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Two cell wall associated peroxidases from *Arabidopsis* influence root elongation

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Abstract Two class III peroxidases from Arabidopsis, AtPrx33 and Atprx34, have been studied in this paper. Their encoding genes are mainly expressed in roots; AtPrx33 transcripts were also found in leaves and stems. Light activates the expression of both genes in seedlings. Transformed seedlings producing AtPrx33-GFP or AtPrx34-GFP fusion proteins under the control of the CaMV 35S promoter exhibit fluorescence in the cell walls of roots, showing that the two peroxidases are localized in the apoplast, which is in line with their affinity for the Ca^{2+} -pectate structure. The role they can play in cell wall was investigated using (1) insertion mutants that have suppressed or reduced expression of AtPrx33 or AtPrx34 genes, respectively, (2) a double mutant with no AtPrx33 and a reduced level of Atprx34 transcripts, (3) a mutant overexpressing AtPrx34 under the control of the CaMV 35S promoter. The major phenotypic consequences of these genetic manipulations were observed on the variation of the length of seedling roots. Seedlings lacking AtPrx33 transcripts have shorter roots than the wild-type controls and roots are still shorter in the double mutant. Seedlings overexpressing AtPrx34 exhibit significantly longer roots. These modifications of root length are accompanied by corresponding changes of cell length. The results suggest that AtPrx33 and Atprx34, two highly homologous Arabidopsis peroxidases, are involved in the reactions that promote cell elongation and that this occurs most likely within cell walls.

Keywords Cell wall \cdot Green fluorescent protein \cdot Pectin \cdot Peroxidase \cdot Root elongation

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Abbreviations CaMV 35S: Cauliflower mosaic virus 35S $promoter \cdot CTPP: C-terminal propeptide \cdot DAPI$: 4° ,6-diamidino-2-phenylindole \cdot GFP: green fluorescent protein ROS : reactive oxygen species $RT-PCR$: reverse-transcriptase polymerase chain reaction \cdot WAK: wall associated kinase

Introduction

The plant specific heme peroxidases belong to a superfamily that contains three different classes of peroxidases (Welinder [1992](#page-9-0)): the intracellular class I (EC 1.11.1.5/.6/ .11), the class II released by fungi during plant–fungi interaction (EC 1.11.1.13/.14), and the secreted class III plant peroxidases (EC 1.11.1.7).

Class III peroxidases are supposed to be involved in a broad range of processes in plants, due to their catalytical versatility and the great number of their isoforms (Passardi et al. [2005](#page-9-0)). In Arabidopsis, they constitute a multigenic family encoding 73 isoenzymes (Tognolli et al. [2002\)](#page-9-0) implicated in diverse activities that are still poorly understood for each single peroxidase isoform. The great diversity of the promoter and intronic sequences partially explains that all kinds of internal or external stimuli regulate the gene expression. On the other hand, the large variability of the putative substrate access channel between the F and G *a*-helices justifies the diversity of activities catalyzed (Gajhede et al. [1997\)](#page-9-0). The flexibility of the regulation and the substrate specificity within this multigenic family could explain the omnipresence of the peroxidases in the plant life cycle.

AtPrx33 and AtPrx34 are two Arabidopsis peroxidases belonging to a cluster of five enzymes containing a putative \widetilde{Ca}^{2+} -pectate binding domain (Dunand et al. [2002\)](#page-9-0) similar to the binding domain of APRX, an anionic peroxidase from zucchini (Carpin et al. [1999](#page-9-0), [2001\)](#page-9-0), which binds specifically to the Ca^{2+} -pectate complex within cell walls (Carpin et al. [1999\)](#page-9-0). APRX transcripts are mainly localized in the elongation zone of

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root and hypocotyl (Dunand et al. [2002](#page-9-0)). The Ca^{2+} pectate binding property could be important in regulating the enzyme activity and localization within the cell wall.

AtPrx33 and AtPrx34 show nearly 95% homology at the protein level, but their promoter and intronic se-quences are strongly divergent (Valério et al. [2004\)](#page-9-0). They can be expected to have similar activity and cellular localization, although the control of their expression and tissular localization could be different. If their cell wall localization is confirmed in planta, what could be their function therein and does the pectin binding play a role in this function?

