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EXPRESSION ANALYSIS OF THE *ARABIDOPSIS* PEROXIDASE MULTIGENIC FAMILY USING NYLON MEMBRANES (MACROARRAY) HYBRIDIZED WITH ^{32}P-RADIOLABELLED cDNA LIBRARIES

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Abstract: Plant peroxidases, also known as class III peroxidases, are proteins containing a hem fraction, encoded by a large number of paralogous genes which form a numerous multigenic family in higher plants having an expression which is particularly sensitive to internal or external events. *Arabidopsis thaliana* genome contains 73 genes encoding peroxidases. Exhibiting homologies ranging from 28% to 93% at the nucleotide level, there is a risk of cross-hybridization which 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 is 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 were blotted on a nylon membrane that was hybridized with radiolabelled cDNA libraries prepared from mRNA of *Arabidopsis* leaves representing three categories of age, namely young, adult and senescent leaves. Many genes were expressed at a low level, often in leaves of all the three cathegories of age, while some of these genes were strongly expressed. 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 generally similar in leaves belonging to different cathegories of age, but it also presented some differences concerning the isoperoxidases bands and those differences could be considered as some new identified isoforms. As far as we know, 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.

Introduction

Higher plants contain a large range of peroxidases belonging to the so called class III (E.C.1.11.1.7.). Each of the isoforms in this class possess a signal peptide, which targets the proteins into the secretory pathway via the endoplasmic reticulum. All these plant peroxidases are involved into a large spectrum of oxidation reactions essential for the cells, using hydrogen peroxide (H_2O_2) as an electron acceptor and several substrates as electron donors (Penel, 2000). Class III peroxidases form a numerous multigenic family in higher plants. One of the most important features of plant peroxidases is their capacity to react to external or internal factors, either by a transcriptional or a post-transcriptional regulation. The *Arabidopsis thaliana* genome includes 73 genes encoding peroxidases [17, 18]. These peroxidases can also oxidize the growth hormones like auxin, or some different substrates [7] and to produce H_2O_2 [1] and hydroxyl radicals [3], two activated oxygen species that are involved in oxidative burst and cell elongation [2, 10, 11, 16].

There may exist a risk of cross-hybridization when measuring the level of transcripts by blotting techniques, using whole cDNA sequences, since they present homologies ranging from 28% to 93% at the nucleotide level. The different isoforms could be implicated in different physiological processes, such as oxidative stress, defence against pathogen attack, salt tolerance,

auxin catabolism, cross-linking of cell wall proteins, suberisation or lignification [8, 13]. Therefore, it could be important to study this multigenic family in order to understand the physiological roles and the characteristics of these genes. We developed a technique to measure the expression level of all the genes responsible for peroxidases synthesis in *Arabidopsis thaliana* on a single membrane, with a high specificity. This method is based on the determination of a short (amplicon between 90-400 bp) specific sequence for each gene, exhibiting at the most 70% homology with any other sequences of the *Arabidopsis* genome. Amplicons specific for each of the 73 peroxidase genes, amplicons for two pseudogenes (1 and 2) used as negative controls and also for a couple of some genes used as positive controls like PR1 (pathogen interaction marker, TAIR (The Arabidopsis Information Resources) database gene number At2g14610),α-TUB (α-tubulin), β-TUB (β-tubulin), CAB (chlorophyll a/b binding protein, TAIR At2g34420), RAB18 (ABA- and drought-induced glycine-rice dehydrin protein), ACT8 (actin 8, TAIR At1g49240), RUBISCO (Ribulose Bisphosphate Carboxylase/Oxygenase), PEX-2 (peroxiredoxine oxidative stress marker, TAIR At1g65970), EF-1 (elongation factor1) have been blotted on a nylon membrane and hybridized with radiolabelled cDNA libraries prepared from mRNA of young, adult and senescent *Arabidopsis* leaves. Some of these genes were expressed at a low level in leaves of all cathegories of age. We have also identified some other strongly expressed genes, differently for every cathegory of age in the analyzed leaves. We found to be expressed some genes with no ESTs (Expressed Sequence Tags) reported in databases, and this was confirmed by RT-PCR. There have been identified ESTs corresponding to only 61 of these peroxidases in the TAIR database (www.arabidopsis.org), suggesting that these genes were transcribed and they could encode a functional enzyme and this was confirmed when some of these peroxidases were expressed in a heterologous system and it was observed that they were able to oxidize guaiacol, a peroxidase substrate (Dunand et al., 2002; Tognolli et al., 2000).

