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Expression analysis of the *Arabidopsis* peroxidase multigenic family

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Abstract

Class III peroxidases form a numerous multigenic family in higher plants, whose expression is particularly sensitive to internal or external events. *Arabidopsis thaliana* genome harbours 73 genes encoding peroxidases. Since they exhibit homologies ranging from 28% to 93% at the nucleotide level, the risk of cross-hybridisation may be important when measuring the level of transcripts by blotting techniques, using whole cDNA sequences. We developed a procedure to assess the expression of all peroxidase genes on one membrane, with a high specificity. The method was based on the determination for each gene of a short specific sequence (amplicon) exhibiting at the most 70% homology with any other sequences of the *Arabidopsis* genome. Amplicons specific for each of the 73 peroxidase genes and two pseudogenes were blotted on a nylon membrane that was hybridised with radiolabelled cDNA libraries prepared from mRNAs of *Arabidopsis* roots, stems, leaves and flowers. Many genes were expressed at a low level, often in all organs, while sixteen genes were rather strongly expressed, in two to four organs. Some genes with no ESTs reported in databases were found to be expressed and this was confirmed by RT-PCR. Isoelectric focusing analysis revealed that the isoperoxidase pattern was similar in leaves, stems and flowers, but was quite different in roots. To our knowledge, only one similar study has been performed on the cytochrome P450 family, using microarrays, but this is the first work describing the expression profile of a whole large multigenic family using specific macroarrays.

Keywords: *Arabidopsis thaliana*; Macroarrays; mRNA/cDNA; Peroxidase; Phylogenetic tree; Specific amplicons

1. Introduction

Land plants contain a large number of class III peroxidases (E.C.1.11.1.7). In this class, all isoforms possess a signal peptide, which targets the proteins into the secretory pathway via the endoplasmic reticulum. These secreted plant peroxidases achieve a great deal of oxidation reactions essential for the cells, using hydrogen peroxide as an electron acceptor and several substrates as electron donors (Penel, 2000). At present, 73 class III peroxidase genes have been identified throughout the *Arabidopsis* genome (Tognolli et al., 2002). ESTs corresponding to 61 of these peroxidases have been identified in the TAIR database (www.arabidopsis.org), which suggests that these genes were transcribed and most likely encode a functional enzyme. Indeed, when some of these peroxidases were expressed in heterologous system, they were able to oxidise guaiacol, a peroxidase substrate (Dunand et al., 2002; Tognolli et al., 2000). Even if a risk of redundancy exists due to the protein sequences homology (between 28% and 100%), the different isoforms could be implicated in quite different physiological processes, such as lignification, suberisation, auxin catabolism, cross-linking of cell wall proteins, defence against pathogen attack, salt tolerance and oxidative stress (Hiraga et al., 2001; Penel et al., 1992). In this way, it will be useful to study this large multigenic family to understand its physiological roles and characteristics.

One of the most important features of plant peroxidases is their capacity to react to internal or external factors, either by a transcriptional or a post-transcriptional regulation. Published works exclusively devoted to peroxidase gene expression in *Arabidopsis thaliana* are rare and often concern one, or a small number of genes. Other studies, using the microarray technique, have

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included some peroxidase genes among the thousands of genes that were examined. The data showed that few peroxidase genes exhibit an organ-dependent expression, while others are active in the whole plant (Tognolli et al., 2002; Welinder et al., 2002; Zhu et al., 2001) or expressed in lignifying tissues (Ostergaard et al., 2000). The treatments that were shown to activate or down-regulate selected peroxidase genes include bacterial infection, methyljasmonate, ethylene, salicylic acid (Schenk et al., 2000), drought or cold stresses (Seki et al., 2001), iron deficiency (Thimm et al., 2001) and light (Ma et al., 2001).

Plants often react to various stimuli through the synthesis or induction of certain peroxidase protein isoforms (Hiraga et al., 2001; Penel et al., 1992). This explains why they were so often used as enzymatic markers to follow the behaviour of plants in a wide range of situations. This has generally been achieved by separating the peroxidases of a plant by various electrophoretic techniques. From the zymograms obtained, it could be seen that the presence of many isoperoxidases is related to a particular plant development stage and/or is dependent on external stimuli such as high or low temperature, light intensity or quality, drought, mechanical stress, air or soil contamination by various pollutants, heavy metals, pathogens, hormonal treatments, oligogalacturonates, etc. (Gaspar et al., 1982; Penel et al., 1992). In some cases, it was observed that following a particular event, different peroxidases appeared or disappeared according to a determined temporal sequence (Gaspar et al., 1985), suggesting that, in a particular situation, certain members of this family may have their own regulation and act sequentially.

In this context, it would be particularly interesting to have an exhaustive expression profile of the *Arabidopsis* peroxidase gene family in different conditions, followed by a protein identification. cDNA microarray analysis has the potential to give information concerning the gene regulation by endo- and exogenous factors. Even if there is not necessarily a quantitative relationship between the transcript level and the protein activity (Dunand et al., 2003), the transcription profile of each peroxidase gene will indicate a putative pattern of the peroxidase enzymatic activity. However, a problem of non-specific hybridisations in a large multigenic family can be associated with the microarray technique. The use of specific short amplicons (90–400 bp DNA sequences obtained by PCR amplification and specific to each peroxidase gene) showing less than 70% homology with the whole *Arabidopsis* genome should allow this problem to be bypassed. Besides, microarray genetic studies are too expensive to allow valid replication numbers, while macroarrays easily allow several replicates to be done in regular laboratory conditions, as recommended previously (Lee et al., 2000).

The results obtained in this work show that it is possible to obtain the expression pattern of all the members of the peroxidase multigenic family in the main *Arabidopsis* organs with good specificity and reproducibility. This opens the way for an extended study of this important gene family.

2. Results

2.1. Verification of amplicon specificity

The characteristics of the amplicons corresponding to each peroxidase gene are given in Table 1. They were designed by comparing independently each 5′UTR + cDNA + 3′UTR peroxidase sequence with the SPADS (Thareau et al., 2003) and CATMA (Crowe et al., 2003) amplicon databases. Sequences identified as being suitable for use as an amplicon were further checked by BLAST analysis (http://www.arabidopsis.org) to verify the percentage of its homology with the whole *Arabidopsis* genome. For some genes, this procedure did not yield specific amplicons. In that case, we performed an alignment between the sequence of interest and the closest homologue (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). A region showing less than 70% homology was then determined and checked again by running a BLAST analysis against the whole *Arabidopsis* genome. Amplicons specific for peroxidases sharing a high gene sequence homology (*AtPrx13*/*AtPrx20*, *AtPrx14*/*AtPrx15*, *AtPrx22*/*AtPrx23*, *AtPrx32*/*AtPrx33*, *AtPrx34*/*AtPrx37*/*AtPrx38*, *AtPrx49*/*AtPrx72* and *AtPrx50*/*AtPrx51*) may show slightly more than 70% homology between them on a short part of the amplicon, sometimes with gaps. It was not possible to discriminate between *AtPrx1* and *AtPrx2*, since they have exactly the same sequence.

