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ORIGINAL ARTICLE

An anionic class III peroxidase from zucchini may regulate hypocotyl elongation through its auxin oxidase activity

Claudia Cosio · Loic Vuillemin · Mireille De Meyer · Claire Kevers · Claude Penel · Christophe Dunand

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Abstract The high number of peroxidase genes explains the description of numerous physiological functions and the fact that the in planta function of a single isoform has never been characterized yet. We analyzed in transgenic *Arabidopsis thaliana* the localization of a zucchini isoperoxidase (APRX), previously purified thanks to its pectin binding ability. We confirmed that the protein is localized near the cell wall, mainly produced in the elongation area of the hypocotyls and respond to exogenous auxin. In addition, the ectopic overexpression of APRX induced changes in growth pattern and a significant reduction of endogenous indole-3-acetic acid (IAA) level. In agreement with these observations APRX showed an elevated in vitro auxin oxidase activity. We propose that APRX participates in the negative feedback regulation of auxin level and consequently terminates the hypocotyl elongation process.

Keywords Auxin oxidase activity · Cell elongation · Cell wall · Pectin · Peroxidase

Abbreviation

APRX	Anionic peroxidase
GFP	Green fluorescent protein
ROS	Reactive oxygen species

Introduction

In higher plants, class III peroxidases (E.C. 1.11.1.7) exist as a large number of different isoforms (isoperoxidases). For example, the *Arabidopsis thaliana* genome contains 73 different genes encoding class III peroxidases (Tognolli et al. 2002). This high number of isoforms probably explains the description of a plethora of physiological functions (Passardi et al. 2005) such as, the formation of lignin in the secondary cell walls (Gabaldon et al. 2006; Sasaki et al. 2006), isodityrosine cross-linking between extensin molecules during normal growth (Iiyama et al. 1994), and auxin catabolism in vitro and in vivo (Lagrimini 1996). The diversity of processes catalyzed by peroxidases as well as the great number of their isoforms suggests the possibility for a functional specialization of each isoform. In addition, the fact that all plant peroxidase sequences contain both

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (http://www.plantcell.org) is: C. Dunand (dunand@scsv.ups-tlse.fr).

The genomic and promoter nucleotide sequence of *APRX* has been submitted to GenBank under the accession number DQ518906.

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C. Cosio · L. Vuillemin · M. De Meyer · C. Penel · C. Dunand
Laboratoire of Plant Biochemistry and Physiology,
University of Geneva, Quai Ernest-Ansermet 30,
1211 Geneva 4, Switzerland

C. Kevers
Unité de Biologie Moléculaire et Biotechnologie Végétales,
Université de Liège, Sart Tilman B22, 4000 Liège, Belgium

Present Address:
C. Cosio
Institut Forel, Université de Genève,
10 rte de Suisse, 1290 Versoix, Switzerland

Present Address:
C. Dunand (✉)
Plant Cell Surfaces and Signaling Laboratory,
University of Toulouse, UPS, CNRS, 24 Chemin de Borderouge,
31326 Castanet-Tolosan, France
e-mail: dunand@scsv.ups-tlse.fr

highly conserved domains (e.g. the catalytic site) and variable parts supports this hypothesis.

In an attempt to identify the function of specific isoforms, several authors reported the generation of transgenic plants with different features (Heggie et al. 2005; Bindschedler et al. 2006). Nevertheless, although in some cases spatial and temporal localization or indirect evidence might indicate their involvement in specific mechanisms (Ostergaard et al. 2000; Jansen et al. 2001), the in planta function of a single isoform has never been clearly characterized. For example, the identification of a tobacco peroxidase whose antisense suppression led to a significantly lower lignin content and vascular tissue modification was reported (Blee et al. 2003). Nevertheless, as the antisense strategy is not totally specific and might also affect the expression of other genes, it would be desirable to complement this study with overexpressing plants and in vitro studies in order to unambiguously asses its function. Another report showed in vitro that a tobacco anionic peroxidase, that was supposed to be involved in lignification (Lagrimini et al. 1997a) also had an auxin oxidase activity (Lagrimini 1996). Surprisingly, further studies in planta failed to show convincing direct evidences of such activities. Indeed, the antisense RNA mutant showed no significant effect on lignification (Lagrimini et al. 1997a) and auxin levels were not affected in the transgenic plants (Lagrimini et al. 1997b). This tobacco peroxidase also had an unexplained role as the transgenic plants had a broad range resistance mechanism to insects (Dowd and Lagrimini 2006). Since it is doubtful that a single peroxidase protein can have several different functions *in planta*, these discrepancies might be explained by their ability to react with numerous plant compounds in vitro, although it is uncertain which of these compounds are the actual in vivo substrates. However, it has to be admitted that in spite of the considerable interest in peroxidases, after over 30 years of investigation little is known of their roles as individual proteins. Moreover, class III peroxidases are generally still monitored through their total activity, encompassing the numerous different isoforms as a single parameter (e.g. Chaoui et al. 2004). It is therefore imperative to study the precise in planta role of each individual isoform for a better understanding of their functions, regulation and also the evolution of this key multifunctional enzyme family.

Many of the functions attributed to plant peroxidases occur in the cell wall. This suggests that different peroxidases may exhibit different interactions with the various constituents of the cell wall. Peroxidase could form phenolic linkages in the wall (Pedreno et al. 1995) and as a consequence restrict the cell growth. Peroxidases can also have an indole-3-acetic acid (IAA) oxidase activity (Gazaryan et al. 1999), and hence may control endogenous rates of auxin. Since growth is auxin dependant in the elongation zone, peroxidases could then regulate the elongation process through the catabolism of auxin in the cell wall (Kawano 2003). Nevertheless, it is generally accepted that the oxidation of IAA by peroxidases also catalyses the generation of reactive oxygen species (ROS). OH· production by this mechanism might be responsible for cell wall cleavage and consequently mediate cell growth (Schopfer et al. 2002). At the same time, the combination of IAA and peroxidase produce hydrogen peroxide (H_2O_2, Kim et al. 2006). H_2O_2 is suspected to be a signal molecule regulating transduction pathway during programmed cell death, plant defense (Neill et al. 2002).

In previous studies the identification and isolation of an anionic peroxidase (initially named APRX and also named CpPrx01 based on the nomenclature of the Peroxibase, Bakalovic et al. 2006) from the apoplast of zucchini was reported. *APRX* mRNA accumulated strongly in hypocotyls (Carpin et al. 1999) particularly in the elongation area (Dunand et al. 2002). APRX was shown to specifically bind a complex of calcium-pectate through a motif of four clustered arginines (Carpin et al. 2001) The localization of APRX related to its binding ability could for example indicate the involvement of APRX in the control of the cell elongation in the hypocotyl. More investigations were nevertheless needed to determine APRX function and regulation *in planta*.

The objective of the present study was to asses if APRX is involved in cell elongation and if so by which mechanism. We carried out a functional characterization of APRX—studying its localization and regulation. We also present evidence implicating APRX in the regulation of auxin levels *in planta*—supported by the fact that APRX has an in vitro auxin oxidase activity.

Materials and methods

Plant material and growth conditions

Dry seeds of zucchini (*Cucurbita pepo* L. cv black Beauty, Botanical garden, Geneva, Switzerland) were imbibed in water for 1 h and germinated in a growth room on wet absorbent paper at 24°C under 12 h light/12 h dark.

Arabidopsis thaliana L. ecotype Columbia was used as wild-type [WT, the European *Arabidopsis* Stock Centre (NASC), University of Nottingham, UK]. Seeds were surface sterilized and put to grow first on 1/2 MS medium (Murashige and Skoog 1962) at 24°C under 16 h light/8 h dark or in complete darkness. Seven-day-old seedlings were then when necessary transplanted to soil.