Material and Methods

The plant material *(Arabidopsis thaliana,* Columbia ecotype) was grown on soil at 24ºC with a photoperiod of 16h. Young leaves (five weeks aged), adult leaves (seven weeks) and senescent leaves (older than seven weeks) were collected and preserved in liquid nitrogen in order to extract total RNA, using the Tri-reagent solution (Sigma, Buchs, Switzerland). mRNA for the cDNA probe synthesis was obtained from 500 μg total ARN with the PolyAtract mRNA Promega Isolation System Kit (Wallisellen, Switzerland). The cDNA was labelled by incorporating 50 μCi of (α)dATP^{32}P during reverse transcription using random primers (ImPromII RT Promega protocol). The probes were radiolabelled using the DNA Polymerase I Large (Klenow) Fragment from Promega. We estimateed the quantity of probe for use in reference to the EST count (TAIR, www.arabidopsis.org) in order to correctly represent the level of transcripts present in the cDNA library. *AtPrx4* amplicon was used as a negative control for the specific assay.

Amplicon design

For all the 73 peroxidases genes and two more pseudogenes (1 and 2) there have been designed gene-specific primers for amplicon synthesis, using different web tools: Specific Primers & Amplicon Design Software (SPADS): http://www.psb.rug.ac.be; Complete Arabidopsis Transcriptome MicroArray (CATMA): http://www.catma.org and manual Basic Local Alignment Search Tool (BLAST): http://www.arabidopsis.org. Short amplicons between 90-400bp obtained by PCR (Polymerase Chain Reaction) show less than 70% homology with the remaining *Arabidopsis* genome sequences. PCR amplifications were done directly from genomic DNA using a Biometra-T thermal cycler (Biolabo, Chatel-St-Denis, Switzerland) and standard PCR techniques. The PCR products were analyzed by gel electrophoresis to verify the product size and to estimate the concentration. To normalize gene expression between different

experiments there have been introduced in the arrays some positive controls corresponding to the genes like PR1, αTUB, βTUB, CAB, RAB18, ACT8, RUBISCO, PEX2, EF1. There was also considered as a negative control a 113bp amplicon corresponding to the peroxidase pseudogene 1 (Tognolli et al., 2002) (apart of *AtPrx4*) after confirmation of its absence of expression by RT-PCR, using the same mRNA samples as for cDNA probe synthesis.

Macroarrays and hybridization

In 20 µl water there were resuspended 50 ng of heat-denatured amplicon PCR products for each of the 73 genes and two extra genes, pseudogenes 1 and 2, and blotted on a Hybond-N+ (Roche, Mannheim, Germany) nylon membrane, using the Bio-Dot apparatus from BioRad (California, USA). The membranes were fixed by baking for 2h at 80ºC. The next step was to hybridize the membranes overnight at 42ºC with the different labelled cDNA probes, using Church buffer (Church and Gilbert, 1984). After hybridization, the membranes were washed two times, 30 min. each time, using 2 x SSC 0,1% SDS, the first wash at 42ºC and the second at room temperature. The radiolabelled membranes were stored between plastic films and exposed to a PhosphorImager screen (BioRad) for 3h.

Image analysis

We used a PhosphorImager Molecular imager FX (BioRad) at a resolution of 50 µm to scan the screen. The spot densities were quantified after normalization to the median (3x3) filter value, using the Quantity One 1-D analysis software (BioRad) and the volume array tool (96 wells, circular shape). The definition of the spot density is as following: the total intensity of all pixels in a defined area divided by this same area. The peroxidase *AtPrx4* (TAIR gene number At1g14540-used as a negative control) spot density value was substracted to each spot density. The expression percentage was calculated considering the average expression level of all the positive controls used as 100%. The expression was considered as highly variable when the standard deviation was equal or superior to the expression percentage minus 1%. Processing in Excel all the final results for the spots on the membranes, eventually we obtained a graphic representing the expression level for all 73 peroxidase genes in *Arabidopsis* in all the three age cathegories, young, adult and senescent leaves. Some of the genes showed to be expressed better in certain leaves, at a certain age.

