Structure and organ specificity of an anionic peroxidase from *Arabidopsis thaliana* cell suspension culture

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Abstract The predominant peroxidase (p*I* 3.5) (E.C. 1.11.1.7) of an *Arabidopsis thaliana* cell suspension culture was purified and partially sequenced. Oligonucleotides were designed and a specific probe was obtained. A cDNA clone was isolated from an *Arabidopsis* cell suspension cDNA library and completely sequenced. The cDNA clone comprised 1194 bp and encodes a 30 residue signal peptide and a 305 residue mature protein (M_r 31 966). The sequence of the mature protein is 95% identical to the well-characterized horseradish peroxidase HRP A2 and has therefore been designated ATP A2. Three introns at positions identical to those found in *Arabidopsis* and horseradish genes encoding cationic peroxidases were identified. RT-PCR analysis revealed root-specific expression.

Key words: Anionic peroxidase, *Arabidopsis thaliana*; Plant cDNA; Peroxidase structure; Root specificity

1. Introduction

Peroxidases have found widespread use in the visualization of specific immunochemical reactions, and in the analysis of many biologically important molecules which are integral to hydrogen peroxide generation in coupled enzymatic assays. The cationic horseradish isoperoxidase HRP C is most often used but there is an increasing demand for peroxidases that are more stable or specific in specialized applications. The anionic HRP A2 isoenzyme has been suggested to be more stable to auto-oxidation during catalytic turnover [1,2]. The kinetic and redox properties of HRP A2 are well-known (reviewed in [1]), and the amino acid sequence and seven sites of *N*-glycosylation have been determined (C.B. Rasmussen, B. Stoffer and K.G. Welinder, in preparation; EMBL accession number P80679). Despite attempts in several laboratories, the A2 type of peroxidase has never been cloned. In the present communication, we describe the properties and cloning of an anionic peroxidase from *Arabidopsis thaliana* cell suspension culture, ATP A2, which is 95% identical to the HRP A2 protein. ATP A2 seems to be root-specific.

2. Materials and methods

2.1. Cell suspension culture

The ecotype of *Arabidopsis thaliana* (L.) Heynh. used was either Landsberg or Columbia. The cells were grown in MS (Blackhall) medium [3], which in addition to organic and inorganic nutrients (ICN Biomedicals, UK) contained 0.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.05 mg/l kinetin, and 3% sucrose, pH 5.7. The cell suspension was subcultured once a week by fivefold dilution into fresh medium.

2.2. Isoelectric focusing

A Phast system (Pharmacia Biotech) and precast isoelectric focusing (IEF) gels, pH 3–9, were used and a crude peroxidase preparation (Boehringer Mannheim, Type II) was used as marker. The peroxidase bands were developed by activity staining (5 min in a mixture of 3% carbazole and 0.5% guaiacol at pH 5, followed by 5–10 min in 0.015% H_2O_2).

2.3. Purification, digestion, and partial amino acid sequencing of ATP A2

The spent medium from a cell suspension culture of *Arabidopsis* (Landsberg) was precipitated with ammonium sulfate (AMS) 40–90% of saturation at 4°C. The peroxidase-containing precipitate was resuspended in buffer A (1.5 M AMS; 50 mM phosphate, pH 7.0) and loaded on a Phenyl Sepharose column (Hic Test kit, Pharmacia Biotech) equilibrated with buffer A at room temperature. Elution was performed with a linear gradient (0–100%) of buffer B (50 mM phosphate, pH 7.0) over 20 column volumes. Acidic peroxidase isoenzymes were purified to homogeneity on a Mono Q column (Pharmacia Biotech) at room temperature (buffer A: 5 mM Tris, 1 mM $CaCl_2$, pH 8.3, and linear gradient of buffer B: 100 mM NaCl, 5 mM Tris, 1 mM $CaCl_2$, pH 8.3).