The plant cell wall is a very dynamic structure, which controls both cell shape and cell elongation. Various enzymatic processes cleave and reassemble the cell wall constituents during cell extension. Changes in the cell wall architecture can be achieved by class III peroxidases through their two catalytic cycles: peroxidative and hydroxylic (Passardi et al. [2004](#page-9-0)). They can stop elongation by forming bonds within the cell wall or favor it by regulating the local concentration of $H₂O₂$ or by generating reactive oxygen species (ROS), which breaks cell wall bonds (Passardi et al. [2004\)](#page-9-0). Indirectly, peroxidases can also control the cell elongation through their auxin oxidase activity. IAA can be oxidized by following two separate mechanisms: a conventional hydrogen-peroxide-dependent pathway and a second one, which is hydrogen-peroxide-independent and requires oxygen (Savitsky et al. [1999](#page-9-0)). By this way, peroxidases might regulate locally auxin concentration. In parallel, peroxidase expression levels are dependent on the endogenous auxin concentrations (Gaspar et al. [1996](#page-9-0)).

In this work, we have combined two different approaches: the RNA silencing technique and the use of T-DNA insertion mutants to get an insight into the function of AtPrx33 and Atprx34. The growth of seedlings lacking AtPrx33 or Atprx34 transcripts was studied. In addition, the localization of the enzymes fused to the green fluorescent protein was analyzed.

Materials and methods

Plant material and growth conditions

The Columbia (Col) and Wassilewskija (Ws) ecotypes of Arabidopsis thaliana were used as wild-type controls. Plants were grown in soil or on $\frac{1}{2}$ MS medium (Murashige and Skoog 1962) at 24° C under 16 h light/8 h dark and 60% humidity. For the in vitro culture, the light intensity was strictly controlled and fixed at 120μ mol photons m⁻² s⁻¹. The T-DNA lines CS10885 mutated for AtPrx33 (in Ws background) and SALK 051769 mutated for AtPrx34 (in Col background) were isolated from the Feldmann collection (Feldmann et al. [1991](#page-9-0)) and from the SALK T-DNA insertion lines (Alonso et al. [2003\)](#page-9-0), respectively. All seed lines are available from the stock center of TAIR (http:// www.arabidopsis.org).

Mutant screening

The collection of T-DNA insertion mutants from K. Feldmann (Feldmann et al. [1991\)](#page-9-0) was screened by PCR using the AtPrx33 sense primer 5'-ATGC AATTCTCTTCATCTTC-3', in combination with the T-DNA-specific primer CD5LB 5'-ATGCAATCGA TAT CAGCAGCCAATTTTA-3'.

A search in the TAIR database (http://www.arabidopsis.org) revealed a T-DNA insertion within the promoter region of AtPrx34 (SALK_051769). The precise location of the insert was determined by DNA sequence analysis of a PCR amplification product that was obtained with the $AtPrx34$ reverse primer 5'-TCCAAGTGGACGATGTTGAAG-3¢ and the T-DNA right border primer CD5RB 5'-GCTCAGGATCC-GATTGTCGTTTCCCGCCTT-3'. PCR amplification was carried out with 0.2 mM dNTPs, $0.4 \mu M$ of each primer, $10 \times Tag$ buffer, and 1 unit of Taq polymerase (Sigma) in a volume of 20 μ . The amplification program consisted of an initial denaturation at 95° C for 1 min followed by 35–40 cycles of denaturation at 94° C for 1 min, annealing at 55°C for 1 min, polymerization at 72 $\rm{°C}$ for 90 s, and a final extension at 72 $\rm{°C}$ for 10 min.

Homozygous insertion mutants in AtPrx33 and AtPrx34 were identified by PCR amplification with gene-specific primers (sense primer for AtPrx33 and reverse primer for AtPrx34 as described above and the reverse primer 5'-TGTTTGTGTGCCATC-3' and sense 5'-TTGAAAGGTCGTCGAGGAGT-3' for AtPrx33 and AtPrx34, respectively).

Imaging of GFP expression

The following constructs (Fig. [1c, d, e\) have been de](#page-2-0)[signed with the purpose of studying the localization](#page-2-0) in planta [of the AtPrx33 and AtPrx34 proteins and the role](#page-2-0) [of the promoter sequence in this localization.](#page-2-0) AtPrx33 and AtPrx34 [coding sequences lacking the stop codon](#page-2-0) [were cloned in frame in pAVA 393 \(von Arnim et al.](#page-2-0) [1998\)](#page-9-0), respectively, with $KpnI/XhoI$ and $NcoI/XhoI$. The AtPrx33-GFP and AtPrx34-GFP constructs were subcloned in pCHF3 binary vector with KpnI and BglII. 3.3 kb PCR fragment containing 1.2 kb of AtPrx33 promoter sequence and the genomic sequence of $AtPrx33$ was cloned with KpnI and XhoI in frame in pAVA 393. The AtPrx33::AtPrxP33-GFP-Term construct obtained was subcloned in pZP222 (Hajdukiewicz et al. [1994](#page-9-0)) with KpnI and BgIII. 3.3 kb PCR fragment containing 1 kb of AtPrx34 promoter sequence and the genomic sequence of AtPrx34 has been cloned with HindIII and NcoI in pAVA 393. The AtPrx34::AtPrx34- GFP-Term construct has been subcloned in pZP211 binary vector with *KpnI* and *PstI*.