RT-PCR analysis

mRNA was isolated from plant tissues as previously described (from young, adult and senescent leaves) and the same samples were used for probe synthesis and RT-PCR analysis. Using random primers from Promega, we synthesized a first cDNA strand, according to the Promega Im-Promp II reverse transcriptase protocol. The following step was to use a pool of cDNA from PCR amplification using primers specific to each peroxidase. Some of the primers were chosen such way they included an intron between them, in order to avoid the possibility of DNA contamination in the RNA samples, allowing observation of a size difference between DNA and cDNA amplification products.

Separation of *AtPrx* by isoelectric focusing (IEF)

Soluble proteins were extracted from three age categories (five, seven and more than seven weeks old) of *Arabidopsis thaliana* leaves by grinding in 20 mM Hepes, pH 7.0, containing 1 mM EGTA (Ethylene Glycol Tetra Acetic acid)(1 ml for each gram of fresh weight). The extract was filtered and centrifuged for 10 min at 10000g. 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 categories of leaves, equal value of peroxidase activity were separated by IEF (Servalyt Precotes 3-10, Wallisellen, Switzerland) performed as described in the section destinated to results and discussion (Penel and Greppin, 1996) and the peroxidase bands were visualized using *o*-dianisidine/hydrogen peroxide.

Results and Discussion

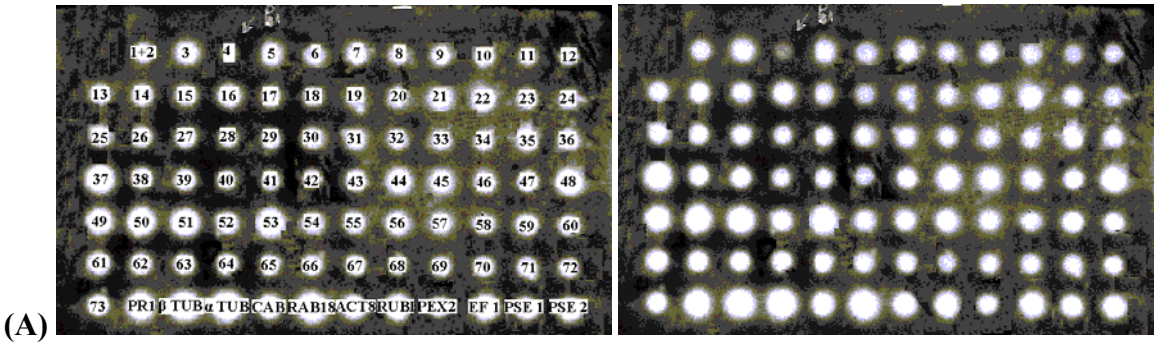
Expression analysis

The expression of peroxidase multigenic family in *Arabidopsis* was analyzed with macroarrays. cDNA probes were prepared from young, adult and senescent leaves using reverse transcription and radiolabelling. The results obtained confirmed that some of the genes have a level of expression which is less than 20%, including the pseudogene 1 and *AtPrx4* used as negative controls (Fig.1B). This expression was verified by RT-PCR, using the same mRNA samples as for cDNA probe synthesis. A PCR analysis using the primers for the "good candidate" genes (genes expressed differently in some leaves than in the others), selected from the graphic, has been further performed, the primers being specific to each peroxidase gene and, when possible, designed in such way that they corresponded to a region of the gene including an intron. This primer design allowed the observation of a size difference between DNA and cDNA amplification products. The results supported the expression profiles obtained with macroarrays (Fig.1A), confirming that the number of peroxidase genes expressed in *Arabidopsis* leaves was higher than the number of ESTs listed in databases (www.arabidopsis.org). According to the expression profiles shown in Fig. 1B, 72,6% of the peroxidase genes (53 genes from a complete range of 73) are expressed in *Arabidopsis* leaves with values higher than 20%.