ATP A2 was reduced with dithiothreitol and carboxymethylated with iodoacetic acid in the presence of 6 M guanidinium chloride, dialyzed, and digested with trypsin in 0.2 M $(NH_4)_2CO_3$, pH 8. Tryptic peptides were separated by reversed-phase HPLC on a C-18 column operating under standard conditions using an Applied Biosystems 130 A Separation System with spectrophotometric detection at 215 nm. Selected peptides were subjected to automatic sequencing.

2.4. Isolation of total RNA from cell suspension culture

The drained cells from 5 ml of a cell suspension culture (Columbia) at 50% confluence were ground in liquid nitrogen. The cell paste was suspended in 3 ml of 7.2 M guanidinium chloride, 0.5 M EDTA, 10 mM dithiothreitol, 1 M MES buffer titrated to pH 6.3 with KOH. After centrifugation, the sample was phenol extracted twice and total nucleic acid precipitated with ethanol. The pellet was dissolved in 500 μl 10 mM Tris-HCl, 1 mM EDTA, pH 8, and RNA was precipitated with 1:10 (v/v) 8 M LiCl. RNA was pelleted, redissolved in 200 μl H_2O and, finally, ethanol precipitated once more.

2.5. RT-PCR

Three degenerate oligonucleotide primers, #A21, #A22, and #A23 (shown in Fig. 3B and corresponding to positions 383–413, 628–598 and 627–653 in Fig. 2A), were designed on the basis of the partial amino acid sequence avoiding putative introns. Primer #A24 (position 994–964) was designed based on a conserved region for plant peroxidases encoding two methionines (Fig. 3B). RT-PCR (Perkin Elmer) was performed on 0.3 μg total RNA and the cycles were run according to the manufacturer's manual. The reaction products were analyzed on a 1.5% agarose gel, purified on a PCR purification column (Qiagen), cloned into the plasmid pUC18 (New England Biolabs) (according to [4]), and sequenced. From the resulting sequences, it was possible to make specific primers #A2c and #A2f (Fig. 3B) and

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produce a DNA fragment of 533 bp (underlined in Fig. 2A) which was subsequently used for screening a cDNA library.

2.6. Screening a λZap library and isolation of a cDNA encoding ATP A2

A cDNA library, AC16H, constructed from *Arabidopsis* (Columbia) cell suspension culture mRNA in λZap (Stratagene), was provided by Dr. D. Tremousaygue [5]. The library was screened with the 533 bp DNA probe (Section 2.5) according to the Stratagene manual. Two positive clones were isolated. Using Taq polymerase (Pharmacia Biotech) in combination with Pfu polymerase (Stratagene), as well as two λ arm-specific primers, the cDNA insert from one of the clones was amplified, digested with *Eco*RI and *Xho*I, and subcloned into the corresponding sites in pUC18. Transformation of *E. coli* strain DH5α was carried out essentially according to [4].

2.7. DNA sequence analysis

Plasmid DNA was isolated using Qiagen spin prep. The cDNA insert subcloned into pUC18 was sequenced with the dyedeoxy method (Perkin Elmer) and reactions analyzed on an ABI Model 373A sequencer (Applied Biosystems, USA). Sequence analyses were carried out using the GeneWorks program and programs SEQED, PUBLISH, TRANSLATE and BESTFIT from the Genetics Computer Group (GCG, version 8.1-unix, Wisconsin Package). The AraClean V 1.1 database, which is an error-corrected and redundancy-reduced database of *Arabidopsis thaliana* sequences extracted from GenBank rel. 87 [6], provided the data for analysis of the translation initiation region and the 3′ untranslated region.

2.8. Isolation of genomic DNA and intron mapping

Genomic DNA was isolated according to [7]. PCR was carried out on genomic DNA and compared to reactions run on ATP A2 cDNA inserted in pUC18. The following primer pairs were employed to establish the presence of introns: #A2a (position 124–149) and #A2b (311–289); #A2c (412–436) and #A2d (619–597); and #A2e (553–576) and #A2f (944–913). Conditions for the PCR cycles were as follows: initial denaturation step at 95°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. Samples were analyzed on a 2% agarose gel.