Amplicon specificity was tested on three strongly homologous sequences, *AtPrx32*, *AtPrx33* and *AtPrx34*. *AtPrx42*, which is quite different, was used as control. The entire cDNAs were blotted on a membrane and probed with a *AtPrx32* cDNA probe (Fig. 1A). The results show that the *AtPrx32* probe hybridised similarly with the three homologous cDNAs, but not with *AtPrx42*. On the other hand, when amplicons specific for *AtPrx32*, *AtPrx33*, *AtPrx34* and *AtPrx42* were blotted on membranes and probed separately with *AtPrx32*, *AtPrx33* and *AtPrx34* whole cDNA probes, the hybridisation was totally specific, demonstrating the selectivity of the amplicons (Fig. 1B).

2.2. Expression analysis

The expression of the peroxidase multigenic family in *Arabidopsis* organs was analysed with macroarrays.

Table 1
Listing of the class III peroxidases

New name	TAIR gene number	Swiss-Prot number	Previously used names	Amplicon size	Homologies superior to 70%	EST	C-Term/target prediction	p*I*/MW
AtPrx1-	At1g05240-	PER1	Atp11a, Atatp11a,	171		32	no/SP	9.3/33322
AtPrx2	At1g05250	PER2	AtP11, Atp12a					
AtPrx3	At1g05260	PER3	Atp7a, Atperox7a, AtPRC, RCI3A	175		15	no/SP	8.8/32446
AtPrx4	At1g14540	PER4	AtP46	115		2	no/SP	7.7/32315
AtPrx5	At1g14550	PER5		190		–	no/SP	8.86/32290
AtPrx6	At1g24110	PER6		300		+	no/SP	6.13/33986
AtPrx7	At1g30870	PER7	AtP30	187		10	no/SP	8.09/33215
AtPrx8	At1g34510	PER8		120		2	no/SP	9.3/31996
AtPrx9	At1g44970	PER9	Atatp418a, Atp18a, AtP18	176		4	no/SP	7.04/33127
AtPrx10	At1g49570	PE10	Atatp5a, Atp5a, AtP5	155		10	no/SP	5.63/32748
AtPrx11	At1g68850	PE11	Atp23a, AtP23	164		7	no/SP	5.1/33879
AtPrx12	At1g71695	PE12	Atprxr6ge, Atp4a, AtP4	264		37	yes/SP	6.49/34782
AtPrx13	At1g77100	PE13		94	*AtPrx20* (74%, 43/58)	–	no/SP	4.95/32283
AtPrx14	At2g18140	PE14		116	*AtPrx15* (73%)	+	no/SP	5.81/33530
AtPrx15	At2g18150	PE15	AtP36	116	*AtPrx14* (73%)	4	no/SP	5.78/33225
AtPrx16	At2g18980	PE16	Atp22a, AtP22	84	*AtPrx45* (66%)	8	no/SP	9.59/33312
AtPrx17	At2g22420	PE17	Atp25a, AtP25	171		3	yes/SP	5.05/34260
AtPrx18	At2g24800	PE18		189		+	no/SP	5.09/32307
AtPrx19	At2g34060	PE19	AtP51	275		2	no/SP	9.15/33651
AtPrx20	At2g35380	PE20	Atp28a, AtP28	187		3	no/VAC	5.25/34715
AtPrx21	At2g37130	PE21	Atprxr5ge, Atp2a, AtP2	398		52	no/SP	7.19/33514
AtPrx22	At2g38380	PE22	Athpreca, prxEa, AtPEa	99	*AtPrx23* (78%, 73/93)	71	yes/SP	5.67/35252
AtPrx23	At2g38390	PE23	AtP34	114	*AtPrx22* (78%, 73/93)	9	yes/SP	8.45/35285
AtPrx24	At2g39040	PE24	AtP47	196		1	no/SP	7.71/33067
AtPrx25	At2g41480	PE25		213		1	no/SP	6.59/32911
AtPrx26	At2g43480	PE26	AtP50	214		1	no/CYT	8.66/33921
AtPrx27	At3g01190	PE27	Atprxr7ge, AtP12	199		17	no/SP	9.05/32301
AtPrx28	At3g03670	PE28	AtP39	180		1	no/SP	4.94/32233
AtPrx29	At3g17070	PE29	AtP40	400		1	no/SP	4.84/32887
AtPrx30	At3g21770	PE30	Atprxr9ge, AtP7	193	*AtPrx3* (70%,)	11	no/SP	9.67/32901
AtPrx31	At3g28200	PE31	AtP41	118	*AtPrx63* (68%)	8	no/SP	9.06/32952
AtPrx32	At3g32980	PE32	Atprxr3ge, AtP16	113	*AtPrx34* (74%)	135	yes/MIT-CHL	6.11/35712
AtPrx33	At3g49110	PE33	Athprxca, prxCa, AtPCa	154		12	yes/SP	7.04/35624
AtPrx34	At3g49120	PE34	Atprxcb, AtPCb	91	*AtPrx33* (75%)	93	yes/SP	7.71/35695
AtPrx35	At3g49960	PE35	Atatp21A, AtP21	248		11	no/SP	9.36/33050
AtPrx36	At3g50990	PE36		300		+	no/SP	4.72/33996
AtPrx37	At4g08770	PE37	AtP38	100	*AtPrx38* (96% 28/30)	10	yes/SP	6.98/35799
AtPrx38	At4g08780	PE38		83	*AtPrx37* (90%), *AtPrx33* (77%)	+	yes/SP	6.98/35682
AtPrx39	At4g11290	PE39	Atatp19a, AtP19	182		25	no/SP	6.58/33094
AtPrx40	At4g16270	PE40		214		+	no/CHL	4.61/32088
AtPrx41	At4g17690	PE41		300		+	no/SP	8.53/33525
AtPrx42	At4g21960	PE42	Atprxr1ge, AtP1	400		340	no/SP	8.35/34289
AtPrx43	At4g25980	PE43		219		+	no/SP	5.36/32629
AtPrx44	At4g26010	PE44	–AtP35	177		17	no/SP	9.99/31362
AtPrx45	At4g30170	PE45	Atp8a, Atperox8A, AtP8	224		43	no/SP	9.40/32811
AtPrx46	At4g31760	PE46	AtP48	196		1	no/SP	4.63/32187
AtPrx47	At4g33420	PE47	AtP32	399		3	no/SP	5.79/31835
AtPrx48	At4g33870	PE48		207		+	no/Other	5.6/33458
AtPrx49	At4g36430	PE49	AtP31	150	*AtPrx72* (78% 33/42)	5	no/SP	8.6/32976
AtPrx50	At4g37520	PE50	Atprxr2ge, AtP9	117	*AtPrx51* (75% with gap)	44	no/SP	8.95/33165
AtPrx51	At4g37530	PE51	AtP37	119	*AtPrx50* (75% with gap)	3	no/SP	8.38/33033