Extraction of RNA, Northern and reverse transcriptase-PCR analysis

Five and 7-day-old seedlings (zucchini and *A. thaliana*) were frozen and ground in liquid nitrogen. Total RNA was extracted using Tri-reagent solution (Sigma, Buchs, Switzerland) according to the instructions of the manufacturer. The RNA concentration was assessed by spectrophotometry. For Northern-blot analysis, RNA (10 µg) was electrophoresed through 1% formaldehyde-containing agarose gels and transferred onto Hybond-N membranes (Amersham, Little Chalfont, UK) with 10× SSC, as described by the manufacturer. RNA deposition and transfer were estimated by ethidium bromide staining and by coloration with a solution containing 0.5 M sodium acetate and 0.04% methylen blue. Hybridization was performed at 50°C in DIG easy hyb buffer (Boehringer Mannheim, Mannheim, Germany) using probe obtained by PCR labeled with DNA labeling kits (DIG, Boehringer Mannheim). For RT-PCR analysis, 1 µg of the crude RNA preparation was treated with 1 U of RNase-free DNase I (Promega, Madison, WI, USA). The resulting RNA was used as a template during reverse transcription as described in the ImPromII RT protocol from Promega. Furthermore, PCR amplification was carried out for up to 40 cycles under the following parameters: denaturation at 95°C (1 min), annealing at 55°C (1 min), polymerization at 72°C (30 s). The final extension was set at 72°C (10 min). The *AtPrx42* transcript was used as loading control as previously reported (Passardi et al. 2006). The primers used to assess the homozygosity have also been used for RT-PCR. The following primers were used for *AtPrx42* cDNA: 5′-GGTCCATCGTTTGTACCCT-3′ and 5′-CCCCTGTCTTTCTCACTTTT-3′ and for *APRX* 5′-ATGCCCAACTCACCGAG-3′ and 5′-CCAGCTCCTGCCTGTAC-3′.

Hormone treatments

Batches of 20 zucchini seedlings were grown 5 days in presence of various concentrations of 2,4-D ranging 0.226–226 µM. After the treatment, hypocotyls for RNA analysis and protein extraction were frozen in liquid nitrogen and processed as described elsewhere in this manuscript.

Seedlings of *A. thaliana* WT or APRX:*GUS* were grown vertically in the dark for 72 h on 1/2 MS medium (Murashige and Skoog 1962) supplemented with 0.09 or 0.9 µM of 2,4-D.

Promoter and intron sequences

The promoter sequence of APRX gene (DQ518906) was obtained by inverse PCR (IPCR). This method described by Ochman et al. (1988) is used for the rapid in vitro amplification of DNA sequences that flank a region of known sequence. The method is based on PCR, but it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon itself to form a circle. Briefly, 1 µg of highly purified zucchini genomic DNA was digested overnight with *Mbo*I. The DNA was precipitated with ethanol, collected by centrifugation and resuspended in 5 µl water. The restriction fragments were diluted to 500 µl with water, 50 µl ligase buffer and 10 µl T4 DNA ligase and incubated at room temperature for 4 h. The DNA was then precipitated with ethanol, collected by centrifugation and resuspended in 8 µl water. Circularized DNA was digested by *Xho*I (10 µl final volume) 2 h. A total of 0.5 µl of the resulting mix was further used in the PCR reaction (annealing temperature: 57°C) with 3'4 5′-GGGAGCATCCTCTAGCAAAA-3′ and 5'4 5′-AATTCCCACTCCAAGCTTCA-3′ primers. The PCR products were further ligated into pGEM-T easy plasmid. The resulting fragments were amplified with T7 and SP6 primers. Positive clones were sequenced. The intronic sequence has been obtained directly from the genomic DNA using 5′ and 3′ primers specific for APRX. The *cis*-element contained in the promoter and introns sequences were analyzing with PLACE database (Higo et al. 1999).

Phylogenetic analysis

Among the high number of peroxidase sequences available in the Peroxibase databases (Passardi et al. 2007), sequences representative of different clades were used to build the global tree. Peroxidase protein sequences were aligned using Clustal W (Thompson et al. 1994). The alignment was further inspected and visually adjusted and realigned with Clustal X. The distance tree was constructed with the Neighbor option of the PHYLIP 3.6a3 package under the JTT substitution frequency matrix, and 1,000 bootstrap replicates were carried out. Njplot software (Perrière and Gouy 1996) was used to visualize phylogenetic trees and BioEdit software to obtain the different consensus sequences.

Overexpression of *APRX* and RNAi construct

The *APRX* cDNA native or mutated for the pectin binding site as previously described by Carpin et al. (2001) have been cloned in pCHF3 binary vector with *Sac*I and *Bam*HI. From the transgenic plants obtained (13 lines 35S:*APRX*, 6 lines 35S:*M3* and 10 lines 35S:*Sens-AntiSens*), the lines *APRX 25.11* and *APRX 45.4* carrying the native sequence and *M3(3)* and *M3(6)* carrying the mutated sequence have been used for phenotype and genetic analysis. The RNAi construct has been obtained by cloning *APRX* cDNA with *Bgl*II and *Sac*I in reverse orientation in pAVA 393 containing

APRX-GFP (described in the following part). Sens-Antisense cassette has been sub-cloned in pCHF3 using *Kpn*I and *Sac*I. The line S-AS 3.7 has been used for further analysis. Presence of the *APRX* sequence within the *A. thaliana* genome has been verified from genomic extract by PCR using the same primers as in RT-PCR experiments.

GUS and GFP constructs

The three following constructions have been designed with the purpose to study the localization in planta of the APRX protein and the role of the N-Term in this localization. *APRX* cDNA with or without (*APRX short*) the signal peptide have been cloned in pAVA 393 (von Arnim et al. 1998) with *EcoR*I and *Nco*I. The two *APRX-GFP* constructs have been subcloned in pCHF3 with *Kpn*I and *Bgl*II. The GFP fluorescence was visualized with an Axioplan2 Zeiss microscope with narrow band excitation (470 ± 20 nm) and emission (510 ± 20 nm) filters (Chroma Technology Corp, Rockingham, VT, USA) and using the Methamorph software (Molecular Devices Corp, Sunnyvale, CA, USA).

APRX promoter region was amplified by PCR with primers containing *Pst*I and *Eco*RI restriction sites (underlined) 5′promoPstI 5′-GTGAGGTTT CTGCAGAATCTT ACCGACGGGGTCTT-3′ and 3′promoEcoRI 5′-TTTTTC AT GAATTCTGTTGTTTGGATTGGTAACG-3′. The amplified fragment was first inserted in pGEM-T easy plasmid (Promega). After digestion by *Pst*I and *Eco*RI, the promoter was further cloned upstream of GUS gene in pCAMBIA1381Xa. Finally the APRX::*GUS* construct was subcloned in the binary vector pZP222 after digestion with *Sac*I and *Xba*I for pZP222 and *Sac*I and *Nhe*I for pCAMBIA APRX::*GUS*.

GUS activity was visualized by X-Gluc staining. X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) was purchased from X-GLUC DIRECT (Wakefield, UK). Staining was performed as described elsewhere (Lariguet et al. 2003). Briefly, 72-h-old seedling grown vertically in the dark were soaked in cold (4°C) acetone 90%, then rinsed and stained (5 min at 4°C and 12 h at 37°C) in a solution containing 2 mM X-Gluc, 2 mM ferrocyanide, 2 mM ferricyanide and 10 mM sodium phosphate buffer.

Plants transformation

Arabidopsis thaliana Columbia ecotypes were transformed with *Agrobacterium tumefaciens* ASE or GV3101 using the spraying technique (Bent 2000). The transformants were selected on 1/2 MS medium containing 50 µg/ml kanamycin or 75 µg/ml gentamycin. The presence of the transgene in plants was confirmed by PCR on genomic DNA using primers designed to amplify specifically *APRX* gene or promoter.