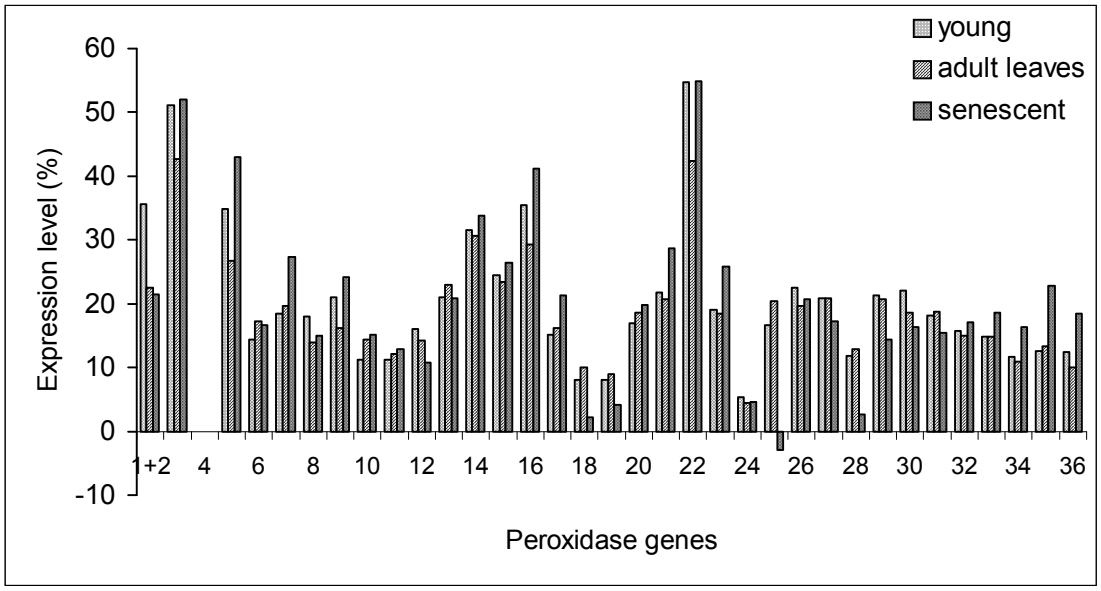
AtPrx separation by IEF

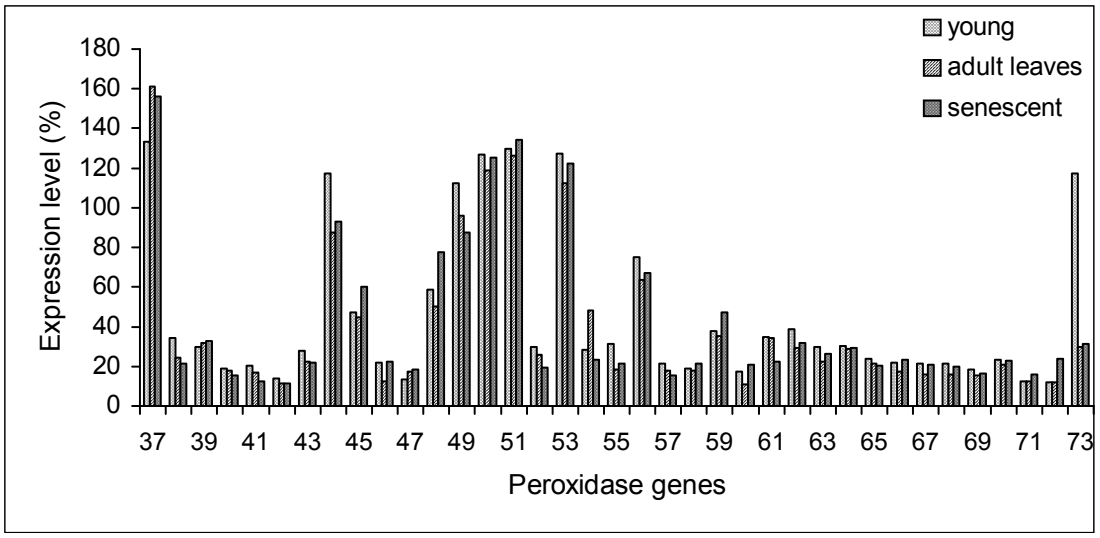
AtPrx proteins present in the three age stages leaves (young, adult and senescent) of *Arabidopsis* were separated by IEF (Fig. 3). The differences between the three profiles revealed the presence of an acidic isoenzyme with a pI value of 5.64, a neutral isoenzyme with a pI of 7.47 and a majority of 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 differently aged leaves and the corresponding macroarrays result (data not shown). There is a difference between the pI values of the peroxidase genes used in this experiment and the pI values obtained through the IEF migration. Even though these values are closely related, they are not identical. In this case, we can allow to say we may have detected some new isoenzymes. Table 1 presents some of the 73 peroxidase genes from the class III whose pI values are very close to the new detected isoenzymes of the IEF migration we have performed.