(*continued on next page*)

Table 1 (*continued*)

New name	TAIR gene number	Swiss-Prot number	Previously used names	Amplicon size	Homologies superior to 70%	EST	C-Term/target prediction	p*I*/MW
AtPrx52	At5g05340	PE52	AtP49	200		2	no/SP	8.84/30961
AtPrx53	At5g06720	PE53	Atpatpa2a, AtPA2	102		4	no/SP	4.82/31695
AtPrx54	At5g06730	PE54	Atp29a, AtP29	190		6	yes/SP	4.54/33938
AtPrx55	At5g14130	PE55	Atatp20a, AtP20	166		6	no/Other	4.91/32409
AtPrx56	At5g15180	PE56	AtP33	229		5	no/SP	8.68/32831
AtPrx57	At5g17820	PE57	Atprxr10g, AtP13	236		13	no/SP	9.99/31636
AtPrx58	At5g19880	PE58	AtP42	162		2	no/SP	5.02/32777
AtPrx59	At5g19890	PE59	AtatPN, AtPN	193		7	no/SP	6.36/32015
AtPrx60	At5g22410	PE60	Atatp14a, AtP14	200		2	no/SP	6.5/33298
AtPrx61	At5g24070	PE61		300		+	no/SP	6.58/33773
AtPrx62	At5g39580	PE62	Atp24a, AtP24	199		9	no/SP	8.7/31594
AtPrx63	At5g40150	PE63	Atp26a, AtP26	215		7	no/SP	8.6/32688
AtPrx64	At5g42180	PE64	Atprxr4ge, AtP17	270		25	no/SP	9.15/32249
AtPrx65	At5g47000	PE65	AtP43	300		5	no/SP	6.8/33560
AtPrx66	At5g51890	PE66	AtP27	183		9	no/SP	9.35/32951
AtPrx67	At5g58390	PE67	AtP44	167		2	no/SP	9.86/32586
AtPrx68	At5g58400	PE68		183		2	no/SP	9.61/32454
AtPrx69	At5g64100	PE69	Atatp3a, AtP3	200		56	no/SP	9.01/31944
AtPrx70	At5g64110	PE70	AtP45	158		2	no/SP	5.86/32328
AtPrx71	At5g64120	PE71	Atpo2, AtP15	208		22	no/SP	8.15/31369
AtPrx72	At5g66390	PE72	Atprxr8ge, AtP6	200		15	no/SP	8.57/34133
AtPrx73	At5g67400	PE73	Atprxr11g, AtP10	204		11	no/SP	9.44/33274
ψ1	AC007454	–	–	113	*AtPrx8* (75%), *AtPrx44* (74%)	–		
ψ2	AC007519	–	–	118		+		

The new proposed nomenclature, the TAIR gene number (the accession number for the two pseudogenes), the Swiss Prot number and some previously used names (Ostell and Kans, 1998; Welinder et al., 2002) are reported, as well as the size of the chosen amplicons. The amplicons that presented the highest homologies to others are specified. The EST column represents the last indexed ESTs and the corresponding counts from TAIR (Huala et al., 2001). When no ESTs were reported, the transcript was controlled individually by RT-PCR and the results are notified as (+) and (−). Some of the peroxidases possess a C-terminal extension (C-Term) which targeting role remains to be determined. The targeting has been predicted using Target P V1.0 (CBS). CHL: chloroplast; CYT: cytoplasm; MIT: mitochondria; SP: secretory pathway; VAC: vacuole. The last column indicates the isoelectric point (pI) and the molecular weight (MW) of each isoform, that were calculated from the deduced amino acid sequence of the mature proteins using ProtParam (Swiss-Prot). ψ1, ψ2: pseudogene 1 and pseudogene 2.

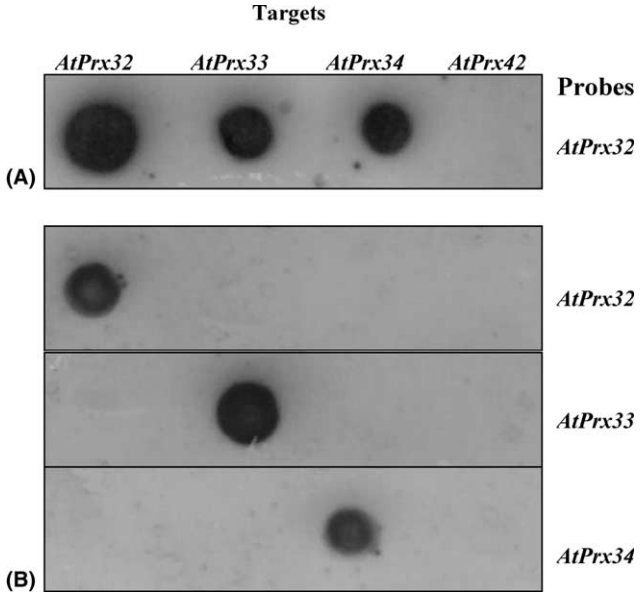

Fig. 1. Illustration of the specificity of the amplicon sequences. Thirty ng of target DNA were blotted on a membrane and hybridised with 5′UTR-cDNA-3′UTR probes. (A) whole cDNA; (B) *AtPrx32*, *AtPrx33*, *AtPrx34* and *AtPrx42* amplicons.

cDNA probes were prepared from roots, leaves, stems and flowers of 6-week old plants, using reverse transcription and radiolabelling (Fig. 2 and Table 2). According to the expression profiles shown in Fig. 2 and summarised in Table 2, 79% of the peroxidase genes are expressed in roots, 61% in stems, 64% in flowers and 78% in leaves. Variable results with high standard deviation values obtained for several genes (Table 2) were considered as being expressed. Stems show the lowest percentage of expressed peroxidase genes, but the expression was strong for many of them, with mean values higher than 100%. Transcripts corresponding to *AtPrx3*, *AtPrx18*, *AtPrx24*, *AtPrx35*, *AtPrx39*, *AtPrx40*, *AtPrx42*, *AtPrx45*, *AtPrx46*, *AtPrx51*, *AtPrx63* and *AtPrx67* are clearly present in all organs, while *AtPrx5*, *AtPrx9*, *AtPrx13*, *AtPrx16*, *AtPrx53*, *AtPrx55*, *AtPrx60* and *pseudogene1* are not expressed. In this last group, only *AtPrx5*, *AtPrx13* and *pseudogene1* have no reported ESTs. *AtPrx12*, *AtPrx14*, *AtPrx17* and *AtPrx65* transcripts are detected only in roots. *AtPrx39*, *AtPrx40*, *AtPrx46*, *AtPrx51* and *AtPrx69* are expressed at least in one organ with a value higher than 200%.