Separation of isoperoxidase by IEF

After confirmation of the root length phenotype, soluble proteins were extracted from *A. thaliana* seedlings grown under 12 h light/12 h dark by grinding in 20 mM Hepes, pH 7.0, containing 1 mM EGTA (2 ml per g of fresh weight). The extract was centrifuged for 10 min at 10,000*g*. Proteins concentration were assayed with Coomassie Blue reagent (Bio Rad, Hercules, CA, USA) following manufacturer instructions. 10 µg of the proteins were separed by IEF performed as described previously (Penel and Greppin 1996) and the peroxidase bands were visualized by staining with *o*-dianisidine/hydrogen peroxide.

Root and hypocotyl length

The length of the hypocotyl and roots was measured on 7-day-old dark/light and dark-grown seedlings, respectively grown vertically. The elongation lengths shown in Figs. 2 and 6 were obtained by measuring 200 hypocotyls and roots of seedlings from five independent batches.

Auxin extraction and determination

The extraction and analytical methods have been performed as described (Nordström and Eliasson 1991). Materials were homogenized in liquid nitrogen. The powder was extracted with 5 mM phosphate buffer (pH 6.5) containing 3-[5(n)-^{3}H] indolylacetic acid as internal standard and butylated hydroxytoluene as antioxidant. After incubation in darkness for 1 h, the extract was filtered through a glass-fiber filter under vacuum. The filtrates were purified through Bond-Elute C18 column conditioned at pH 6.5. The pH of the eluates was adjusted to 2.5 using 2.5 M phosphoric acid and then applied to C18 columns (chromabond, Macherey-Nagel, Düren, Germany) pre-conditioned at pH 2.5. The columns were washed with distilled water, followed by acidic ethanol (ethanol/glacial acetic acid/water, 20/2/78, by vol.). A second purification of the last eluates was performed on a second C18 column at pH 2.5. Auxins were eluted from the second C18 columns with 2×300 µl aliquots of 80% methanol. A total of 50 µl of the methanolic extract were injected in an automated Merck–Hitachi HPLC system in the same conditions of elution pattern as those previously described (Heloir et al. 1996): Lichrospher 100-RP18 column, 12.5 cm × 4 mm internal diameter, 5 µm particle size; column and solvent at 30°C; flux 1 cm^3 min^{-1}; mobile phase acetonitrile: glacial acetic acid: water (10:2:88, by vol.); detection by fluorescence detector

(absorbance 292 nm, emission 360 nm). Free IAA and aspartate-conjugated IAA were monitored separately.

Auxin oxidase activity

Two purified peroxidases were tested: zucchini APRX, prepared as described elsewhere (Penel and Greppin 1994) and horseradish HRPC (Fluka, Buchs, Switzerland). Enzyme concentration was assessed as described in Penel and Greppin (1994). The incubation medium contained 10 mM IAA in 20 mM phosphate buffer pH 6.1 and 0.1 μM peroxidase. The remaining IAA was quantified after various times by briefly measuring the fluorescence (excitation 285 nm, emission 357 nm). Controls without peroxidases were also performed to take into account IAA auto-oxidation. Experiment was performed at room temperature.

Results

Identification and analysis of the intronic and promoter sequences of *APRX*

Intronic and promoter sequences are essential for fine regulation of gene transcription. The *APRX* gene presents the classical pattern of four exons/three introns shared by the majority of class III peroxidase genes (Tognolli et al. 2002). Exhaustive data mining has been performed to detect APRX othologs. Two isoforms with 70–86% identity were identified in *C. melo* and *C. sativus* (Bakalovic et al. 2006). The Cucurbitaceae APRX homologues formed a well supported branch clearly separated from other species peroxidases (Fig. 1a). The percentage of identity decreased significantly with sequences from *Populus* and other Eudicotyledons. In *A. thaliana* the closest ortholog was AtPrx54 with 53% identity to the APRX protein sequence. Notably AtPrx54 lacks the tetra-arginine motif responsible for the binding of APRX to calcium–pectate complexes (Fig. 1b, c; Carpin et al. 2001). These results suggested that APRX sequence is largely conserved among Cucurbitaceae taxonomic group and that there is no direct close homologue to APRX in *A. thaliana*.

A 900 bp promoter sequence upstream of the ATG codon was obtained by inverse-PCR performed on zucchini genomic DNA. This *APRX* promoter sequence was assessed with the Place database (Higo et al. 1999) to detect *cis*-regulatory elements. Such an analysis could indicate potential regulatory mechanisms for *APRX* and thus potential insights to its function. Numerous light- and auxin-response *cis*-elements were identified (Table 1).

APRX mRNA is regulated by auxin treatment in zucchini

To investigate the possible regulation of the *APRX* transcription by phytohormones, the level of *APRX* mRNA from 5 day-old zucchini seedlings treated with different concentrations (0.226–226 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D) was assessed by Northern blot. Treatment with the lowest 2,4-D concentration tested here (0.226 μM) significantly increased the accumulation of *APRX* transcripts compared with the untreated seedling, whereas the highest concentration (226 μM) significantly decreased the accumulation of *APRX* transcripts (Fig. 2b). In all the other treatments *APRX* mRNA levels were not affected by 2,4-D treatment. In a previous study *APRX* mRNA transcripts were mainly found in the hypocotyl elongation zone of zucchini (Dunand et al. 2002). Therefore, in parallel, we also monitored the effect of auxin treatments on growth. At all the concentrations tested, auxin dramatically inhibited the development of seedling shoots (Fig. 2a) and roots (data not shown). At the lowest concentrations tested, 5 day-old zucchini seedlings show a 70% reduction in hypocotyl length compared to an untreated seedling. At the highest concentrations seedling growth is dramatically delayed, and is accompanied by the development of a very dense hairy root system. To further investigate the regulation of *APRX* gene and also the location of APRX protein, we designed several constructs with *APRX* gene and its cloned promoter sequence (Fig. 3) for further analysis in *A. thaliana*. Indeed, although zucchini is a good plant model for peroxidase analysis at the biochemical level, it is very limited for molecular biology. Because there is no close ortholog of APRX in *A. thaliana* we heterologously expressed APRX in the plant model for molecular biology analysis.

APRX protein is localized in cell walls and gene expression is found in the elongation zone of hypocotyls in *A. thaliana*

In *A. thaliana*, an APRX-GFP construct of the entire APRX coding sequence containing its signal peptide was localized at the cell wall, confirmed by its co-localization with propidium iodide staining of the cell wall (Fig. 4f, g). A second APRX-GFP construct lacking the signal peptide resulted in a diffuse GFP signal in the cytoplasm and in the nucleus (Fig. 4b-e). Thus the cell wall localization of APRX was due to its signal peptide, as expected from the targeting prediction of APRX to the secretory pathway (TargetP 1.1, Emanuelsson et al. 2007) and hence the extracellular space and cell wall (Carpin et al. 2001).