Fig. 1 Schematic structures of the gene cassettes. a CaMV 35S:: 43xrPtA-linker-AtPrx34 RNAi construct used to obtain the 4.1 line. b CaMV 35S::AtPrx34 overexpression construct used to obtain the 30.2 line. c CaMV 35S::GFP contruct for the localization of GFP alone. d CaMV 35S::AtPrx33/34-GFP contructs for cellular localization of the peroxidases. (e) AtPrx33/34 endogenous promoter::AtPrx33/34-GFP contructs for subcellular localization of the peroxidases

A third construct used as control has been obtained by cloning 35S::GFP-Term in pCGN1547 (McBride and Summerfelt [1990](#page-9-0)).

The sequences of the individual primers are as follows (engineered restriction sites are underlined): AtPrx33, 5¢-GGGGTACCGCTTGGTTTGGTTTCCATTG-3¢, 5¢- GGGGTACCATGCAATTCTCTTCATCTTC-3' and 5¢- CCGTTCTCGAGACATAGAACTTACAAAGTC -3' and AtPrx34, 5'-CCCAAGCTTTGGATTTCTTC-3¢, 5¢-CCGCTCGAGATGCATTTCTCTTCGTCTT-3¢ and 5'-CATGCCATGGGCATAGAGCTAACAAAG-TC-3'. For the GFP analysis, seedlings were counterstained with 10 μ g/ml propidium iodide and placed on slides in a drop of water. The GFP fluorescence was imaged with an Axioplan2 Zeiss microscope with narrow band excitation $(470 \pm 20 \text{ nm})$ and emission $(510 \pm 20 \text{ nm})$ filters (Chroma Technology Corp, VT, USA) and using the Metamorph software (Molecular Devices Corp, CA, USA).

Overexpression of Atprx34 and RNAi constructs

For the overexpression experiment, $AtPrx34$ cDNA sequence under the control of CaMV 35S (Fig. 1b) was cloned in pCHF3 vector using KpnI and BamHI. From the transgenic plants that have been obtained, only the line ''30.2'' has been used for an extensive phenotype study.

The RNAi construct with the whole AtPrx34 cDNA (Fig. 1a) has been obtained in three successive steps in pBluescript KS+. The cDNA has been cloned in the antisense orientation with EcoRI and BamHI. A linker of 700 bp has been cloned into the previous plasmid with HindIII and XhoI. The final 43xrPtA-linker-AtPrx34 construct has been obtained by a three-way ligation: the $43xrPtA$ -linker opened with XbaI and XhoI and the *AtPrx34* opened with *XhoI* and *PstI* was subcloned into pCHF5 with XbaI and PstI. The capacity of

the construct to form a hairpin loop has been controlled before plant transformation using an exonuclease after denaturation and quick renaturation. The construct was used to transform the *atprx33* insertion knock-out mutant, thus resulting in a double-transgenic line called $"4.1"$.

Complementation assays have been performed by transforming atprx33 T-DNA mutant with the AtPrx33::AtPrxP33-GFP-Term construct. Root and root cell lengths were determined in homozygous complemented lines. RT PCR using specific primers spanning the junction site Atprx33-GFP verified the level of AtPrx33 transcript.

Plant transformation

All plasmids were introduced into Arabidopsis Col ecotype (except 4.1 double mutant in Ws) by Agrobacterium-tumefaciens-mediated transformation using the spraying technique (Bent [2000](#page-9-0)). Agrobacterium tumefaciens strain ASE was used in all cases. The transformants were selected on $\frac{1}{2}$ MS medium containing 50 μ g/ml kanamycin or 40 μ g/ml Basta, depending on the binary vector used. The presence of the transgenes in all transgenic plants was confirmed by genomic PCR using primers specific in each case.

Root and cell length measurements

The length of the roots and cells was measured on oneweek old seedlings grown in square boxes placed in vertical position. Root length was measured on 40–50 seedlings from several independent batches. Cell length was determined by measuring 200 cells in the root hair zone from six independent seedlings. For this purpose, roots were stained with 10^{-4} M DAPI and observed at a $10\times$ magnification by fluorescence with an Axioplan2 Zeiss microscope (excitation: 359 ± 50 nm, emission: 460 ± 50 nm), using the Metamorph software for measurement. Differences between wild-type and transgenic seedlings were evaluated for every measurement assay using a 1-way between subjects ANOVA test (Analyse-it Software, v.1.71).