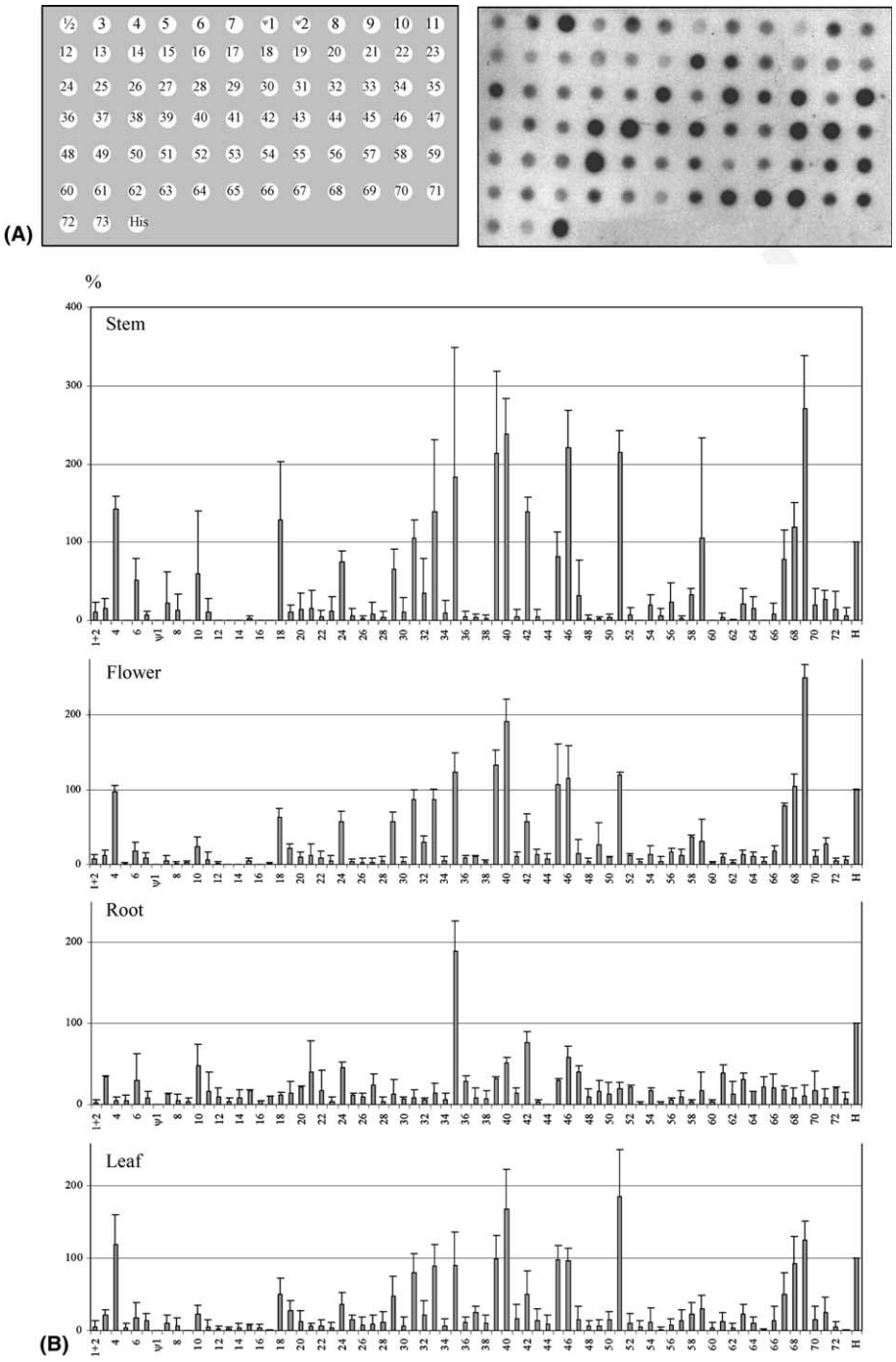

Fig. 2. (A) Peroxidase amplicon macroarray membrane with labelled leaf cDNA. 50 ng of each amplicon were blotted on a nylon membrane and hybridised with ^{32}P-radiolabelled leaf cDNA. Image acquisition was performed using a PhosphorImager and the Quantity One 1-D analysis software (BioRad). (B) Peroxidase gene expression profile. The means of three determinations obtained with different membranes minus the value of pseudogene 1 value were plotted on the histogram after conversion to percentage using the histone expression level as 100%. Standard deviation was indicated for each gene. ψ1, ψ2: pseudogene 1 and pseudogene 2.

The amplicon selectivity, demonstrated for the *AtPrx32*, *AtPrx33* and *AtPrx34* cluster (Fig. 1), was further confirmed by macroarray, since these genes showed different expression profiles (Fig. 2). This was also the case for other pairs of homologous genes, such as *AtPrx14*/*AtPr15*, *AtPrx22*/*AtPrx23*, *AtPrx31*/*AtPrx63*, *AtPrx37*/*AtPrx38*, *AtPrx50*/*tPrx51* and *AtPrx53*/*AtPrx54*, in one or several organs. Therefore, macroarrays proved to be a useful technique to assess the expression of members of a multigenic family, even those exhibiting a great homology.