Tissue specificity of *APRX* gene expression was examined using an *APRX* promoter:*GUS* reporter fusion gene containing the 900 bp-long 5′ upstream region of the *APRX* gene. GUS staining was localized in the

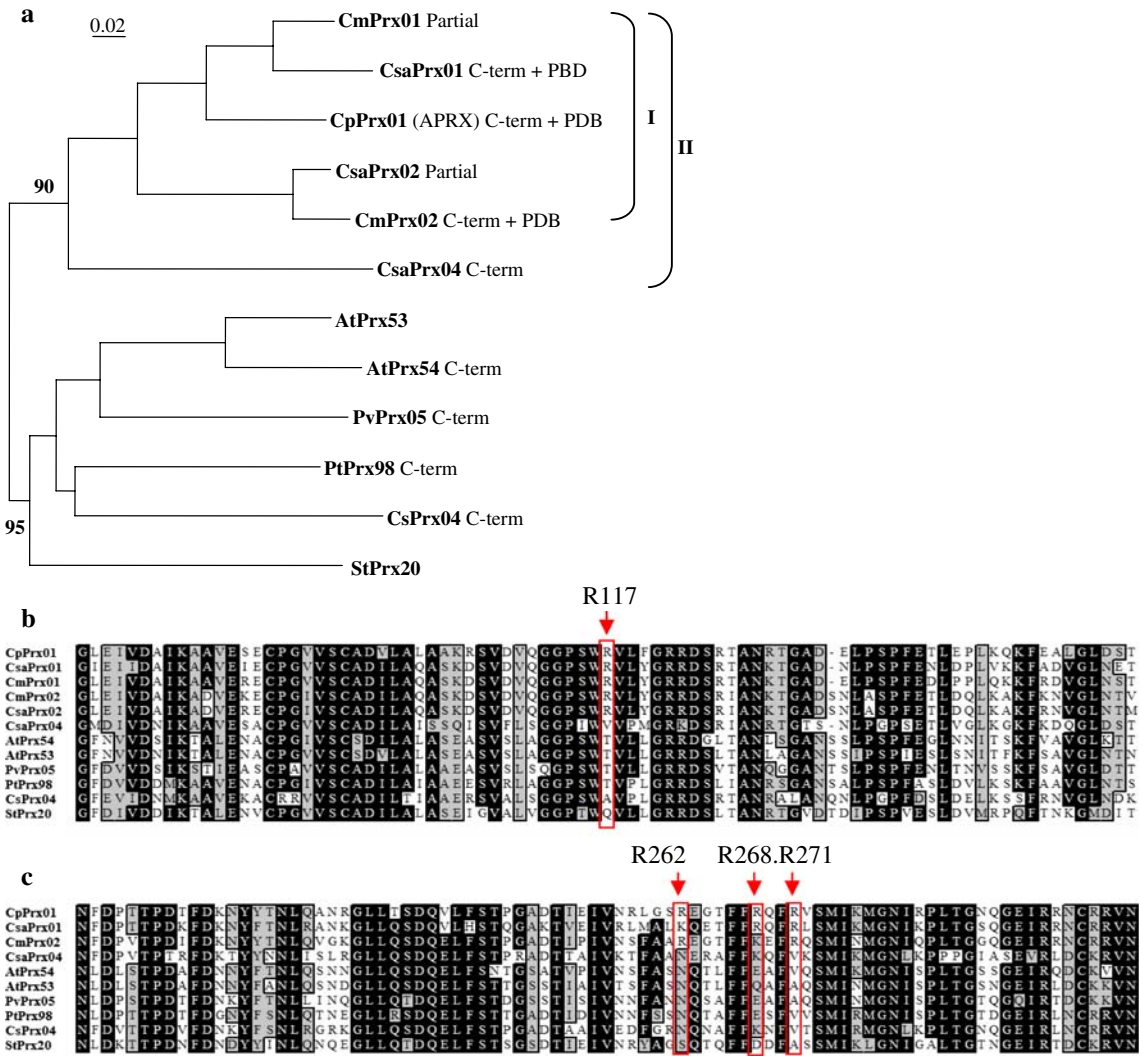

Fig. 1 CpPrx01 (APRX) forms part of a Cucurbitaceae specific cluster. **a** Neighbor-joining (*NJ*) tree of 12 class III peroxidases amongst a representative sample of sequences. Bootstrap values were obtained by the NJ method. (I) labels the pectin-binding domain cluster and (II) the Cucurbitaceae peroxidase cluster. Incomplete sequences in databases are labeled as partial, the presence of C-term and pectin binding domain (PBD) are indicated. **b**, **c** Selected parts of the multiple sequence alignment: areas around the non essential arginine residue (R117, **b**) and around the pectin binding triad (R262-R268-R271, **c**). The residue positions are based on the mature protein sequence of CpPrx01 (APRX)

elongation area of the hypocotyls (Fig. 5). A higher accumulation of GUS staining in the external part of the hypocotyl curvature was observed, as seen previously in zucchini (Dunand et al. 2002). No GUS activity was detected in roots.

APRX gene expression is regulated by auxin in *A. thaliana*

Seedlings containing the *APRX* promoter:*GUS* reporter fusion gene were treated with exogenous 2,4-D. GUS staining showed a significant increase within the hypocotyl elongation zone (Fig. 5c, d). These results were consistent with the regulation of *APRX* transcription by auxins observed in zucchini seedling (Fig. 2b).

APRX controls elongation and intracellular auxin levels in *A. thaliana* transgenic lines

To asses in detail the function of APRX during elongation processes and the role of its pectin binding activity, *A. thaliana* was transformed with two different constructs both under the control of the CaMV 35S promoter to overexpress (OE) APRX (Fig. 3). *APRX* total sequence (*APRX*) and *APRX* mutated at its pectin binding site (*M3*) were tested. For root length phenotype 13 lines 35S:*APRX*, and 6 lines 35S:*M3* were screened (data not shown). For both constructs identical phenotypes (see description below) were observed, dependent on the expression of *APRX* but not due to the presence of the pectin binding site (Fig. 6a–c). For

Table 1 Main *cis*-regulatory elements present in the 900 bp promoter region upstream of the *APRX* gene

Motif	Sequence	Function	Position
ABRE	YACGT	Abscisic acid response	18, 259, 260 (−)
AuxRE	ctcCTCAataac	Auxin response element	376 (−)
AuxRR	GGTCca	Auxin response	366 (−), 685 (−)
DPBF	ACACNNG	Abscisic acid response	729 (−)
G-box	CACGTG	Light response	268
GA-motif	ATAGA	Light response	47 (−), 425, 550
GATA-box	GATA	Light response	61(−), 271 (−), 302 (−), 422, 615 (−), 626, 754
I-Box	GATAAGR	Light-regulated genes	274 (−), 422
LAMP	ccgaATCCa	Light response	129
LTR	CCGAC	Low-temperature and abscisic acid response	434, 840
MYB	WAACCA	Drought and abscisic acid response	41, 578 (−)
P-box	CCTTttc	Gibberellin response	118
W-box	TTGACY	Salicylic acid response	144 (−), 802 (−), 807 (−)

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

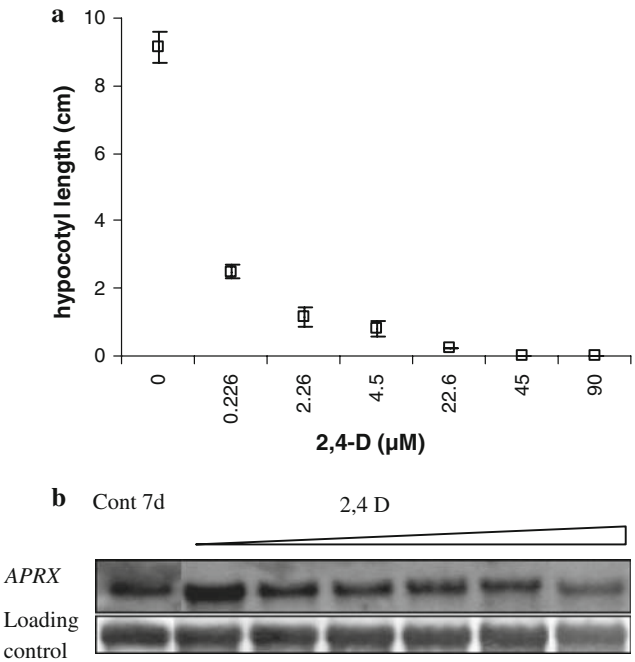

Fig. 2 **a**, **b** Effect of auxin on zucchini hypocotyl growth and *APRX* mRNA content. **a** Hypocotyl length of zucchini. **b** Northern-blot analysis of *APRX* transcripts after treatment with 2,4-D concentration ranging from 0.226 to 226 µM (**a**, **b**). Total RNA was extracted from the hypocotyl of 5-day-old seedlings