2.9. Expression analysis by RT-PCR

Total RNA was purified from whole plants at different stages of development and from different organs by the RNeasy kit from Qiagen. Cell suspension mRNA was isolated as described above. RT-PCR was performed as in Section 2.5 using 65°C for annealing. The quality of total RNA was checked by RT-PCR performed with primers directed against the mRNA for eukaryotic elongation factor 1α (eEF-1α) [8]. eEF-1α is a member of the translational apparatus, expected to be present at all stages and in all tissues. RT-PCR experiments with eEF-1α were done in parallel with the ATP A2 mRNA analyses.

3. Results and discussion

3.1. Purification and characterization of ATP A2

IEF of spent medium from *Arabidopsis* suspension culture indicated the presence of both anionic and cationic peroxidases (Fig. 1). The predominant peroxidase from spent medium has a p*I* of 3.5, which is similar to that of HRP A2 present in the marker. From 250 ml of spent medium from a Landsberg culture 45 μg of homogeneous anionic peroxidase of RZ (A_{403}/A_{280}) 3.1 was obtained after three steps of purification, namely (i) AMS precipitation, (ii) Phenyl Sepharose chromatography (retaining phenolics and partially fractionating isoperoxidases) and (iii) Mono Q ion exchange chromatography. The amino acid composition and the absorption spectra were similar to those of HRP A2. Reduced and carboxymethylated peroxidase was digested with trypsin and selected peptides were subjected to sequence analysis (Fig. 2A). Again the similarity to HRP A2 was striking, and the *Arabidopsis* peroxidase was therefore designated ATP A2.

3.2. Cloning and sequence analysis of ATP A2 encoding cDNA

A specific 533 bp DNA probe was generated based on the partial amino acid sequence information. 200 000 plaques from the *Arabidopsis* cell culture cDNA library AC16H were screened and two positive clones isolated. One was completely sequenced on both strands. The cDNA of the isolated clone is 1194 bp excluding the poly(A) tail (Fig. 2A). It contains an open reading frame of 1008 bp encoding 335 amino acids.

We have assigned the putative start codon to position 49–51 as the site of translation initiation for ATP A2. The Kozak context around the AUG determined in vertebrates [9] might be different in plants [10] and our statistical analysis of 129 *Arabidopsis* genes from the AraClean database verify that C at +5, which is present in the mRNA for ATP A2, is used almost as frequently (46%) as A at −3 (60%), which is absent in ATP A2 mRNA.

The polyadenylation signal in the 3′ untranslated region of plant mRNAs also differs frequently from the otherwise highly conserved AAUAAA sequence [11]. In ATP A2 mRNA, hexanucleotides at positions 1172–1177, 1181–1186, and 1182–1187 differ only at one position compared to the consensus. Various findings ([12,13], L. Østergaard, unpub-