AtPrx5, *AtPrx6*, *AtPrx13*, *AtPrx14*, *AtPrx18*, *AtPrx36*, *AtPrx38*, *AtPrx40*, *AtPrx41*, *AtPrx43*, *AtPrx48* and *AtPrx61*, as well as *pseudogene1* and *pseudogene2*,

Table 2
Peroxidase expression level in *Arabidopsis* roots, stems, flowers and leaves evaluated using macroarrays

Peroxidase	Root	Stem	Flower	Leaf	Peroxidase	Root	Stem	Flower	Leaf
AtPrx1–2					*AtPrx39*				
AtPrx3					*AtPrx40*				
AtPrx4					*AtPrx41*				
AtPrx5					*AtPrx42*				
AtPrx6					*AtPrx43*				
AtPrx7					*AtPrx44*				
AtPrx8					*AtPrx45*				
AtPrx9					*AtPrx46*				
AtPrx10					*AtPrx47*				
AtPrx11					*AtPrx48*				
AtPrx12					*AtPrx49*				
AtPrx13					*AtPrx50*				
AtPrx14					*AtPrx51*				
AtPrx15					*AtPrx52*				
AtPrx16					*AtPrx53*				
AtPrx17					*AtPrx54*				
AtPrx18					*AtPrx55*				
AtPrx19					*AtPrx56*				
AtPrx20					*AtPrx57*				
AtPrx21					*AtPrx58*				
AtPrx22					*AtPrx59*				
AtPrx23					*AtPrx60*				
AtPrx24					*AtPrx61*				
AtPrx25					*AtPrx62*				
AtPrx26					*AtPrx63*				
AtPrx27					*AtPrx64*				
AtPrx28					*AtPrx65*				
AtPrx29					*AtPrx66*				
AtPrx30					*AtPrx67*				
AtPrx31					*AtPrx68*				
AtPrx32					*AtPrx69*				
AtPrx33					*AtPrx70*				
AtPrx34					*AtPrx71*				
AtPrx35					*AtPrx72*				
AtPrx36					*AtPrx73*				
AtPrx37					*ψ2*				
AtPrx38					*ψ1*				

The transcription level is symbolized according to the percentage of expression as following: ⩽ 5%; highly variable expression; 6–49%; 50–100%; 101–200%; >200%. Results were obtained based on the average of three independent hybridization experiments for each organ. ψ1, ψ2: *pseudogene 1* and *pseudogene 2*.

have no reported ESTs until now (Huala et al., 2001; (www.arabidopsis.org). The results obtained here (Table 2) confirmed that *AtPrx5*, *AtPrx13* and *pseudogene1* are not expressed. However, the other peroxidase genes listed above appeared to be expressed in some or all organs, sometimes strongly, like *AtPrx18* and *AtPrx40*. This expression was verified by RT-PCR, using the same mRNA samples as for cDNA probe synthesis. A mix of

cDNA from root, leaf, stem and flower was used for PCR amplification, the primers being specific to each peroxidase gene and, when possible, designed in such a way that they correspond to a region of the gene including an intron. This allows to observe a size difference between DNA and cDNA amplification products. The results supported the expression profiles obtained with macroarrays (Fig. 3), confirming that the number of peroxidase genes expressed in *Arabidopsis* was higher than the number of ESTs listed in databases (www.arabidopsis.org). On the other hand, some genes having known ESTs did not appear to be expressed in the conditions of this work. This is the case of *AtPrx9*, *AtPrx16*, *AtPrx53*, *AtPrx55* and *AtPrx60*.

2.3. AtPrx separation by IEF

AtPrx proteins present in the four *Arabidopsis* organs were separated by IEF (Fig. 4), revealing the presence of a few acidic isoenzymes with pI ranging between 4.8 and 5.6, neutral and slightly basic isoenzymes (6.6–7.9), and a majority of isoforms present at higher pHs (8.0–10.0). Taking into account the theoretical isoelectric points, several putative correlations could be suggested between the IEF protein profiles observed for the different organs and the corresponding macroarray results (data not shown). Leaf, flower, and stem profiles appear to be quite similar, while root pattern is very different.

2.4. Phylogenetic relationships between peroxidases

A phylogenetic tree including all *Arabidopsis* peroxidases is presented in Fig. 5. It is not based on the alignment of the entire protein sequences, like the tree already published by Tognolli et al. (2002), but on 90 amino acid residues sequence, including F′ and F″ α-helices and two conserved cysteines involved in a disulphide bridge. This domain is known to be variable and contain residues important for the access of the substrate to the catalytic site (Gajhede et al., 1997). It could therefore allow to build a tree taking into account the putative substrate preferences of the various peroxidases. A comparison of both trees (Fig. 5 and Tognolli et al., 2002) shows conservation concerning the homologies between pairs or small clusters of genes. This is the

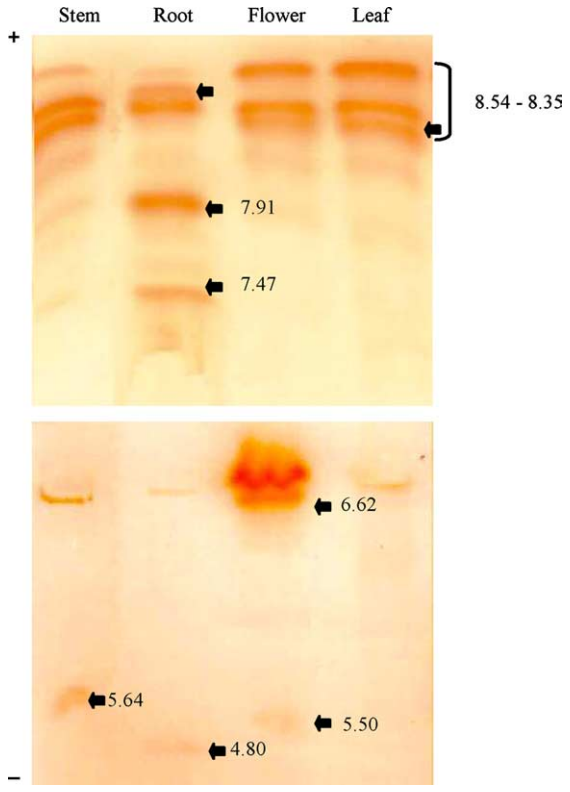

Fig. 4. Isoperoxidase pattern of *Arabidopsis* organs. Isoelectric focusing (IEF) separation (pH 3.0–10.0) was performed on extracts from six week-old plants. The same samples were used for the IEF and the macroarray probe synthesis. Two independent IEF have been performed respectively for the acidic and basic pI for a better resolution. The peroxidase bands were visualised using *o*-dianisidine/hydrogen peroxide. The arrows indicate the most significant band differences between organs. Isoelectric points are indicated for those bands.