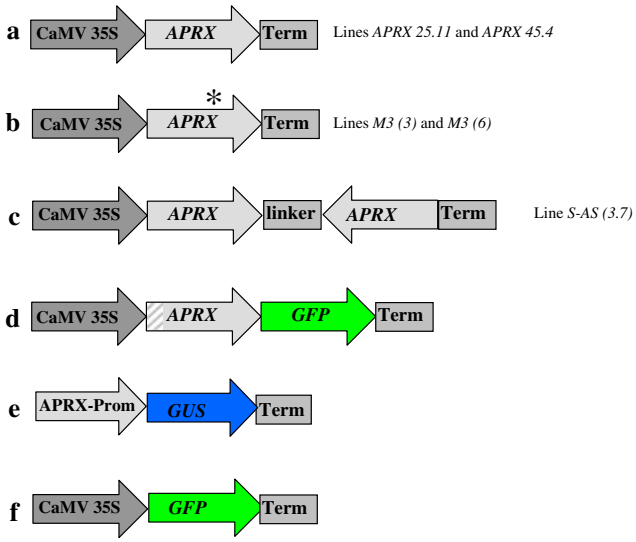

Fig. 3 Schematic structures of the various genetic constructs used in this study and resulting transgenic lines. **a** CaMV 35S:*APRX* construct, line *APRX 25.4* and *APRX 45.4*. **b** CaMV 35S:*APRX* with a mutation of the pectin binding domain (*asterisk*), line *M3 (3)* and *M3 (6)*. **c** 35S:*APRX-linker-XPRA* RNAi construct, line *S-AS (3.7)*. **d** CaMV 35S:*APRX-GFP* with or without the signal peptide (hatched zone) for cellular localization. **e** APRX endogenous promoter:*GUS* construct for subcellular localization. **f** CaMV 35S:*GFP* used as control for cellular localization

clarity purpose, we therefore present the description of *APRX* and *M3* together in this section. In the over-expressing lines (namely *OE-APRX*, observed in e.g. *APRX 25.11* and *M3(6)* plants) both transcript as well as protein APRX levels were ectopically over-expressed (Supplementary material S1). The plants display root and hypocotyl lengths longer than wild-type (WT, Fig. 6a, Supplementary material S2). In silenced lines (namely *S-APRX*, observed in e.g. *APRX 45.4* and *M3(2)* plants) *APRX* gene was present in the plants but was not expressed. The plants display on the contrary root lengths smaller than the WT, but shoot length was not significantly affected when compared to WT (Fig. 6a). The latter phenotype was also observed in plants silenced using the RNAi construction (*S-AS*; Fig. 3). Although certain variability of phenotypes has been observed, in all lines it correlated well with expression

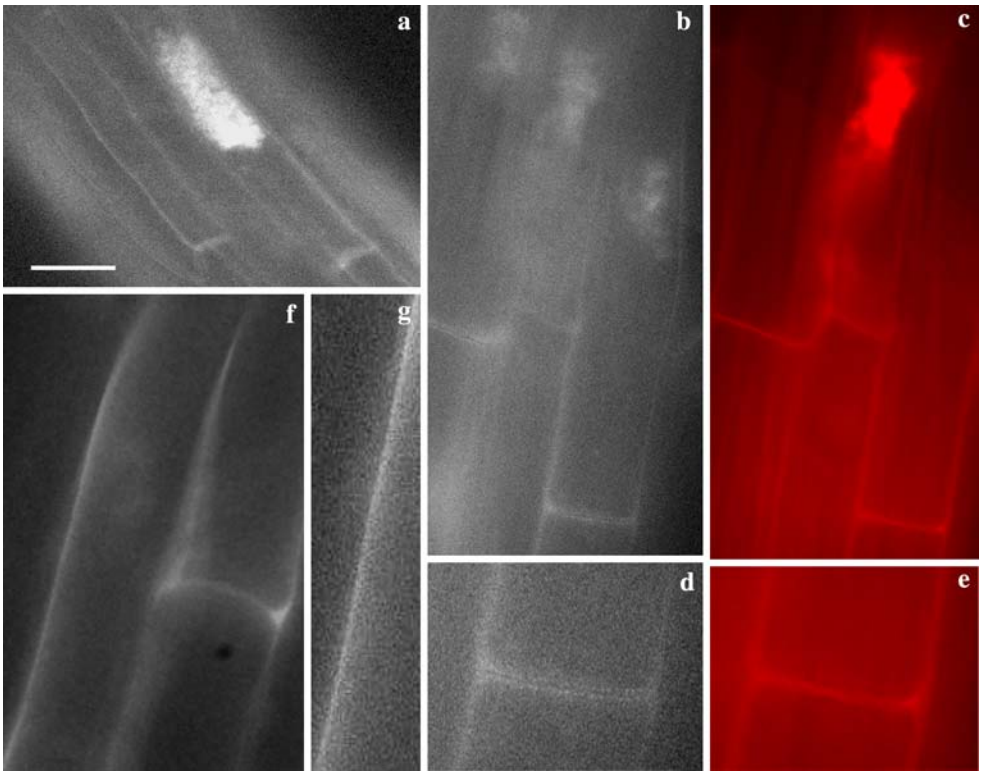

Fig. 4 Detection of GFP fluorescence in roots of *A. thaliana* seedlings. **a** Plants transformed with the construction 35S:*GFP*. **b–e** Plants transformed with the construction 35S:*APRX short-GFP*. **f**, **g** Plants transformed with the construction 35S:*APRX-GFP*. **c**, **e** Staining with 10 μg/ml propidium iodide. *Bar* 50 μm (**a**), 25 μm (**b–f**)

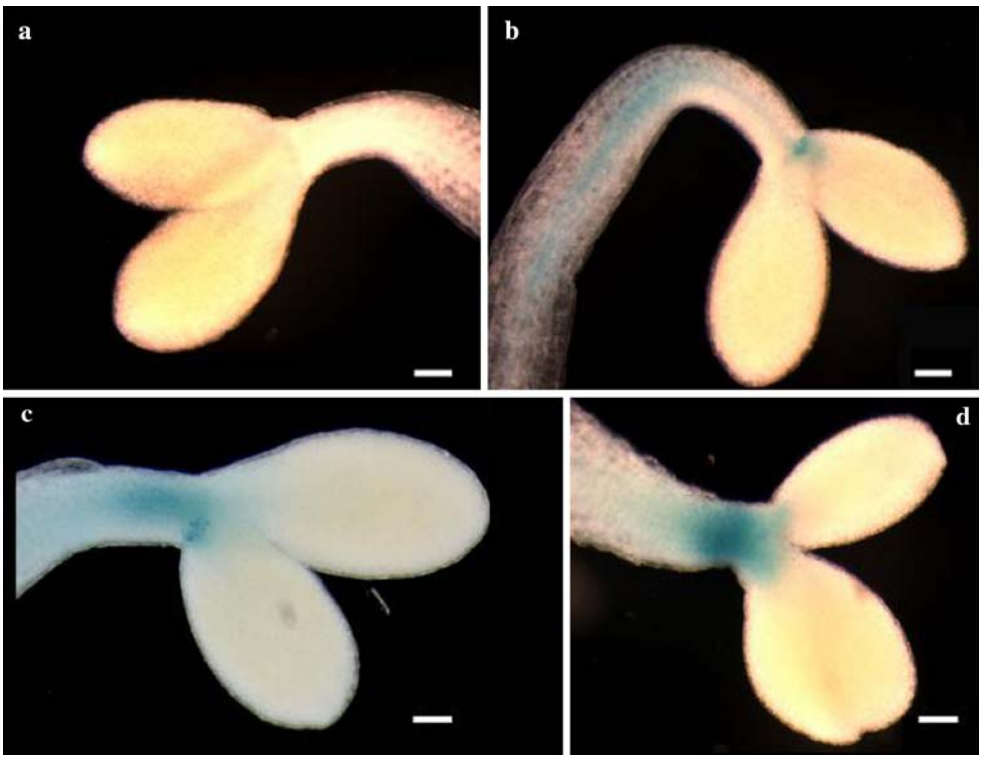

Fig. 5 Detection of GUS activity (X-Gluc staining) in WT (**a**) and in transformed with the APRX:*GUS* construct seedlings (**b**). Seedlings were grown vertically 72 h in the dark on MS (**a**, **b**) or in the presence of 2,4-D 0.1 μM (**c**), or 1 μM (**d**). *Bar* 100 μm