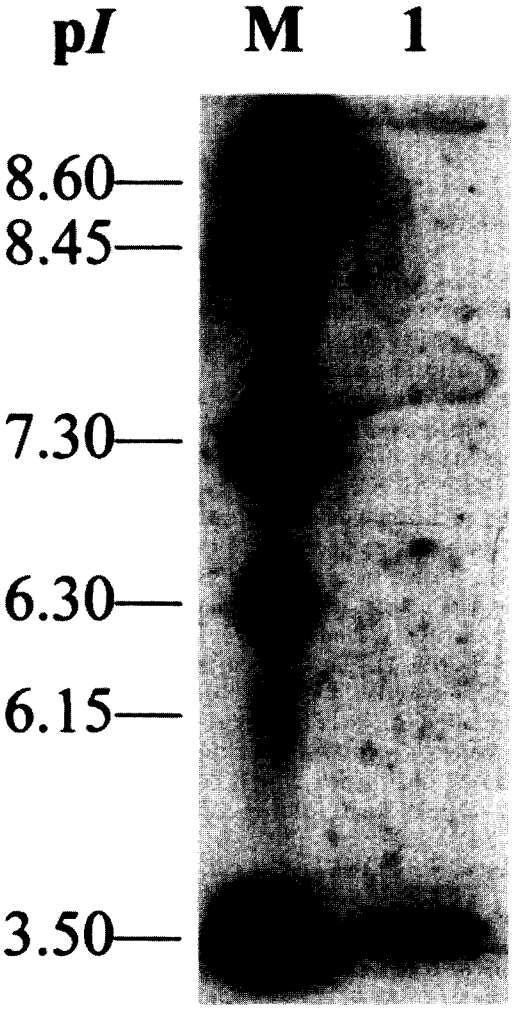
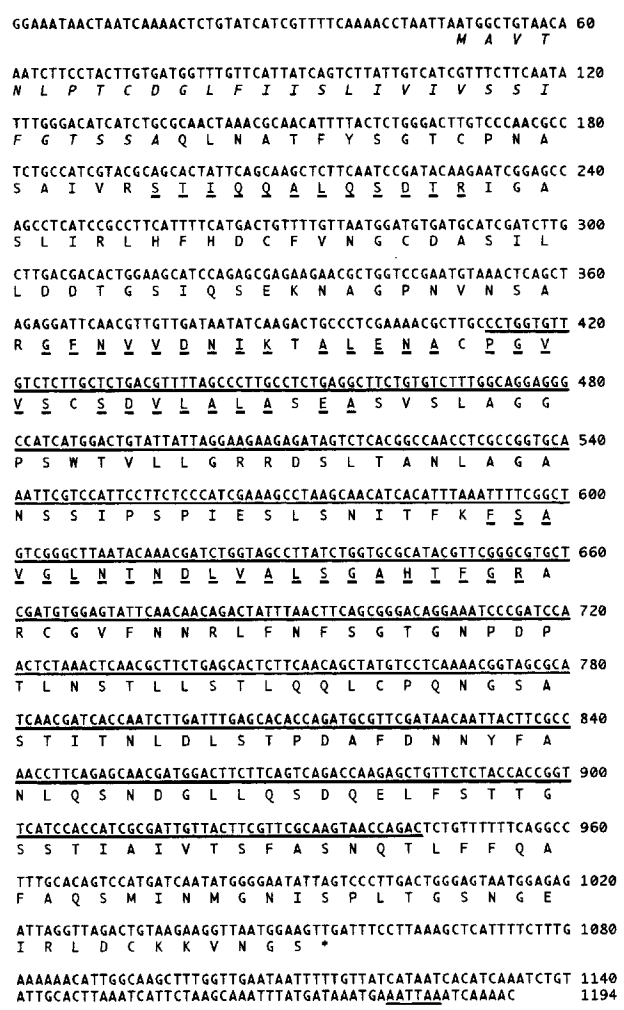


Fig. 1. Activity stained isoelectric focusing gel pH 3–9 of peroxidases from *Arabidopsis thaliana* (Columbia) cell suspension culture at 50% confluence. p*I* marker bands of horseradish isoperoxidases (lane M) and spent medium (lane 1).



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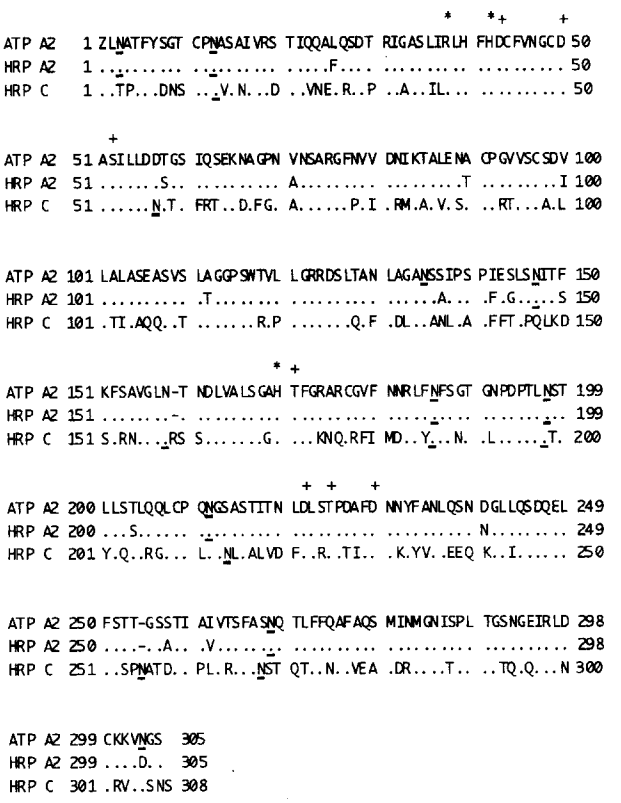