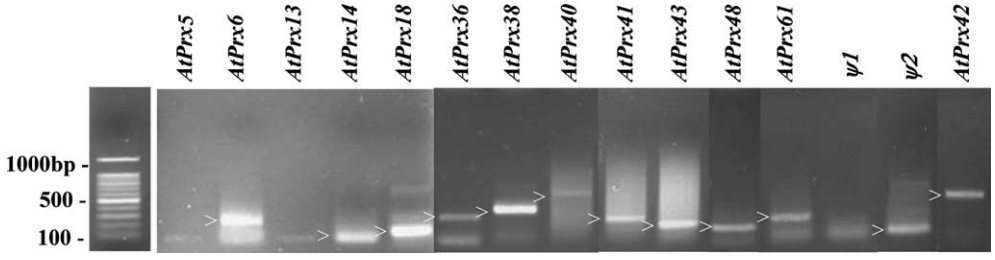

Fig. 3. RT-PCR analysis of expression of *A. thaliana* peroxidases without reported ESTs in databases. A pool of cDNA from roots, stems, flowers and leaves was used for PCR amplifications. Reaction products are compared to a standard of DNA bands of known sizes (first lane) through agarose gel electrophoresis. The predicted sizes of the amplification products (indicated with an arrow) are 768 bp for *AtPrx5*, 300 bp for *AtPrx6*, 94 bp for *AtPrx13*, 116 bp for *AtPrx14*, 189 bp for *AtPrx18*, 300 bp for *AtPrx36*, 390 bp for *AtPrx38*, 659 bp for *AtPrx40*, 300 bp for *AtPrx41*, 219 bp for *AtPrx43*, 207 bp for *AtPrx48*, 300 bp for *AtPrx61*, 113 bp for *pseudogene1* (ψ1), 118 bp for *pseudogene2* (ψ2) and 577 bp for *AtPrx42*. The smaller size bands represent traces of primers. The strongly expressed peroxidase *AtPrx42* was used as a positive control. The expected product sizes for *AtPrx5*, *AtPrx38*, *AtPrx40*, *AtPrx61* and *AtPrx42* suppose the excision of an intron placed between the primer positions, confirming the absence of genomic DNA and the correct amplification of cDNA.

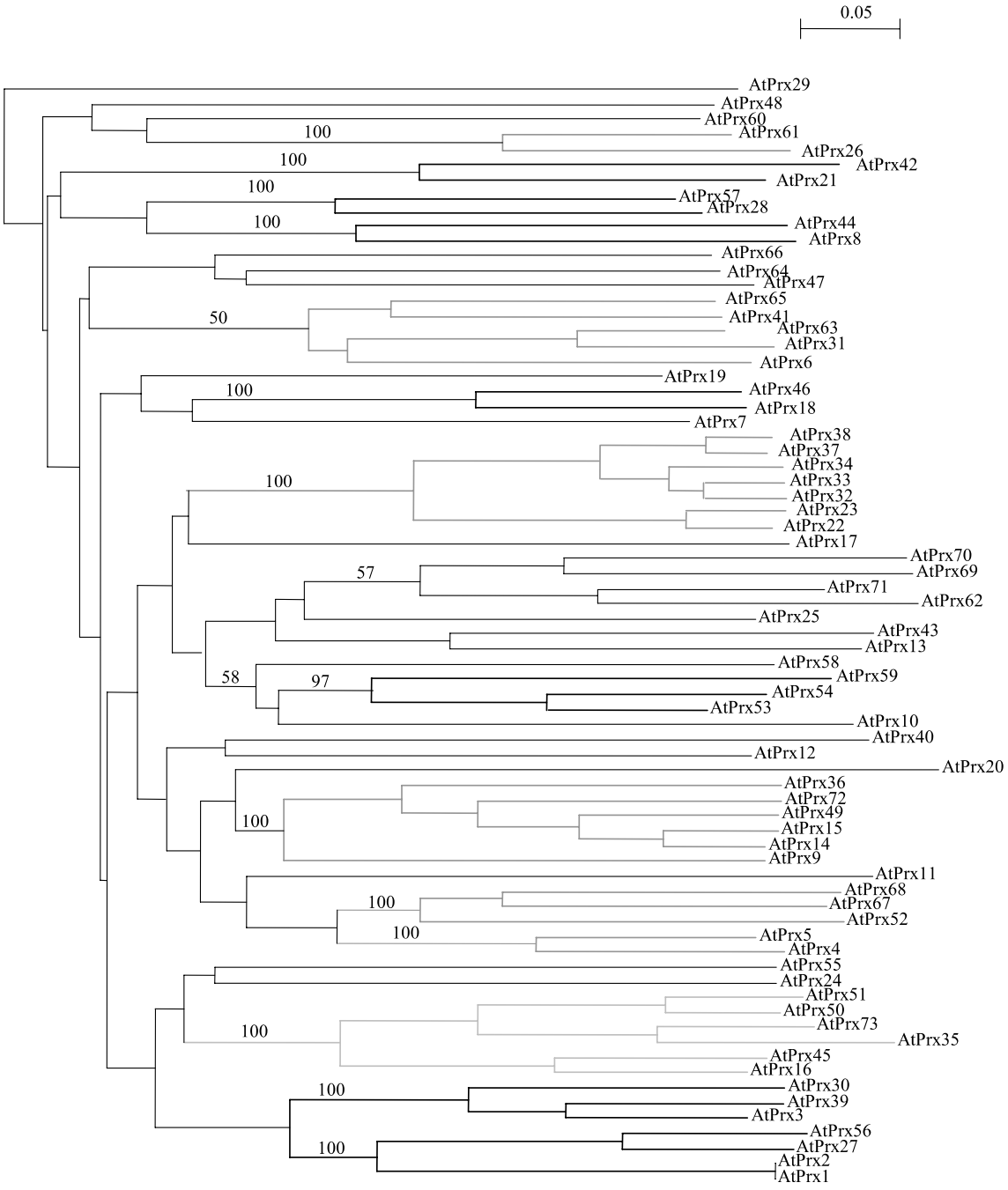

Fig. 5. Phylogenetic tree of the *A. thaliana* peroxidase proteins based on sequence alignments of the putative substrate access channel. All branches are drawn to scale and the scale bar represent 0.05 substitution per nucleotide.

case of *AtPrx1*/*AtPrx2*, *AtPrx27*/*AtPrx56*, *AtPrx8*/*AtPrx44*, *AtPrx28*/*AtPrx57*, *AtPrx62*/*AtPrx71*, *AtPrx50*/*AtPrx51*, *AtPrx22*/*AtPrx23* and *AtPrx49*/*AtPrx14*/*AtPrx15*. Macroarray experiments showed that the expression profiles of the pairs *AtPrx27*/*AtPrx56*, *AtPrx8*/*AtPrx44*, *AtPrx22*/*AtPrx23* and of *AtPrx49*/*AtPrx14*/*AtPrx15* did not differ much (Table 2). In contrast, the expression observed for *AtPrx28*/*AtPrx57* and *AtPrx62*/*AtPrx71* was different, and *AtPrx50*/*AtPrx51*, that encode two peroxidases with 86% sequence identities, had completely opposite expression profiles. This was also the case of homologous genes arranged in tandem (*AtPrx4*/*AtPrx5*, *AtPrx33*/*AtPrx34*, *AtPrx37*/*AtPrx38* and *AtPrx67*/*AtPrx68*).