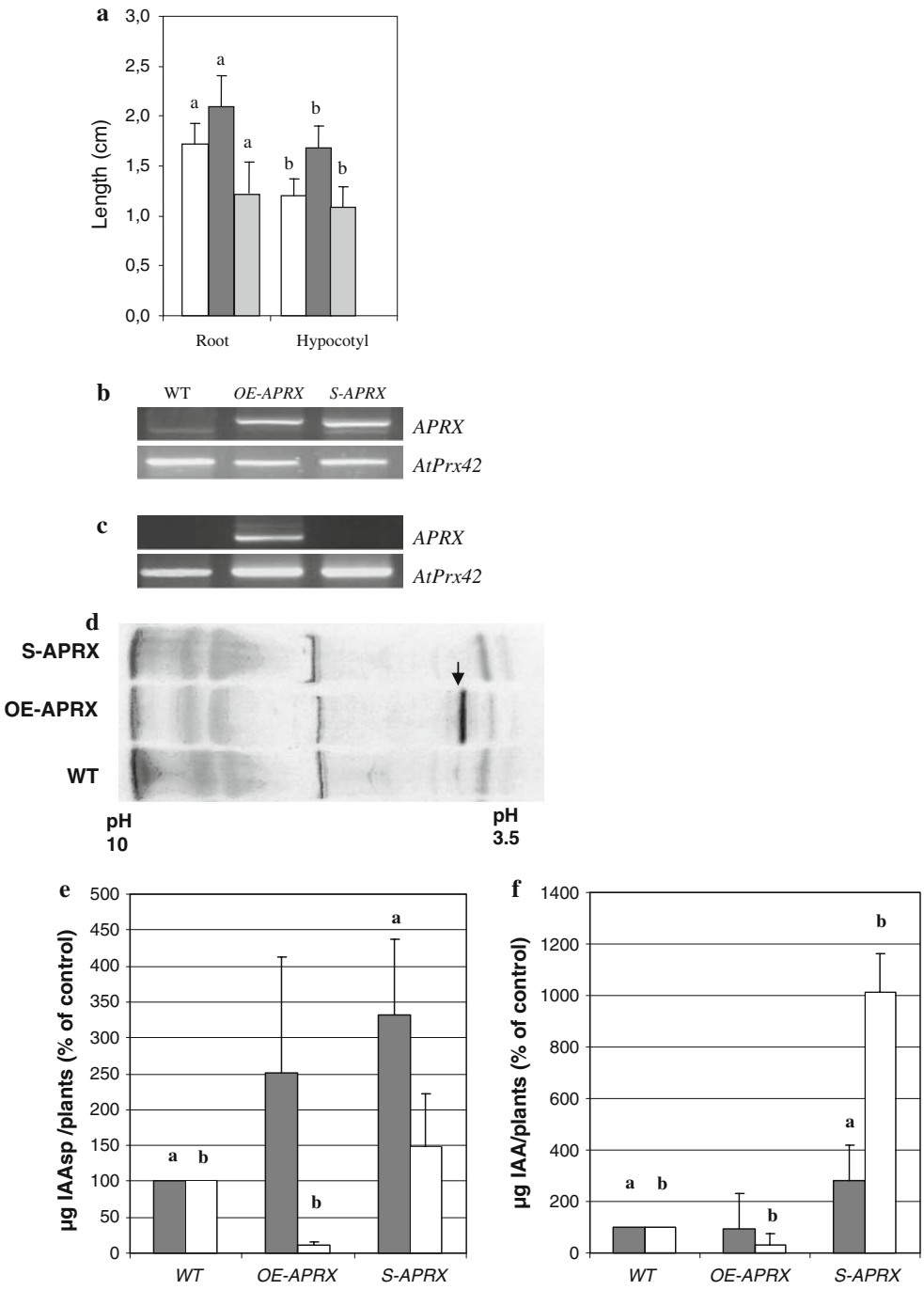

Fig. 6 Growth observation, expression of *APRX* genes and auxin content in different transgenic seedlings. **a** Root and hypocotyl lengths of WT (*white bars*) and transgenic *OE-APRX* (*dark grey bars*) and *S-APRX* (*grey bars*) 10-day-old seedling. Lengths are the average of 100–200 measures coming from 5 independent batches. **b** Genomic DNA from WT, over-expressing *APRX* (*OE-APRX)* and silenced *APRX* (*S-APRX)* was used for detection of *APRX* gene by PCR amplification with specific *APRX* primers. **c** *APRX* transcript level in WT, *OE-APRX* and *S-APRX* visualized by RT-PCR and showing a specific mRNA silencing in silenced lines. **d** Separation of isoperoxidases based on their isoelectric points. The peroxidase bands were visualized using o-dianisidine/H_2O_2. Direct dosage of aspartate-conjugated IAA (**e**) and free IAA (**f**) in shoots (*grey bars*) and roots (*white bars*) of 7-day-old seedlings. The differences between WT and mutant seedlings were analyzed by 1-way ANOVA test. *Same letters* indicate significant differences with *P* values: $P < 0.0001$ (**a**) and $P = 0.01$ (**b**). Lengths are the average of 100 measures coming from 5 independent batches. Auxin quantification represents 3 independent experiments of 200 plants. Standard deviation is indicated by *t bars* in all graphs

level of APRX (Supplementary material S3). The two phenotypes *OE-APRX* and *S-APRX* were observed with several different and independent T-DNA lines, excluding the possibility that they could originate from a positional effect of the T-DNA insertion in *A. thaliana* genome, rather than from *APRX* gene insertion. However, to further discard the possibility of a mutation due to a gene disruption that would be responsible for the phenotype; we assessed the inheritance of the mutation by crossing homozygous mutant plants to WT Columbia. All the heterozygous F1 progeny showed the adequate phenotypes. This confirmed that the phenotype observed was only due to the *APRX* (or mutated *APRX)* gene.

Two forms of auxin were also assayed in *A. thaliana* transgenic plants (Fig. 6e, f): free auxin (IAA) and aspartate-conjugated auxin (IAA-Asp). The latter species is probably inactive and represents a major product of auxin degradation (Woodward and Bartel 2005). It is usually found at high levels in tissues such as expanding leaves and roots that also contain the highest free IAA levels

(Kowalczyk and Sandberg 2001). Indeed in the samples tested here, the level of IAA-Asp varied in a similar manner than free IAA. In *OE-APRX* transgenic lines a 90% reduction in root IAA concentration was observed compared to WT plants, indicating a possible role for APRX in auxin catabolism. Due to an elevated standard deviation no significant difference was observed for the shoot part. On the contrary, in the S-*APRX* transgenic lines, a twofold and tenfold increase of the free IAA concentration was observed in roots and shoots, respectively (Fig. 6f). The observation of a higher auxin concentration suggested that the silencing of *APRX* induced the silencing of *Arabidopsis* endogenous peroxidase genes involved in auxin catabolism. Indeed, a detailed observation on IEF presented in Fig. 6 revealed that at least 1 basic and 1 acidic band were missing in S-APRX and that several other bands were fainter when compared to WT. Nevertheless, this is only indicative because only major bands are visible on the IEF. It has to be taken in consideration that from the 73 peroxidase gene present in *A. thaliana* only the more active are visible and/or also that one band—particularly the thicker ones—might be formed by several isoperoxidases of close pI.