Reverse transcriptase PCR

Reverse transcriptase-PCR was used as a semiquantitiative method to assess the expression of peroxidase genes. Leaves, stems, flowers, and roots from 5-week old plants were harvested and frozen immediately in liquid nitrogen. Approximately, 100 mg of tissue sample was ground in liquid nitrogen, and total RNA was extracted with the Tri-reagent solution (Sigma) according to the instructions of the manufacturer. After quantification of the concentration by spectrophotometry and confirmation by electrophoresis, 1μ g of the crude RNA preparations was treated with one unit of RNase-free DNase I (Promega). The DNA-free RNA was then used as a template during reverse transcription according to the ImPromII RT protocol from Promega. PCR amplification was conducted for up to 40 cycles using the following thermal profile: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 30 s, with a 10 min terminal extension step at 72° C. To determine whether comparable amounts of RNA from the different tissues had been used for RT-PCR, the level of AtPrx42 transcript was used as a constitutive control. Indeed, AtPrx42 is strongly expressed in all Arabidopsis organs (Tognolli et al. [2002;](#page-9-0) Valério et al. [2004](#page-9-0)), it is the most abundant peroxidase in Arabidopsis (505 ESTs were found from a total of 353 000 ESTs indexed in TIGR) and its expression was found strong and stable independently of the age of the plants, various treatments and growth conditions in reverse transcription assays (data not shown). Reactions without RT were used to rule out contamination by genomic DNA. Primers used to determine the homozygosity have also been used for RT-PCR. The following primers were used for $AtPrx42$ cDNA: 5'-GGTCCATCGTTTG-TACCCT-3' and 5'-CCCCTGTCTTTCTCACTTTT-3'.

Quantification of the RT-PCR bands on agarose was performed with the software QuantityOne (BioRad). The results shown in Fig. 4 [represent the sum of four](#page-5-0) [bands resulting from four independent experiments for](#page-5-0) [each condition \(light/dark\).](#page-5-0)

Expression in baculovirus-insect cell system and binding activity

The cDNA sequences encoding AtPrx33, AtPrx34 and AtPrx37 without the signal peptide have been cloned, respectively, with BamHI/PstI, BamHI/EcoRI, and BamHI/XbaI into pVL1392 vector (Pharmingen, San Diego, CA, USA) and expressed in baculovirus-insect cells (Carpin et al. [2001\)](#page-9-0). After purification, the resulting recombinant proteins were tested for their binding capacities. The binding of peroxidases to the $Ca²⁺$ -pectate gel was assessed by centrifugation as already described (Penel and Greppin [1996](#page-9-0)). APRX, an anionic peroxidase from zucchini (Carpin et al. [2001\)](#page-9-0) and AtPrx32 from Arabidopsis (Dunand et al. [2002\)](#page-9-0) have been used as positive controls. A horseradish peroxidase mixture containing mainly the HRPC isoform (Fluka, Buchs, Switzerland) was used as a negative control for the binding activity. The assays were performed in a total volume of 100 µl containing the same level of guaicol peroxidase activity $(0.025 \text{ OD}_{470nm}/\text{min}).$

Sequence analysis

The 1,000-bp region upstream each peroxidase gene was analyzed using PLACE and PlantCARE softwares (http://www.dna.affrc.go.jp/htdocs/PLACE and oberon.rug.ac.be:8080/PlantCARE).

Results

Identification of the atprx33 and atprx34 T-DNA tagged mutants

To obtain plant lines with a disruption in the AtPrx33 and AtPrx34 genes, we screened the collection of T-DNA insertion mutants of Feldmann (Feldmann et al. [1991\)](#page-9-0) and the TAIR knockout facility (http:// www.arabidopsis.org). We used a PCR strategy with primers specific for T-DNA borders and gene-specific sequence in order to identify T-DNA mutants for $AtPrx33$ or $AtPrx34$ genes. A homozygous line with insertions in intron 1 was obtained in the case of atprx33, leading to gene disruption 660 bp downstream of the ATG initiation codons (Fig. [2\). A homozygous](#page-4-0) [line with insertion 200 bp upstream of the ATG initia](#page-4-0)[tion codons was obtained for](#page-4-0) atprx34 (Fig. 2). The po[sition of the insert was verified using T-DNA specific](#page-4-0) [primers, the wild-types \(Col and Ws\) being used as](#page-4-0) [negative control. The homozygosity was confirmed by](#page-4-0) [PCR with gene-specific primers flanking the T-DNA](#page-4-0) [insert. No amplification was observed for the T-DNA](#page-4-0) mutants (Fig. [3a\). The presence of a single insert and the](#page-4-0) [homozygosity were confirmed with the ratio of resistant](#page-4-0) [plants for both lines \(data not shown\).](#page-4-0)