In addition, two other phylogenetic trees, one including the 1000 bp promoter sequence upstream of the ATG codon of each gene and the other based on intronic sequences were drawn up (data not shown), revealing a low level of homology for the promoter and intronic sequences. Moreover, little correlation can be

observed between the protein sequences alignment and the non-coding sequence alignments.

3. Discussion

The functions of class III peroxidases in plants have been roughly identified and appear to be important in many essential processes, like cell elongation, cell wall differentiation or defence against pathogens (Gaspar et al., 1982; Penel et al., 1992). However, the determination of the precise role of each member of this multigenic family remained mostly elusive until now. It is supposed that there is an important functional redundancy among these enzymes, but this remains to be demonstrated. One of the key questions to be addressed concerns the control of the expression of the numerous paralogous genes encoding a peroxidase, in order to determine where, when or following which stimulus every gene is switched on or off. The procedure presented here appears to be suitable to unravel this problem. The identification of the corresponding proteins and of their catalytic functions should also be achieved. The macroarray method described here has the advantage over other techniques in that all the products of the peroxidase genes are measured in parallel, after having been processed together in a single preparation, which limits the fluctuations due to separate experiments. mRNAs present in each preparation were copied into radioactively labelled cDNA by reverse transcriptase, preserving the relative abundance of individual transcripts. The intensity of the hybridisation signal for a given gene was proportional to the abundance of the corresponding transcript. The procedure was standardized as far as possible. To overcome variations in array spotting, hybridisation and evaluation of the results, we deposited the amplicons on several membranes at the same time allowing a correct comparison after hybridisation. Three membranes were separately hybridised for each analysed *Arabidopsis* organ. Additionally, the intensities of the spots were quantified digitally. The reliability of the macroarray was also ensured by a careful evaluation of the specificity of each amplicon, based on the genomic sequences and not on EST sequence. Since EST clones are often incomplete, their specificity to a particular gene may be uncertain. This is particularly important for genes belonging to large families, where a full length clone may cross-hybridise to other family members, as shown in Fig. 1.

Our main requirement when designing the amplicons was that they should be as long as possible, while showing no more than 70% homology to any other part of the *Arabidopsis* genome. Hybridisation experiments on nylon membranes have shown that cross-reactivity is possible when two different gene targets share 77–100% sequence identity (Vernier et al., 1996). A careful evaluation of our results indicates that the macroarray procedure is likely to give reliable informations on the respective expression of each member of the peroxidase multigenic family. This is illustrated by the fact that pairs of very homologous genes exhibited a quite different expression among the four organs (Table 2, Fig. 5). The use of amplicons ensures a satisfactory specificity for the assay of peroxidase gene products, their small size not being detrimental to the sensitivity of the assay (Kane et al., 2000).

The results obtained by this technique can be compared to those of previous studies on the same subject. Tognolli et al. (2002) already used a cDNA macroarray procedure to study the expression of 23 peroxidase genes, spotting full length cDNA sequences on the membranes, which reduces the specificity of the results. This probably explains why our results do not completely crosscheck their data. For example, *AtPrx42* was by far the most intensively expressed gene in their study, but not in the present work (Table 2). On the contrary, we found that *AtPrx35* transcripts were abundant in root, stem and flower, while this previous study hardly detected them in the root. A previous work on *AtPrx21* and *AtPrx42* have also shown the constitutive expression of this two genes (Kjærsgård et al., 1997). An other study specifically devoted to peroxidase gene expression in *Arabidopsis* was realized on 33 genes with a gene-specific RT-PCR approach (Welinder et al., 2002). The results were similar to ours for 70% of the genes. The major differences concern *AtPrx35*, *AtPrx39*, *AtPrx62* and *AtPrx69*. Rather few peroxidase genes were found to be transcribed in only one organ, as for our results. The present study shows that the strongest peroxidase gene transcription occurs in floral stem, while former studies concluded that *Arabidopsis* root was the organ where peroxidase gene expression was the highest (Shah et al., 2004; Tognolli et al., 2002; Welinder et al., 2002). This discrepancy may be related to differences in plant growth conditions. It is also known that the expression of some plant genes, including some peroxidases, is extremely sensitive to even very gentle mechanical perturbations (Moseyko et al., 2002).

Macroarray analysis revealed that 30 of the 73 peroxidase encoding genes are expressed at different levels in the four organs of *Arabidopsis*. The product of 8 genes could not be detected in any organ with this method. The other genes were expressed in two or three different organs. Organ-specific expression was finally observed only for three genes active in root. This suggests that the expression of most genes does not depend on the nature of the organ, but would be rather tissue-specific or regulated by endogenous regulators or external stimuli. Confirming the importance of the transcriptional regulation, a lack of homology for promoters and intronic sequences can explain the diversity of expression profiles of paralogues.

Possible differences among organs at the peroxidase protein level were also assessed by performing IEF separations (Fig. 4). The similarity of the isoperoxidase pattern obtained for leaf and flower is striking, as well as the singularity of the root profile. Thus, despite the fact that a majority of genes that are transcribed in root are also active in leaf (59 genes) or in flower (51 genes), many isoperoxidases found in root are obviously different from those active in leaf and flower. This probably means that either the regulation at the translational level plays a predominant role in the determination of the amount of peroxidase present in tissues or that the peroxidases are differently inactivated in different organs. At this stage, we could find putative correlations between the expression profiles of the genes and the isoperoxidase patterns. However, it has been shown that the amount of a particular isoperoxidase does not necessarily correspond to the level of expression of its encoding gene (Dunand et al., 2003).

4. Concluding remarks

The macroarray technique used in this work proved to be suitable to analyse the regulation of a multigenic family like peroxidases, which encodes closely related proteins. It will allow to better understand the role of peroxidases in various situations, but also to identify genes reacting to particular physiological events or stimuli. It will be possible to further study the role of the peroxidases encoded by those genes by using knock-out mutants that do not produce them. Peroxidase enzymes were extremely often used as markers in relation to many treatments of developmental stages (Gaspar et al., 1982). The identification of peroxidase gene(s) up- or down-regulated by a given factor (chemical treatment, specific pathogens) will be available as markers for diagnostic purposes. Macroarrays can also allow the identification of promoters exhibiting interesting characteristics that could be used in plant transformation, to have a transgene expressed in certain conditions.