APRX has an auxin oxidase activity

To assess the role of APRX in the regulation of auxin levels, APRX was purified from zucchini seedling based on its pectin binding ability (Carpin et al. 1999). The purified protein was assayed for its auxin oxidase activity. Commercial HRP extract (HRPC enriched) was used as a positive control (Beffa et al. 1990). The degradation of auxin by HRPC can occur without the addition of any cofactor, and either in the presence or absence of H_20_2. In the conditions tested here—absence of cofactors and H_20_2, APRX showed an initial auxin oxidase activity similar than an equal quantity of HRPC and took longer time to reach a plateau (Fig. 7).

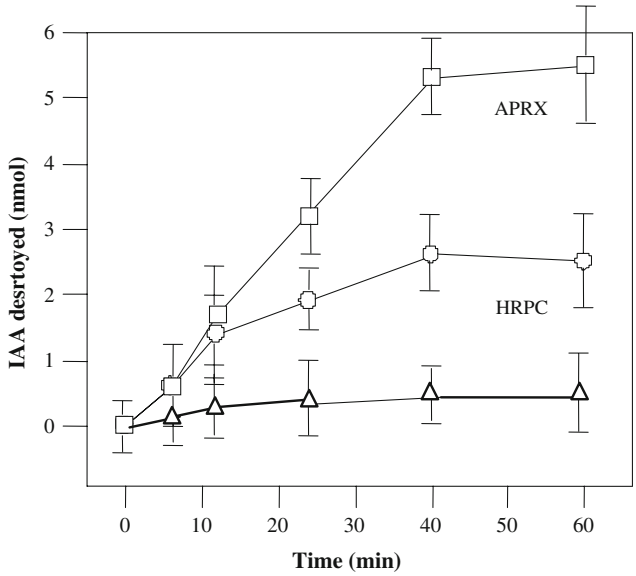

Fig. 7 APRX presents an IAA oxidase activity. Catabolism of IAA has been monitored in presence of 0.1 μM of APRX (*open square*) or HRPC (*open circle*) and without protein (*open triangle*). Each value was the mean of at least four separate experiments done in duplicate

Discussion

In previous studies, the purification of APRX from zucchini based on its capacity to form an insoluble complex in the presence of Ca^{2+} and pectin was reported (Carpin et al. 2001). Transcription of *APRX* was shown to be highest in hypocotyls of zucchini (Dunand et al. 2003). Localization of transcripts seemed to indicate a role in the elongation process. Nevertheless a precise analysis of the regulation of this peroxidase was still lacking. Moreover its function had not been clearly assessed, although several hypotheses had been formulated. We report here the elucidation of the regulation and in planta function of APRX, a class III peroxidase. This function was further supported by the in vitro assessment of the protein activity.

APRX is a peroxidase peculiar to Cucurbitaceae

APRX gene sequence was compared with other known peroxidase sequences. *APRX* gene showed a classical pattern shared by the majority of the class III peroxidase genes. The protein sequence is largely conserved among cucurbital taxonomic group, but not in other Eudicotyledons, indicating that APRX might be a Cucurbitaceae specific peroxidase. *Cucumis* organellar genomes are unusually labile and numerous accumulations of repetitive sequences as well as major rearrangements are thought to have taken place during the evolution of *Cucumis* (Renner et al. 2007). Although no direct evidence has demonstrated a similar evolution of genomic DNA, the existence of specific peroxidase encoding sequence could be the proof of such phenomena. Moreover, we also observed that the mutation of the pectin binding site did not affected APRX activity in *A. thaliana* since the mutated constructs induced the same phenotypes as the non-mutated ones. This observation further supports the idea that the binding specificity of APRX might be a Cucurbitaceae specific peroxidase with no close homolog in *A. thaliana*. Moreover none of the peroxidases known to have some ability to bind to pectin in *A. thaliana* (Shah et al. 2004) have significant sequence similarity to APRX.

APRX is regulated by auxin levels

In this study, the promoter sequence of *APRX* was identified and analyzed. Analysis of *cis*-elements contained in

this sequence revealed numerous light and auxin response elements. Both light and auxin are essential and often associated in the regulation of hypocotyls growth (Tian and Reed 2001). Indeed, many of the developmental processes that occur as a result of light signals are dependent, at least in part, on the action of phytohormones. Several works had shown that light alter the levels of IAA (Behringer and Davies 1992). In order to asses if the presence of these *cis*-elements could be associated to the regulation of APRX transcription, we performed analysis upon auxin treatments.

APRX transcription level in zucchini was significantly up-regulated by a low concentration of auxin and was down-regulated in high concentration treatments. Further experiments in *A. thaliana* with the *APRX* promoter:*GUS* transgenic lines demonstrated that treatments with low auxin concentration visibly increased the detected GUS activity. These results were consistent with a regulation of *APRX* transcription by auxin. Curvature of the growing organ is known to rise from regulated lateral auxin gradients (Went and Thimann 1937). *APRX* transcripts are present in the external part of the hypocotyl curvature in the zone of highest auxin concentration of the growing hypocotyls. An enhancement of peroxidase activity after treatments with auxin and a relationship between the distribution of IAA and peroxidase activity have previously been reported in lupin hypocotyls (Ferrer et al. 1991). Other studies contrarily reported a decrease of total peroxidase activity in oat coleoptiles treated with 40 μM IAA, but they nevertheless also observed the induction of a specific unidentified isoform in the intercellular fraction upon IAA treatment (Gonzalez et al. 1999). Both studies are in agreement with the results presented here, suggesting the probable existence of a direct hormonal control of specific peroxidases by IAA.

APRX is involved in auxin catabolism

Free IAA and Asp-conjugated IAA levels in transgenic lines over-expressing (*OE-APRX*) or silencing (*S-APRX*) *APRX* were monitored. Asp-conjugated IAA should be considered as catabolite rather than storage forms of IAA (Woodward and Bartel 2005). Both IAA types varied in a similar manner in the different mutants studied here. These results are in agreement with other reports in which the modification of endogenous free IAA level caused similar modification of the auxin catabolites and conjugates (e.g. Barlier et al. 2000). A significant reduction of the auxin level due to the ectopic overexpression of APRX was observed in *OE-APRX* roots. Silenced lines (*S-APRX*) showed an increased level of auxin in both roots and shoots, probably due to a reduction of IAA catabolism by peroxidases. Since APRX is a heterologous protein its suppression should not result in any phenotype. Therefore, the observed phenotype can only results from affected endogenous proteins. The antisens strategy is known to be unspecific. For example *A. thaliana* plants expressing the heterologous French bean peroxidase type 1 (FBP1) resulted in the silencing of at least two endogenous isoforms in leaves (Bindschedler et al. 2006). Because peroxidase gene sequences contain highly conserved domain, it is highly probable that the silencing of *APRX* in *A. thaliana* also affected the transcription of other *Arabidopsis* peroxidases involved in auxin catabolism and therefore result in a higher endogenous auxin content in S-*APRX* seedlings. Nevertheless more studies are required to confirm this hypothesis, identify the concerned isoforms and assess their function.