Reverse transcription-PCR experiments on RNA from atprx33 seedlings indicated the absence of transcripts from this gene in the mutant background, whereas amplification products were readily detectable in the wild-type seedlings (Fig. 3b). Atprx34 [transcripts](#page-4-0) [could be slightly detected in the](#page-4-0) atprx34 mutant, but [their level was decreased when compared to the wild](#page-4-0)[type level in 2-week-old seedlings \(Fig.](#page-4-0) 3c). This means [that the transcription of](#page-4-0) $AtPrx34$ was not completely

Fig. 2 AtPrx33 (M58380) and AtPrx34 (X71794) gene structures and insertion sites of the T-DNA. Grey boxes represent exons and empty boxes introns (numbered from I to IV); bold lines are the predicted and observed 5'and 3'UTR. RB right border, LB left border

abolished by the presence of the insert in the promoter region.

Expression profile of AtPrx33 and AtPrx34 peroxidase genes

High level of AtPrx33 transcripts was detectable in the roots of 5-week-old plants and at a lower extent in leaves

Fig. 3 Molecular

characterization of the atprx33 and atprx34 T-DNA tagged mutants. a Control of the position and homozygosity of the insert. Genomic DNA from atprx33 and atprx34 mutants and from the corresponding wild-types (Ws and Col, respectively) was used for PCR amplification with primers specific for the T-DNA insert or for the peroxidase genes. b AtPrx33 transcript levels in 2-week-old atprx33 and wild-type (WT) seedlings. RT-PCR with AtPrx33 gene-specific primers (lanes 1, 3 and 5); with $AtPrx42$ primers (lanes 2, 4 and 6). c AtPrx34 transcript levels in 2-week old atprx34 and wild-type seedlings. RT-PCR with AtPrx34 gene-specific primers (lanes 1, 3 and 5); with $AtPrx42$ primers (lanes 2, 4, and 6). $RT-PCR$ products shown for AtPrx33 and AtPrx34 were amplified for 40 cycles from 1μ g of total RNA. In b and c, the AtPrx42 transcript level was used as a constitutive control

and stems. Some transcripts were also found in flowers. In contrast, low levels of $AtPrx34$ transcripts were detected only in roots (Fig. 4a). $AtPrx33$ [transcripts in](#page-5-0)[creased with the age of seedlings, both in light and in the](#page-5-0) dark (Fig. 4b). $AtPrx34$ [transcripts, present at a much](#page-5-0) [lower level seemed to increase as well. The expression of](#page-5-0) AtPrx33 [was largely lower in the dark than under light](#page-5-0) (Fig. [4b\). In all cases, independently of the organs, the](#page-5-0) [light quantity and the age of the plants,](#page-5-0) $AtPrx33$ tran[scripts were more abundant than](#page-5-0) $AtPrx34$ ones. The [equivalent efficiency of](#page-5-0) $AtPrx33$ and $AtPrx34$ primer [annealing was verified with genomic templates \(data not](#page-5-0) [shown\).](#page-5-0)

Subcellular localization of the AtPrx33 and AtPrx34 in planta

Plants were transformed with Atprx33-GFP and Atprx34-GFP constructs under the control of the CaMV 35S promoter. Endogenous promoters were also used, but in that case the expression was too low and the fluorescence could not be observed. Under the control of CaMV 35S, AtPrx33-GFP, and AtPrx34-GFP fusion proteins accumulated in cell walls (Fig. [5c-g\)](#page-6-0). GFP [protein alone had a nuclear and cytoplasmic localization](#page-6-0)

Fig. 4 Expression level of $AtPrx42$ (1), $AtPrx33$ (2), and $AtPrx34$ (3) genes assessed by RT-PCR. a Flowers (Fl), leaves (L), stems (St), and roots (R) from 5-week-old plants; 7- day-old whole seedlings from Columbia wild-type (Col), and *atprx34* mutant. **b** Columbia seedlings grown for 3, 7, and 14 days under a 16 h photoperiod. c Columbia seedlings grown for 3, 7, and 14 days in

darkness. In b and c, a representative gel and a graph corresponding to the mean of four independent experiments are shown. Total RNA $(1 \mu g)$ was used for RT-PCR with primers specific for $AtPrx42$ (1), $AtPrx33$ (2), and $AtPrx34$ (3) genes. The RT-PCR products shown were amplified for 40 cycles. The AtPrx42 transcript level was used as a constitutive control