5. Experimental

5.1. Designation of arabidopsis peroxidases

It appeared necessary to rationalize the identification of the numerous *A. thaliana* peroxidases, since in recent years several names have been given to each gene, impeding the comparison of the data published on peroxidase genes. We adopted the nomenclature already used by Shah et al. (2004), with the letters "At" for *A. thaliana* and "Prx" for peroxidase, followed by a number reflecting the position of the gene on the chromosomes (Tognolli et al., 2002). Table 1 shows the correspondence of the standardised names with other names, available at the TAIR (http://www.arabidopsis.org) or Swiss-Prot (www.expasy.ch) databases, and the names that appear in NCBI (Ostell and Kans, 1998) or in previous works (Welinder et al., 2002).

5.2. Plant material and probe synthesis

Arabidopsis thaliana plants (ecotype Columbia) were grown on soil at 24 °C under a 16-h photoperiod. Different plant organs used for total RNA extraction were collected after 6 weeks of growth and immediately frozen in liquid nitrogen. mRNA for the cDNA probe synthesis was obtained from about 500 μg total RNA with the PolyAtract mRNA Isolation System kit from Promega (Wallisellen, Switzerland), the total RNA being extracted using the Tri-reagent solution (Sigma, Buchs, Switzerland). The cDNA was labelled by incorporating 50 μCi of (α)dATP^{32}P during reverse transcription using random primers, according to the ImPromII RT protocol from Promega. The specificity of the amplicons was assessed with three homologous genes. For this purpose, AtPrx32, AtPrx33 and AtPrx34 probes were obtained by PCR from the corresponding cDNA sequence comprising the 5′ and 3′ UTR and radiolabelled using the DNA Polymerase I Large (Klenow) Fragment from Promega, following the manufacturer instructions. The quantity of probe to use was estimated in reference to the EST count (TAIR,www.arabidopsis.org), in order to correctly represent the level of transcripts theoretically present in the cDNA library. *AtPrx42* amplicon was used as a negative control for the specificity assay.

5.3. Amplicon design

Gene-specific primers for amplicon synthesis have been designed for the 73 peroxidases genes and two pseudogenes, using different web tools: (i) Specific Primers & Amplicon Design Software (SPADS): http://www.psb.rug.ac.be, (ii) Complete Arabidopsis Transcriptome MicroArray (CATMA): http://www.catma.org and (iii) manual BLAST: http://www.arabidopsis.org. The short (90–400 bp) specific DNA sequences obtained by PCR (amplicons) show less than 70% homology with the remaining *Arabidopsis* genome sequences. PCR amplifications were done directly from genomic DNA using a Biometra T-Personal thermal cycler (Biolabo, Chatel-St-Denis, Switzerland) and standard PCR techniques. Following amplification, PCR products were analysed by gel electrophoresis to verify the product size and estimate the concentration. A positive control corresponding to a 140 bp sequence from the constitutively expressed *A. thaliana* putative histone H4 (accession AY142651) was also introduced in the arrays to normalise gene

expression between different experiments. The 113 bp amplicon corresponding to the peroxidase pseudogene I (Tognolli et al., 2002), after confirmation of its absence of expression by RT-PCR, was considered as a negative control.

5.4. Macroarrays and hybridisation

50 ng of the heat-denatured amplicon PCR products were resuspended in 20 μl of water, carefully blotted onto a Hybond-N+ nylon membrane (Roche, Mannheim, Germany) using the Bio-Dot apparatus from BioRad (California, USA) and fixed by baking for 2 h at 80 °C. Hybridisation of the membranes with the different labelled cDNA probes was performed over-night at 42 °C in three replicates, according to standard techniques, using the Church buffer (Church and Gilbert, 1984). Hybridisation was followed by two 30 min washes using 2× SSC 0.1% SDS, the first at 42 °C and the second at room temperature. Radiolabelled membranes were finally stored between plastic films and exposed to a PhosphorImager screen (BioRad) for 3 h.

5.5. Image analysis

The screen was scanned with a PhosphorImager Molecular imager FX (BioRad) at a resolution of 50 μm. Using the Quantity One 1-D analysis software (BioRad) and the volume array tool (96 wells, circular shape), the spot densities were quantified after normalisation to the median (3×3) filter value. The spot density is defined as the total intensity of all pixels in a defined area divided by this same area. The peroxidase pseudogene I spot density value was subtracted to each spot density and the expression percentage was calculated considering the histone H4 value as 100%. The expression was considered as highly variable when the standard deviation was equal or superior to the expression percentage minus 1%.

5.6. RT-PCR analysis

mRNA was isolated from plant tissues as described above and the same samples were used for probe synthesis and RT-PCR analysis. A first-strand cDNA was synthesised according to the Promega Im-Promp II reverse transcriptase protocol, using random primers from Promega. A pool of cDNA from root, leaf, stem and flower was used for PCR amplification using primers specific to each peroxidase. To eliminate the possibility of DNA contamination in the RNA samples, some primers were chosen to include an intron between them, allowing observation of a size difference between DNA and cDNA amplification products.

5.7. Separation of AtPrx by isoelectric focusing (IEF)

Soluble proteins were extracted from the main organs of 6-week old plants by grinding in 20 mM Hepes, pH 7.0, containing 1 mM EGTA (1 ml for each gram of fresh weight). The extract was filtered and centrifuged for 10 min at 10 000*g*. Proteins were assayed with the Coomassie Blue reagent (BioRad) and each extract was assayed for total peroxidase activity using guaiacol/hydrogen peroxide. Due to the large variation of the protein concentrations between the different organs, equal values of peroxidase activity were separated by IEF (Servalyt Precotes 3–10, Wallisellen, Switzerland) performed as described previously (Penel and Greppin, 1996) and the peroxidase bands were visualised using *o*-dianisidine/hydrogen peroxide.

5.8. Sequence and phylogenetic analysis

A phylogenetic tree based on a particular region of the 73 peroxidase proteins was constructed. This region included up to 90 amino acid residues and included the α-helices F′ and F″ and the conserved cysteine residues C_6 and C_7 (corresponding to cysteines 177 and 209 in horseradish peroxidase C) The 1000 bp sequence upstream of the ATG codon and the intronic sequences of all peroxidase encoding genes were also aligned. Multiple alignment analysis was done using the neighbor-joining (N-J) method implemented in ClustalW software (Saitou and Nei, 1987; Thompson et al., 1994) (http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html). Alignments were reconciled and adjusted visually to minimise insertion/deletion events using GeneDoc software.

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