The functions of class III peroxidases in plants have been roughly identified until now and they seem to have a large importance in some of the essential physiological processes like cell elongation, defence against pathogens, cell wall differentiation, growth hormenes oxidation (Gaspar et al., 1982; Penel, 1992). The macroarray method here described was used as a mean of finding more about the precise role of each member of this multigenic family, about the expression control of numerous paralogous genes encoding a peroxidase in purpose of knowing better where, when or following which stimulus every gene is switched on or off. This technique presents the advantage of measuring in paralell all the products of the peroxidase genes, after having been processed together in a single preparation. This way the fluctuations due to individual experiments are very much diminished. mRNAs present in every preparation were copied into radioactively labelled cDNA by reverse transcription, preserving the relative abundance of individual transcripts. The intensity of the hybridization signal for each gene was proportional to the amount of the corresponding transcript. In order to have a correct comparison analysis after hybridization and to avoid variations in array spotting we blotted the amplicons on several membranes at the same time. To improve the quality of the results we digitally quantified the intensity of the spots obtained. The specificity of each amplicon was carefully evaluated based on the genomic sequences and not on EST sequence, since EST clones are often incomplete and having been supposed that their specificity to a particular gene may be uncertain. The main requirement when we designed the amplicons was that they should be as long as possible, but showing no more than 70% homology to any other part of the *Arabidopsis* genome.



(A)





(B)

Fig. 1: (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 were plotted on the histogram after convertion to percentage using the expression of positive control genes like PR1, aTUB, bTUB, CAB, RAB18, ACT8, RUBISCO, PEX2, EF1expression level as 100%.

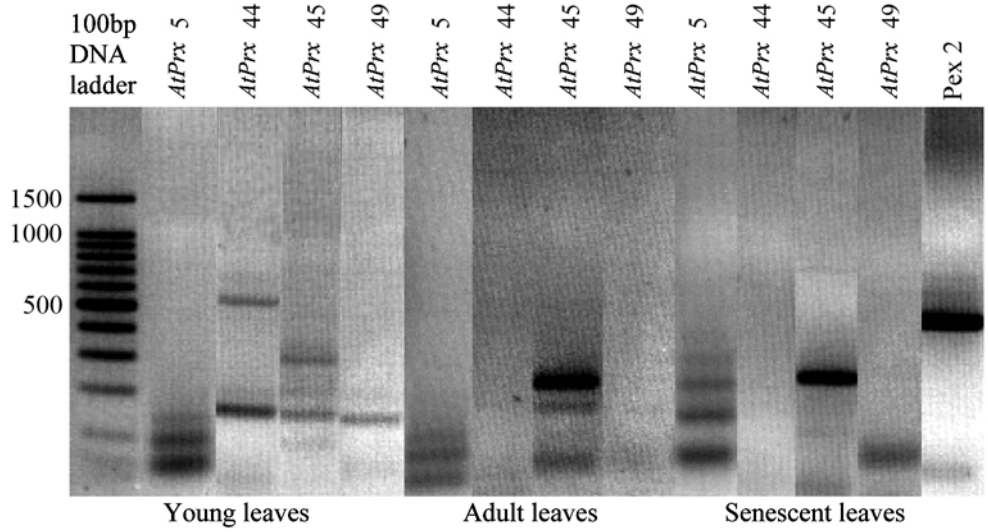


Fig. 2: RT-PCR expression analysis of *A.thaliana* peroxidases without reported ESTs in databases. A pool of cDNA from young, adult and senescent leaves was used for PCR amplification. Reaction products were compared to a standard of DNA bands of known sizes (first lane) through agarose gel electrophoresis. The predicted size of the amplification products are as following: 190 bp for *AtPrx5*, 177 bp for *AtPrx 44*, 224 bp for *AtPrx45, 150 bp for AtPrx49 and 482 bp for Pex 2*. The smaller size bands represent traces of primers. The strongly expressed *Pex 2* product was used as a positive control.