[\(von Arnim et al.](#page-9-0) 1998) (Fig. [5a, b\). In vitro, re](#page-6-0)[combinant AtPrx33, AtPrx34, and AtPrx37, produced](#page-6-0) [by the baculovirus insect cell system, showed some](#page-6-0) [capacity](#page-6-0) [to](#page-6-0) [bind](#page-6-0) to [the](#page-6-0) Ca^{2+} Ca^{2+} -pectate complex, which is [mainly found in the middle lamella. Their binding](#page-6-0) [activities were much lower than the binding capacity of](#page-6-0) [AtPrx32 and zucchini APRX, but significant when](#page-6-0) [compared to the lack of binding of horseradish peroxi](#page-6-0)dase (Fig. [6\). AtPrx32 and HRPC both show high](#page-6-0) [homology at the protein level with AtPrx33/34 \(over](#page-6-0) [87%\), but only the former bears the putative pectate](#page-6-0) [binding domain.](#page-6-0)

Root length and peroxidase gene expression

The length of roots was measured on various transgenic seedlings, including atprx33, atprx34, the AtPrx34 overexpressor 30.2, and the 4.1 RNAi double mutants. Since the mutants were obtained in two different backgrounds, Columbia and Wassilewskija Arabidopsis were also analyzed. The 4.1 double mutants do not contain AtPrx33 transcripts and exhibit a reduction of AtPrx34 mRNA (Fig. 7a). Seedlings containing a T-DNA inserted in the AtPrx34 promoter sequence (atprx34) showed a reduced expression of $AtPrx34$ and the seedlings containing the 35S::AtPrx34 construct (30.2 line) an accumulation of AtPrx34. atprx34

seedlings had no significant root length variation when compared to the wild-type plants, whereas the 30.2 mutant had clearly longer roots (Fig. 7b). In knockedout atprx33 plants and in the double mutant (4.1 line), a significant reduction of the root length could be observed (Fig. [7b\). The root length reduction is additive](#page-7-0) [when both genes are affected. The fully elongated cells](#page-7-0) [present in the root hair zone were also measured. It](#page-7-0) [appeared that the length of these cells was also signif](#page-7-0)icantly reduced in atprx33 [and 4.1 mutants and in](#page-7-0)[creased in 30.2 mutant.](#page-7-0)

In order to determine the heritability of the mutation in mutant lines, homozygous transgenic plants were crossed with corresponding wild-type ecotypes. The F1 (for overexpressor lines) and the F2 progenies (for the atprx33 mutant) showed the appropriate root phenotype (data not shown). The co-segregation of the resistance and of the phenotype confirmed that the mutations related to AtPrx33 and AtPrx34 are directly responsible for the modification of the root length.

Finally, complementation assays were performed by introducing the AtPrx33::AtPrxP33-GFP-Term construct in knocked-out *atprx33* plants. A significant increase of root and root cell lengths was observed and compared to *atprx33* plants in a complemented line. There was no significant difference between this complemented line and the Ws wild-type control (Fig. [8\).](#page-7-0)

Fig. 5 Detection of GFP fluorescence in roots of seedlings transformed with a CaMV 35S::GFP construct (a, b), with a CaMV 35S:: $AtPrx33-GFP$ construct (c–e) and with a CaMV

Discussion

Analysis of the nucleotide sequences

The structure of AtPrx33 and AtPrx34 corresponds to a classical peroxidase gene with three introns and four exons (Tognolli et al. [2002\)](#page-9-0). The first 100 bp upstream

Fig. 6 Binding of recombinant peroxidases to Ca^{2+} -pectate. The assays were performed in the presence of 10μ g of polygalacturonic acid and 2 mM CaCl₂ in a total volume of 100 µl with the same level of peroxidase activity (Penel and Greppin [1996\)](#page-9-0). After centrifugation, the peroxidase activity was measured in the Ca^{2+} -pectate pellets (() and in the corresponding supernatant ((). The data are the means of three independent experiments ± SD

35S:: $AtPrx34-GFP$ construct (f, g). Staining with 10 µg/ml propidium iodide (f). Cell wall (unstained zone), nucleus, and cytosol (stained zones) are indicated by arrows in B

the ATG codon of the two genes are highly conserved. This short sequence contains the TATA box and is sufficient for a basal expression, but probably does not allow a fine and specific regulation of expression as shown by T-DNA insertion in the AtPrx34 promoter. Indeed, the insertion 200 bp upstream of the ATG should remove light response and root localization. Known cis-elements found in the promoter of AtPrx33

