedia*. *Plant Molecular Biology* **35**: 303–311.
 30. **Schöning B, Vieths S, Petersen A, Baltes W**. 1995. Identification and characterization of allergens related to *Bet v 1*, the major birch pollen allergen, in apple, cherry, celery and carrot by two-dimensional immunoblotting and N-terminal microsequencing. *Journal of Science and Food Agriculture* **67**: 431–440.
 31. **Siebert PD, Chenchick A, Kellogg DE, Lukyanov KA, Lukyanov SA**. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Research* **23**: 1087–1088.
 32. **Somssich IE, Schmelzer E, Bollmann J, Hahlbrock K**. 1986. Rapid activation by fungal elicitor of genes encoding 'pathogenesis-related' proteins in cultured parsley cells. *Proceedings of the National Academy of Sciences, USA* **83**: 2427–2430.
 33. **Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S**. 1999. Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of *Mal d 1*, the major apple allergen, and *Bet v 1*, the major birch pollen allergen. *European Journal of Nutrition* **38**: 201–215.
 34. **Swoboda I, Scheiner O, Heberle-Bors E, Vicente O**. 1995. cDNA cloning and characterization of three genes in the *Bet v 1* gene family that encode pathogenesis-related proteins. *Plant, Cell and Environment* **18**: 865–874.
 35. **Swoboda I, Scheiner O, Kraft D, Breitenbach M, Heberle-Bors E, Vicente O**. 1994. A birch gene family encoding pollen allergens and pathogenesis-related proteins. *Biochimica et Biophysica Acta* **1219**: 457–464.
 36. **Vanek-Krebitz M, Hoffmann-Sommergruber K, Laimer da Câmara Machado M, Susani M, Ebner C, Kraft D, Scheiner O, Breiteneder H**. 1995. Cloning and sequencing of *Mal d 1*, major allergen from apple (*Malus domestica*), and its immunological relationship to *Bet v 1*, the major birch pollen allergen. *Biochemical and Biophysical Research Communications* **214**: 538–551.
 37. **van Loon LC, Pierpoint WS, Boiler T, Conejero V**. 1994. Recommendation for naming plant pathogenesis-related proteins. *Plant Molecular Biology Reporter* **12**: 245–264.
 38. **van Loon LC, van Strien EA**. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology* **55**: 85–97.
 39. **Vidya CSS, Manoharan M, Sita GL**. 1999. Cloning and characterization of salicylic acid-induced, intracellular pathogenesis-related gene from tomato (*Lycopersicon esculentum*). *Journal of Biosciences* **24**: 287–293.
 40. **Vieths S, Jankiewicz A, Schöning B, Aulepp H**. 1994. Apple allergy: the IgE-binding potency of apple strains is related to the occurrence of the 18-kDa allergen. *Allergy* **49**: 262–271.
 41. **Vieths S, Schöning B**. 1996. Characterization of *Mal d 1*, the 18-KDa major apple allergen, at the molecular level. In: Wüthrich B, Ortolani C, eds. *Highlights in Food Allergy. Monographs in Allergy*, Vol. 32. Basel: Karger, 63–72.
 42. **Walter MH, Liu JW, Wünn J, Hess D**. 1996. Bean ribonuclease-like pathogenesis-related protein genes (*Ypr10*) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. *European Journal of Biochemistry* **239**: 281–293.
 43. **Wang CS, Huang JC, Hu JH**. 1999. Characterization of two subclasses of PR-10 transcripts in lily anthers and induction of their genes through separate signal transduction pathways. *Plant Molecular Biology* **40**: 807–814.
 44. **Warner SAJ, Scott R, Draper J**. 1993. Isolation of an asparagus intracellular PR gene (*AoPRI*) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. *The Plant Journal* **3**: 191–201.
 45. **Wen J, Vanek-Krebitz M, Hoffmann-Sommergruber K, Scheiner O, Breiteneder H**. 1997. The potential of *Bet v 1* homologues, a nuclear multigene family, as phylogenetic markers in flowering plants. *Molecular Phylogenetics and Evolution* **8**: 317–333.
 46. **Xu Y, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA**. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *The Plant Cell* **6**: 1077–1085.