B

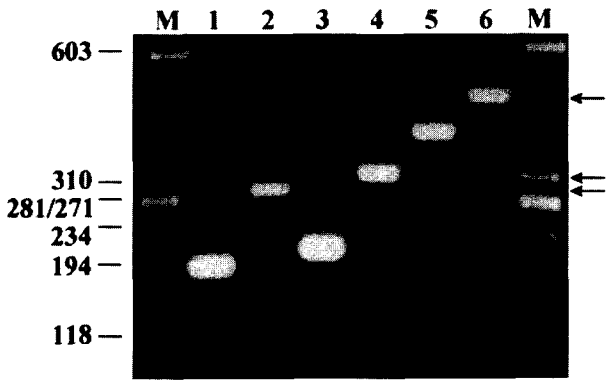
Fig. 2. A: Nucleotide and deduced amino acid sequence of a cDNA encoding *Arabidopsis* ATP A2 peroxidase. The putative signal peptide sequence is shown in italics and residues observed by amino acid sequencing of tryptic peptides are underlined. The probe used for screening (pos. 412–944) and the putative polyadenylation signal are underlined. B: Alignment of the amino acid sequence of the putative mature ATP A2 and the completely sequenced HRP A2 (C.B. Rasmussen, B. Stoffer and K.G. Welinder, in preparation) and HRP C [18]. Identical amino acids are indicated by dots. *N*-Glycosylation sites observed in HRP A2 and HRP C and predicted in ATP A2 are underlined. In the first line, active site residues are indicated with (*) and putative Ca^{2+}-binding side chains by (+).

lished) suggests that AAUUAA at position 1181–1186 is a potential polyadenylation signal in ATP A2 mRNA.

3.3. Structure of ATP A2

All known classical plant peroxidases have an N-terminal signal peptide and are targeted to the secretory pathway via the endoplasmic reticulum. Some, like HRP C and barley grain peroxidase BP 1, have a carboxy-terminal propeptide which directs them to the vacuole [14]. The deduced amino acid sequence of ATP A2 indicates that it contains an N-terminal signal peptide of 30 residues. This is based on the presence of glutamine in amino acid position 31 which becomes pyroglutamate (Z1 in the mature sequence, Fig. 2B) as observed for HRP A2 and HRP C. The open reading frame encoding ATP A2 stops at a position corresponding to the C-terminus of mature plant peroxidases [15] indicating that it will follow the default pathway out of the cell. This is in agreement with the presence of ATP A2 in the medium of the cell culture.

Mature ATP A2 consists of 305 amino acid residues and is 95% identical to the anionic HRP A2, but only 58% identical to the cationic HRP C (Fig. 2B). The catalytic residues arginine 38 and histidine 42 at the open distal site of the heme group, and histidine 169 coordinated to the heme iron as well as the eight cysteine residues forming four disulfide bridges 11–91, 44–49, 176–208, and 97–299, which are conserved throughout the plant peroxidase superfamily [16,17], are found in ATP A2. Furthermore, the amino acid side chains which can bind a distal Ca^{2+}, Asp-43, Asp-50, and Ser-52, and a proximal Ca^{2+}, Thr-170, Asp-221, Thr-224, and Asp-229, are conserved. HRP A2 contains seven *N*-linked glycans and HRP C eight glycans [18]. ATP A2 seems to contain two glycans in addition to those observed in HRP A2. All nine putative glycans in ATP A2 are located on the surface of the molecule using the peanut peroxidase crystal structure as a model [17]. One glycan, at Asn-135, is close to the presumed site for binding aromatic donor molecules [19]. It is notable that this site is glycosylated in another anionic peroxidase, HRP A1 (C.B. Rasmussen and K.G. Welinder, unpublished). Excepting the possible steric effect of this glycan on reaction