Fig. 7 Expression of AtPrx33, AtPrx34 and AtPrx42 genes and the measurement of root length and cell root length in various transgenic Arabidopsis seedlings. a Expression of the three peroxidase genes in aprx34 and overexpressor 30.2 mutants in a Columbia (Col) background and in atprx33 and RNAi 4.1 mutants in a Wassilewskija (Ws) background, assessed by RT-PCR. The AtPrx42 transcript level was used as a constitutive control in the various seedlings. b Root lengths and cell root lengths of 7-day-old seedlings from the plant lines described in a. The differences between wildtype and corresponding mutant seedlings were analyzed by 1-way ANOVA test. Same letters indicate significant differences with \overline{P} values: $P < 0.0001$ (a,b,c,g); $P = 0.0046$ (d); $P=0.001$ (e); $P=0.0297$ (f); $P = 0.0121$ (h)

and Atprx34 genes have been listed in Table. [1. There is](#page-8-0) [a similar distribution of cis-elements related to the](#page-8-0) [control by light and the expression in root. This](#page-8-0) in silico [analysis is in agreement with the actual expression pat](#page-8-0)[tern, which showed a preferential transcription in the](#page-8-0)

Fig. 8 Complementation assays. Root and cell root lengths were determined for 7-day-old seedlings from Wassilewskija (Ws), atprx33 and atprx33 complemented line (comp). The differences between the seedlings were analyzed by a 1-way ANOVA test. Same letters indicate significant differences with P values: $P < 0.0001$ (a, c, d); $P = 0.0007$ (b)

[roots and an up-regulation by light \(Fig.](#page-5-0) 4). In contrast, [large differences exist for the abscissic acid response](#page-5-0) [elements \(seven elements in](#page-5-0) AtPrx33 and none in $AtPrx34$ [\) and for the salicylic acid response elements \(six](#page-5-0) elements in AtPrx34 and none in AtPrx33[\), which is in](#page-5-0) [line with the idea of a similar activity but a different](#page-5-0) [regulation for the two peroxidases.](#page-5-0)

AtPrx33 and AtPrx34 are cell wall associated and involved in cell elongation

AtPrx33 and AtPrx34 are members of a same gene cluster and show a high level of homology with 90% of identity. Both peroxidases are localized in cell walls (Fig. [5\). They have an amino acid motif including three](#page-6-0) [lysine residues, which is similar to the well-characterized](#page-6-0) Ca^{2+} -pectate binding site of the zucchini peroxidase [APRX \(Carpin et al.](#page-9-0) 1999, [2001;](#page-9-0) Dunand et al. [2002\)](#page-9-0). Purified AtPrx34 has also been shown to bind to Ca^{2+} pectate (Shah et al. [2004\)](#page-9-0) and recombinant AtPrx33 and AtPrx34 produced by insect cells exhibited some affinity for Ca^{2+} -pectate (Fig. [6\). This means that, their locali](#page-6-0)[zation](#page-6-0) [within](#page-6-0) [the](#page-6-0) [cell](#page-6-0) [walls](#page-6-0) [could](#page-6-0) [be](#page-6-0) [modulated](#page-6-0) [by](#page-6-0) Ca^{2+} Ca^{2+} , [as already demonstrated for APRX \(Carpin,](#page-9-0) 2001).

AtPrx33 and AtPrx34 sequences possess a signal peptide and a C-terminal extension. The signal peptide targets the protein to endoplasmic reticulum and the secretory pathway, whereas the C-term extension (CTPP) may address the protein to the vacuole

Table 1 Main cis-elements present in the 1,000 bp upstream the $AtPrx33$ and $AtPrx34$ genes

Motif	Sequence	Function	Position for <i>AtPrx33</i>	Position for AtPrx34
ABRE	YACGTGGC	Abscissic acid responsiveness	$46(-), 47, 144(-),$ $\overline{222(-)}$, 539(-), 540, 898(-)	
ARF/AuxREs	TGTCTC	Auxin response element	$618(-)$	$247(-)$
AuxRR	GGTCCAT	Auxin response	$938(-)$	
ERE	ATTTCaaa	Ethylene-responsive element	647	5, 295, 337
$G-box$	CACGTG	Element involved in light responsiveness	45, 538	
GA-motif	ATAGATAA	Part of a light responsive element	$273(-), 477(-)$	725
GATA-box	GATAGa	Part of a light responsive element	$65(-), 652$	244 (-), 636, 790
LAMP-element	CTTTATCA	Part of a light responsive element		303
LTR	CCGAaa	Low-temperature responsiveness	$616, 76(-)$	585
MBS	CAACTG	MYB binding site involved in drought-inducibility	$247(-)$	
$P-box$	CCTTTTG	Gibberellin responsive element		$833(-)$
Root motif	ATATT	Root localization	$289(-), 407(-), 410, 645,$ 791(-), 792, 852	$6(-), 406(-), 407, 518(-), 522,$ $561, 616, 644(-)$
TGA-element	AACGAC	Auxin response element	808	
W-box	TTGACC	Salicylic acid responsiveness	903	378(-), 579, 672(-), 813, 903, 979

The data have been obtained with PlantCare and PLACE. Indicated positions are relative to the ATG codon. Underlined positions correspond to the underlined part of the cis-element