In order to clarify the putative implication of APRX in auxin level regulation, purified APRX protein was assayed for its auxin oxidase activity and compared with HRPC. In the condition tested here APRX showed an initial auxin oxidase activity comparable to HRPC. These results eventually supported in vitro the in planta observations of a role for APRX in auxin catabolism. It should be noted though that if the appropriate phenolics are present most peroxidases can catalyze IAA oxidation, and only the isoperoxidases such as APRX that are able to oxidize IAA directly without a cofactor are putative in vivo IAA oxidases (Gazaryan et al. 1996). Nevertheless over-expression of APRX reduced global auxin pool only in roots and not in shoots in the transgenic *Arabidopsis* line studied here. On other hand in silenced line auxin pool was affected in both roots and shoots. These discrepancies probably indicated that APRX is one of several proteins involved in auxin homeostasis. The modification of the expression of a single gene often results in no discernable mutant phenotype, because it is compensated by other genes acting in a redundant manner. This is particularly true for members of large gene families such as class III peroxidases. Auxin oxidase activity of peroxidase proteins has been already reported in Tobacco, in vitro (Gazaryan and Lagrimini 1996) and in vivo (Lagrimini 1996). Nevertheless, auxin levels were not affected in the overexpressing transgenic plants (Lagrimini et al. 1997b) and the protein was not considered as having a role in auxin catabolism anymore (Dowd and Lagrimini 2006). The generation of transgenic plants with different features has been performed in attempt to identify the function of specific peroxidase isoforms (Heggie et al. 2005; Bindschedler et al. 2006). However, to our knowledge, amongst the many peroxidases expressed in plants, no studies have unambiguously shown until now a particular isozyme to be both in vitro and in planta specifically involved in auxin catabolism or another specific mechanism.

APRX may participate in the negative feedback regulation of auxin level

In this work, we observed that the *APRX* transcription was under the control of auxin. APRX was additionally associated with a specific function in auxin catabolism. The phytohormone auxin is involved in diverse developmental processes, many of which depend on regulated auxin gradients (Went and Thimann 1937). For example, a gradient in auxin concentration from tip to base is believed to be responsible for differential elongation rates in different regions of shoots (Sánchez-Bravo et al. 1992). Strict control of the endogenous IAA concentration is therefore of great importance. The balance between auxin biosynthesis and metabolism, including conjugation, deconjugation, and catabolism, determines the level of the active hormone (Bartel 1997). In *A. thaliana* several genes involved in auxin homeostasis are known to contain auxin response elements in their promoters (Barlier et al. 2000). Furthermore recent studies have revealed that specific auxin-mediated responses are controlled via a negative (auto)feedback loop (Tatematsu et al. 2004). As *APRX* gene is auxin-inducible but that the protein is also an auxin oxidase, APRX may participate in the local negative feedback regulation of auxin levels. A feedback circuit such as this would allow for precise spatial and temporal control of auxin related mechanisms.

APRX is localized in the cell wall and the hypocotyl

In order to precisely determine the function of APRX, we analyzed the localization of APRX protein and transcript in transgenic *A. thaliana*. Different GFP constructs were used in order to asses the APRX cellular localization. APRX was detected in the cell wall as expected based both on the fact that APRX was first isolated from the apoplast (Carpin et al. 1999) and its capacity to bind the calcium-pectate complex (Carpin et al. 2001). Nevertheless, mutation in the pectate binding site did not modified the effect of APRX ectopic expression in the *Arabidopsis* transgenic lines studied here, suggesting that its binding ability is not essential to APRX function.

Experiments in *A. thaliana* with the *APRX* promoter:*GUS* transgenic lines revealed that the GUS protein accumulated in the elongation area of the hypocotyl. In precedent studies these localization was also observed in zucchini (Dunand et al. 2002). It was suggested that this localization could indicate the involvement of APRX—as other peroxidases (Passardi et al. 2006) in the control of the cell elongation in the hypocotyl. In zucchini *APRX* mRNA was in addition detected in root tip (Dunand et al. 2002). Nevertheless GUS activity could not be detected in roots in the *Arabidopsis* transgenic lines studied here. It is probable, as the *APRX* promoter used was only partial and from zucchini, that a weaker expression resulted in *A. thaliana*. It would be favourable to fuse this promoter sequence with 35S enhancer sequences to see if we could detect *APRX* localization in roots. Since APRX was temporally and spatially correlated with the elongation zone of hypocotyls, a localization in the meristem—the elongation zone of the roots—would be consistent with the hypothesis that APRX is involved in the elongation process (Dunand et al. 2002). However, taken together our data confirm that APRX is associated to the cell wall in the elongation zone of the hypocotyl.

APRX has a role in elongation processes

We observed in zucchini that the reduction of *APRX* expression due to auxin treatment might be correlated to the reduction of seedlings growth. In *A. thaliana*, over-expressing *APRX* lines showed longer root and shoot lengths. Silenced *APRX* lines showed on the contrary shorter roots. Interestingly, root growth is strongly inhibited by auxin concentrations that promote elongation in stems and coleoptiles (Davies 1995). Application of auxin (>1 μM) stops root growth and causes alkalinization (Evans et al. 1980) and stiffening (Büntemeyer et al. 1998) of cell wall in the growing zone. A significant reduction of the auxin level due to the ectopic overexpression of APRX in roots could therefore be responsible for the longer roots. On the contrary an increase of endogenous IAA in the shoots and roots of silenced lines—due to a reduction of IAA catabolism by peroxidases—could have no detectable effect on shoot growth but could affect root growth. These data eventually supported a putative role for APRX in the elongation process through its auxin oxidase activity. It is possible that peroxidase isoforms with other functions that auxin catabolism were also affected by the silencing originating from APRX overexpression and were also responsible of the phenotypes observed. However, further investigation will be necessary to conclude.

According to the literature (Rayle and Cleland 1992), auxins may directly stimulate the early phases of cell elongation by activating cell wall-loosening proteins of the elongation region. When the cell wall is loosened by the action of auxins, this now-less-rigid wall is expanded by cell turgor pressure, which presses against the cell wall. Acidification of the extracellular space (Cosgrove 2000) is in addition expected to facilitate transport of the protonated form of auxin into the cell and so further stimulate the cell response (Leyser 2005). Kawano (2003) described three overall IAA signalling pathways: (1) a transmembrane, (2) an intracellular and (3) an extracellular signalling pathway. According to this author, IAA signalling in the extracellular pathway might be mediated by peroxidases. IAA may be utilized for the generation of ROS, or merely degraded by

the peroxidase. It is indeed generally accepted that peroxidases catalyze the generation of ROS coupled to the oxidation of IAA. ROS may have a direct role in cell-wall loosening (Schopfer et al. 2002) or as signalling molecule (Neill et al. 2002). However, since the proof of these peroxidase functions have not been demonstrated in planta and are still a matter of debate, based on its in vitro and in planta auxin oxidase function, spatial and temporal localization in shoots, we propose that APRX could oxidize auxin in the cell wall region of hypocotyl elongation zone. Consequently, APRX could participate in the local decrease of auxin stimulation of the cell that eventually leads to the cessation of the elongation process. Indeed, the level of endogenous auxin is nearly optimal for growth in the subapical region of the stem where the elongation region is located. When the endogenous source of auxins is removed—e.g. by APRX—the growth rate rapidly decreases to a low basal rate. In leaves several studies have reported an association between increasing peroxidase activity and the timing or location of cessation of cell expansion (MacAdam et al. 1992).

IAA represents one of the most important plant hormones, regulating many aspects of plant growth and development, from seed germination to fruit ripening (Davies 1995). Auxin regulation is certainly controlled by multiple systems (Normanly and Bartel 1999). This multiplicity of transcriptional regulators certainly accounts for the pleiotropic character of auxin action. The data presented here fits well in this idea; yet, additional studies will be required to decipher the details by which this particular peroxidase isoform cooperates with other proteins to precisely and properly regulate growth and auxin catabolism. Future research will fill in knowledge gaps and identify new genes and proteins relevant to auxin action. However, peroxidases involved in auxin catabolism could certainly take place in the control of local auxin homeostasis necessary for the fine tuning of the elongation process. In conclusion the cell wall of the elongation zone might be an appropriate place to locally control auxin homeostasis.

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