Table 1: Listing of some of the class III peroxidase genes pI values

New name	TAIR gene name	Swiss-Prot number	Previously used names	Amplicon size	Similarity higher than 70%	EST	p*I*/MW
AtPrx22	At2g38380	PE22	Athpreca, prxEa, AtPEa	99	AtPrx23 (78%, 73/93)	71	5.6735252
AtPrx23	At2g38390	PE23	AtP34	114	AtPrx22 (78%, 73/93)	9	8.45/35285
AtPrx24	At2g39040	PE24	AtP47	196		1	7.71/33067
AtPrx34	At3g49120	PE34	Atprxcb, AtPCb	91	AtPrx33 (75%)	93	7.71/35695
AtPrx41	At4g17690	PE41		300		+	8.53/33525
AtPrx42	At4g21960	PE42	Atprxr1ge, AtP1	400		340	8.35/34289
AtPrx48	At4g33870	PE48		207		+	5.6/33458
AtPrx51	At4g37530	PE51	AtP37	119	AtPrx50 (75% with gap)	3	8.38/33033

The new proposed nomenclature, the TAIR gene name, the SwissProt 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 transcipt was controlled individually by RT-PCR and the results are notified as (+). The last column indicates the isoelectric point (pI) and the molecular weight (MW) of the isoforms, calculated from the deduced amino acid sequence of the mature proteins using ProtParam (Swiss-Prot).

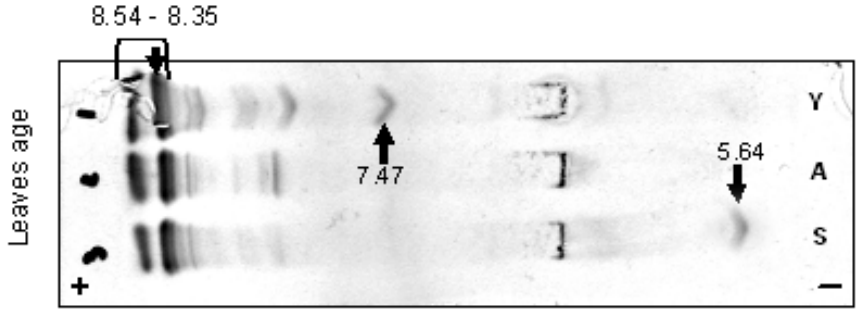


Fig. 3: Isoperoxidase pattern of *Arabidopsis* leaves. Isoelectric focusing (IEF) separation (pH 3.0-10.0) was performed on extracts from five (Y-young), seven (A-adult) and more than seven (S-senescent) weeks old leaves. The same samples were used for the IEF and the macroarray probe synthesis. The peroxidase bands were visualised using *o*-dianisidine/hydrogen peroxide. The arrows indicate the most significant band differences between leaves. The values represent isoelectric points.

As it was previously remarked (Vernier et al., 1996) cross-reactivity is possible when two different gene targets share sequence identity between 70-100%. According to the experiments of Tognolli (Tognolli et al., 2002) spotting full length cDNA sequences on the membranes reduces the specificity of the results. Regarding the results we obtained, we can conclude that there was a difference of expression between several genes in different leaf-developmental stages. It was proved that peroxidase genes like *AtPrx1, AtPrx2,AtPrx42, AtPrx44, AtPrx49, AtPrx53, AtPrx56,* AtPrx73 were best expressed in young leaves, *AtPrx37*, *AtPrx54* showed best expression in adult leaves, while genes like *AtPrx5*, *AtPrx7*, *AtPrx 16*, *AtPrx23*, *AtPrx35*, *AtPrx36*, *AtPrx* 45, *AtPrx* 48, *AtPrx51*, *AtPrx59* and *AtPrx72* had best representation in senescent leaves. (some of the underlined examples can be observed in the attached picture– Fig.2). Possible differences between leaves of three cathegories of age that have been analysed at the peroxidase protein level were also assessed by performing IEF separations (Fig.3). Some of the isoperoxidases found in leaves of one cathegory of age could not be localised in the others. The meaning of this could be that either the regulation at the translational level has a predominant role in the determination of the amount of peroxidases present in the leaves or that the peroxidases are differently inactivated in leaves of different age. However, it has been demonstrated that the amount of a particular isoperoxidase does not necessarily correspond to the level of expression of its encoding gene (Dunand et al., 2003).