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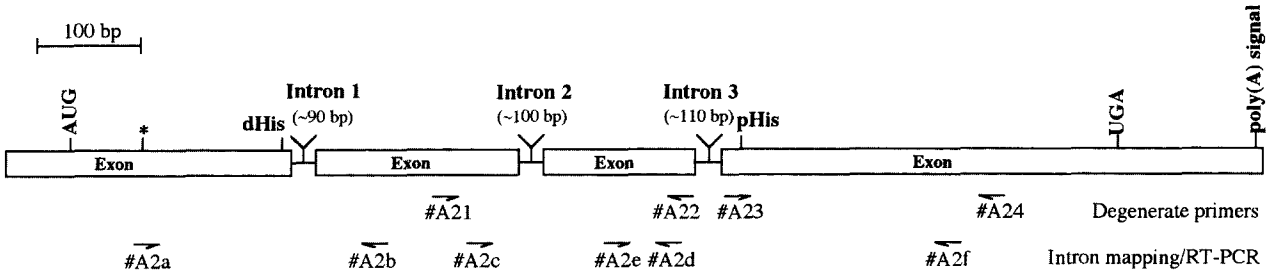

B

Fig. 3. Intron mapping of the *atp A2* gene. Lanes M show size markers. A: Lanes 1, 3, and 5 show PCR reactions run on plasmid DNA harboring ATP A2 cDNA, and lanes 2, 4, and 6 show PCR reactions run on genomic DNA from *Arabidopsis*. Primer pairs were #A2a and #A2b (lanes 1 and 2); #A2c and #A2d (lanes 3 and 4), and #A2e and #A2f (lanes 5 and 6). Arrows on the right indicate the products generated from genomic DNA. B: Schematic organization of the *atp A2* gene. The asterisk indicates the position of the codon encoding the first amino acid of the native protein, and the regions for the distal and proximal histidines are symbolized by dHis and pHis.

rates, the favorable enzymatic and stability properties of HRP A2 [2] are also expected for ATP A2.

3.4. Intron mapping

Three pairs of primers were designed to probe the presence of the two or three introns observed in other plant peroxidase genes. Positions of the primers are shown in Fig. 3B and should result in band sizes of 188 bp (#A2a+#A2b), 208 bp (#A2c+#A2d), and 392 bp (#A2e+#A2f), when PCR is carried out on ATP A2 cDNA. Fig. 3A shows that this is indeed the case and that PCR performed on genomic DNA gives rise to bands of larger size for all three primer pairs. The introns are estimated to be approximately 90 bp, 100 bp and 110 bp, which is sufficient to form the lariat structure in the splicing process [6]. Moreover, the average intron length found in 146 *Arabidopsis* genes with more than two introns is 146 bp, while the shortest is 59 bp [20]. Thus, these data indicate that the three introns and four exons found in genes encoding cationic horseradish and *Arabidopsis* peroxidases [21,22] are also present in the *atp A2* gene.

3.5. Expression analysis

In a systematic study of the repertory of peroxidases in *Arabidopsis*, we now know that more than 37 different isoperoxidases [23] are present, based largely on information obtained from the dbEST (database for expressed sequence tags) [24]. Since peroxidases are characterized by their general lack of substrate specificity and, furthermore, produce highly reactive radical products which continue in non-enzymatic reactions, this great repertory of peroxidase genes is a mystery, although one which expression analysis may shed some light on.

To exclude cross-reactions with other peroxidase mRNAs in the RT-PCR reaction, a mixture of plasmid DNA containing all peroxidase cDNAs found in dbEST so far was employed in a PCR reaction with primers #A2c and #A2f. This reaction did not yield any products (data not shown), suggesting that the primer pair is specific for ATP A2 mRNA.