(Neuhaus [1996](#page-9-0)). However, a recent study cast some doubt on the validity of this hypothesis in the case of a horseradish peroxidase (Kis et al. [2004](#page-9-0)), and so far there has been no evidence suggesting that the CTPP is cleaved from HRPC1a for the cell wall targeting (Matsui et al. [2003\)](#page-9-0). Similarly to APRX, which also has a C-term extension but was found in apoplast (Carpin et al. [1999\)](#page-9-0), AtPrx33 and AtPrx34 are localized in the cell wall (Fig. [5\). Even if both proteins transit via the vacuole](#page-6-0) [\(Carter et al.](#page-9-0) 2004), they finally end in the apoplast. The same transitory targeting has been observed for a cellwall-associated kinase 1 (WAK1) found in the vacuole (Carter et al. [2004\)](#page-9-0) but known to bind the Ca^{2+} -pectate complex (Decreux and Messiaen [2005](#page-9-0)). All WAK family members (WAK1–5) exhibit a similar protein length without apparent C-term extension. Thus, vacuole targeting could be transient before a final localization in the cell wall regulated by the presence of the CTPP but not related to the cleavage of this extension. Regarding At-Prx33/34 GFP fusion protein, substantial evidence shows that the fusion proteins are correctly folded and active: GFP fluorescence was detected in the plants containing AtPrx33/34-GFP, and the AtPrx33-GFP construct can complement the atprx33 mutant phenotype.

The peroxidase cellular localization could be associated with a particular activity in relation to cell wall elongation. AtPrx33 and AtPrx34 are highly homologous and most likely have similar enzymatic properties in the cell wall. On the other hand, we have observed important difference concerning the transcription profile. These variations might explain the presence of two very close isoforms, performing similar tasks, but differentially transcribed in response to various stimuli.

Knocking out AtPrx33 gene expression resulted in the reduction of root elongation. However, this did not correspond with a significant modification of the total peroxidase activity (data not shown). The high number of peroxidase isoforms present in Arabidopsis can easily explain this. Even if the overall peroxidase activity was not affected in *atprx*33 or 4.1 mutants, the AtPrx33 protein was most likely absent since there were no detectable transcripts. This absence resulted in a reduction of growth, thus suggesting that this peroxidase is involved in growth promoting reactions. This was confirmed by the additional growth reduction observed following *AtPrx34* downregulation in 4.1 double mutant, the restoration of normal root growth in atprx33 mutant complemented with Atprx33::Atprx33-GFP construct and the stimulation of root growth brought about in the 30.2 mutant overexpressing AtPrx34. Another peroxidase, horseradish prxC1a peroxidase, was already reported to stimulate growth when expressed in hybrid aspen (Kawaoka et al. [2003](#page-9-0)). In the present work, there was a correlation between the root length and the length of root cells (Fig. [7\), suggesting that the](#page-7-0) [observed modifications of root growth are due to the](#page-7-0) [changes in cell elongation. This means that AtPrx33 and](#page-7-0) [AtPrx34 would be involved in reactions promoting cell](#page-7-0) [elongation, for example free radical production that may](#page-7-0) [loosen cell wall, as proposed by Schopfer \(2001\)](#page-9-0). The level of AtPrx33 and AtPrx34 transcripts was light- and age-dependent (Fig. [4\), which can be related to the](#page-5-0) [stimulating effect of light and of the stage of develop](#page-5-0)[ment on root growth. The light regulation of both genes](#page-5-0) [is also in line with the presence of numerous light related](#page-5-0) elements in their promoter sequences (Table 1), and with observations reporting a phytochrome-dependent regulation of peroxidases in other plant species (Kim et al. [1989;](#page-9-0) Casal et al. [1994\)](#page-9-0).

The assessment of the transcript levels by RT-PCR indicated that $AtPrx33$ gene was expressed at a much higher rate than $AtPrx34$. This is not in agreement with a previous northern blot analysis (Shah et al. [2004](#page-9-0)). This latter discrepancy could be explained by the specificity of the primers used for the PCR amplification versus the lower specificity of the cDNA probes used previously for the northern blot analysis. However, both techniques showed that $AtPrx33$ and $Atprx34$ mRNAs accumulated mainly in roots. Concerning the EST counts, it is probable that the developmental stage at which we studied our plants was not one in which AtPrx34 expression was the highest. Further studies are needed to clarify this point.

The results obtained in this work as well as the presence of auxin related cis-elements in the promoter region of AtPrx33 and Atprx34 genes (Table [1\) argue in](#page-8-0) [favor of an active role of the two encoded peroxidases in](#page-8-0) [root cell elongation.](#page-8-0)

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