Concluding remarks

The macroarray technique we used for this experiment showed itself to be suitable to analyse the regulation of a multigenic family like peroxidases, encoding for closely related proteins. This study facilitates a better understanding of the role of peroxidases in various situations and the identification of the genes which react to particular physiological events or stimuli. In this way it will be possible to study the role of the peroxidases which are encoded by these genes using the knock-out mutants which do not produce them. The peroxidase enzymes have been often used as markers related to many treatments linked to different stages of plants development (Gaspar et al., 1982). Macroarrays can also be used to identify the promoters exhibiting interesting characteristics that could be used in plant transformation in order to obtain transgenes to be expressed in speciffic conditions.

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ANALIZA EXPRIMĂRII FAMILIEI MULTIGENICE DE PEROXIDAZE DE LA *ARABIDOPSIS* UTILIZÂND MEMBRANE DE NYLON (MACROARRAY) HIBRIDIZATE CU BĂNCI DE ADNc MARCATE CU P^{32} RADIOACTIV

(Rezumat)

Peroxidazele vegetale, cunoscute şi sub denumirea de peroxidaze din clasa III, sunt proteine care conţin o fracţiune hem, codificate de un număr mare de gene asemănătoare care formează la plantele superioare o familie multigenică numeroasă, familie ale cărei gene au o exprimare sensibilă îndeosebi la factori externi şi interni. Genomul de *Arabidopsis thaliana* coţine 73 de gene care codifică peroxidaze. Manifestând omologii cuprinse între

28% şi 93% la nivelul nucleotidelor, există riscul apariţiei unei cross-hibridizări care ar putea avea importanţă în cazul măsurării nivelului de transcriere prin tehnicile de blotting (imprimare), folosindu-se secvenţe întregi de ADNc. Noi am dezvoltat o metodă pentru a evalua, cu o specificitate crescută, exprimarea tuturor genelor pentru peroxidaze pe o singură membrană. Metoda se bazează pe determinarea în cazul fiecărei gene a unei secvenţe specifice scurte (amplicon) care manifestă o omologie de cel mult 70% cu oricare altă secvenţă din genomul de *Arabidopsis*. Ampliconii specifici ai fiecăreia dintre cele 73 de gene pentru peroxidaze au fost imprimaţi (blotted) pe o membrană de nylon care a fost hibridizată cu bănci de ADNc radioactiv, preparate din ARNm extras din frunze de *Arabidopsis* tinere, adulte şi senescente. Multe din aceste gene s-au exprimat la un nivel redus, adesea în frunze din toate cele trei categorii de vârstă, dar au fost şi gene care au avut un nivel ridicat de exprimare. Au fost exprimate şi unele gene pentru care în bazele de date nu există nici un EST (sau ORF-open reading frame), fenomen care a fost confirmat prin RT-PCR. Analiza IEF (isoelectric focusing) arată că profilul izoperoxidazelor din frunze aparţinând celor trei categorii de vârstă este în general similar, dar există, de asemenea, şi unele diferenţe notabile în ce priveşte apariţia unor benzi noi de izoperoxidaze, diferenţe care ar putea fi interpretate drept identificare de noi izoforme. Din câte se ştie, până în present s-a efectuat doar un singur studiu similar la familia citocromului P450, folosindu-se tehnica microarray, dar studiul pe care îl prezentăm acum este primul care descrie profilul de exprimare pentru o întreagă familie multigenică prin utilizarea tehnicii macroarray.