The results of RT-PCR on total RNA isolated from cell suspension culture and whole plant material are shown in Fig. 4A. Positive signals were detected at all stages except from day 2. The RT-PCR is not a quantitative assay, however, due to competition in the PCR reaction the presence of a product originating from the genomic sequence at similar intensity indicates an initial amount of the mRNA in the same range as the chromosomal copy. Thus, the level of ATP A2 mRNA seems higher at day 4 and day 6 than at any other stage tested. RT-PCR was carried out on total RNA from root, leaf, and stem from 35 and 59 day old plants and expression was detected only in roots. Results from 35 day plant organs are shown in Fig. 4B. Commercial HRP A2 is purified from horseradish roots, but its expression has not been studied.

We therefore conclude that expression of the *atp A2* gene is developmentally regulated and organ-specific. Moreover, expression in the same organ for ATP A2 and HRP A2 as well as the high degree of identity between them suggests that these two peroxidases have the same enzymatic properties and that

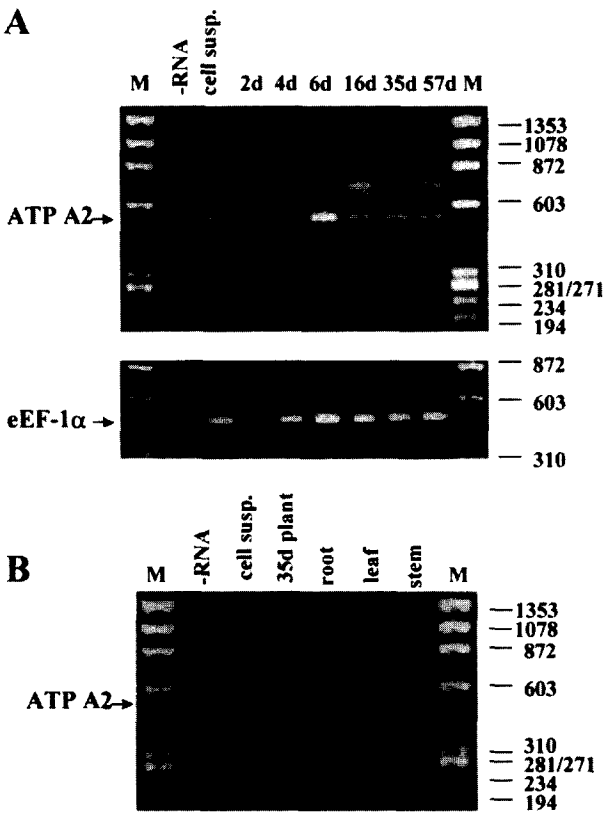

Fig. 4. Expression analysis of mRNA encoding ATP A2 using RT-PCR. Reactions were carried out on total RNA from (A) cell suspension culture and whole plant material at age 2, 4, 6, 16, 35, and 57 days and (B) cell suspension culture, whole plant material, root, leaf and stem from day 35. A positive internal control for mRNA encoding eEF-1α is shown below A. M indicates marker bands with sizes indicated on the right. −RNA indicates negative control with no RNA present. Bands corresponding to PCR products from genomic DNA (approximately 200 bp larger) were seen in reactions with no initial reverse transcriptase step (data not shown) as well as in some of the reactions shown here.

this type of peroxidase existed in a common ancestor before *Arabidopsis* and horseradish separated into different genera.

4. Conclusions

This is the first report of the cloning of an anionic A2 type of peroxidase. The A2 protein was isolated from the spent medium of an *Arabidopsis* cell suspension culture and cloned from a corresponding cDNA library. The *atp A2* gene seems only to be expressed in root, and the ATP A2 protein is present in the extracellular space or is attached to the cell wall. Its function is as yet unknown. The mRNA for ATP A2 is so rare that it has not yet appeared in the dbEST, in contrast to data for 37 other *Arabidopsis* isoperoxidases.

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