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Isolation and Characterization of Four Ascorbate Peroxidase cDNAs Responsive to Water Deficit in Cowpea Leaves

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• *Background and Aims* Abiotic stresses stimulate formation of active oxygen species in plant tissues. Among antioxidant mechanisms, H_2O_2 detoxication by ascorbate peroxidases (APX) plays an important role. Several APX isoforms exist in plant cells, and they have rarely been studied separately. The aim of this work was to study changes in cytosolic, peroxisomal, stromatic and thylakoid APX gene expression in response to progressive drought, rapid desiccation and application of exogenous abscisic acid in the leaves of cowpea (*Vigna unguiculata*) plants.
• *Methods* Two cowpea (*V. unguiculata*) cultivars, 'EPACE-1' which is drought-tolerant and '1183'which is drought-sensitive, were submitted to drought stress by withholding irrigation. Detached leaves were air-dried or treated with exogenous abscisic acid. APX cDNAs were isolated by PCR and cloned in plasmid vectors. Changes in gene expression were studied using reverse-transcription PCR.
• *Key Results* Four new *V. unguiculata* cDNAs encoding putative cytosolic, peroxisomal and chloroplastic (stromatic and thylakoidal) APX were isolated and characterized. In response to the different treatments, higher increases in steady-state transcript levels of the cytoplasmic and peroxisomal APX genes were observed in '1183' compared with 'EPACE-1'. On the other hand, the expression of the chloroplastic APX genes was stimulated earlier in the tolerant cultivar when submitted to progressive drought.
• *Conclusions* Water deficit induced differences in transcript accumulation of APX genes between the two cultivars that were related to their respective tolerance to drought. Chloroplastic APX genes responded early to progressive water deficit in the tolerant plant, suggesting a capacity to efficiently detoxify active oxygen species at their production site. The more sensitive '1183' was also able to respond to drought by activating its whole set of APX genes.

Key words: Active oxygen species, drought tolerance, ascorbate peroxidase, abscissic acid, gene expression, *Vigna unguiculata*.

INTRODUCTION

Water deficit (drought and desiccation) is known to generate active oxygen species (AOS). Among these, H_2O_2 is produced mainly in the chloroplasts and mitochondria of stressed cells and is the source of important cell damage (Foyer *et al*., 1994; Dat *et al*., 2000). Protection against AOS involves water-soluble and lipophilic antioxidants, as well as the ascorbate–glutathione cycle. In this cycle, ascorbate peroxidase (APX; EC 1.11.1.11) catalyses the first reaction between H_2O_2 and ascorbate, giving rise to monohydroascorbate and H_2O. APX is responsible for H_2O_2 detoxication in green leaves (Foyer and Harbinson, 1994; Chaudière and Ferrari-Iliou, 1999) and is considered as a key antioxidant enzyme in plants (Orvar and Ellis, 1997). APX activities are located in chloroplasts, cytosol, mitochondria and peroxisomes, each cellular compartment possessing one or several APX isoforms. APX cDNAs have now been isolated and characterized from several plant species (for review see, Shigeoka *et al*., 2002). The isoforms are named cytosolic APX (cAPX), peroxisomal or microbody APX (pAPX), chloroplastic, i.e. thylakoidal and stromatic APX (tAPX and sAPX, respectively). In arabidopsis, the same protein is dually targeted to mitochondria and chloroplast stroma (Chew *et al*., 2003). Recent studies have focused on changes in activity and gene expression for APX isozymes in higher plants subjected to environmental stresses, such as ozone, high light, extreme temperatures and salt (Shigeoka *et al*., 2002). As far as is known, the only report concerned with the effect of water deficit on the expression of different APX isoform genes is in air-desiccated spinach leaves (Yoshimura *et al*., 2000) and there is not one dealing with plants submitted to progressive drought.

To understand better the role of the different forms of APX under water deficit, comparative studies were carried out using two *Vigna unguiculata* (*Vu*) cultivars: 'EPACE-1' which is drought-tolerant and '1183' which is drought-sensitive. When submitted to drought, the two cultivars showed different physiological responses: EPACE-1 closed its stomata at lower leaf water potentials and therefore maintained photosynthetic activity longer than 1183 did (Cruz de Carvalho *et al*., 1998). At the cellular level,

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The nucleotide sequence data are registered in Genbank under the accession numbers: *cAPX*, U61379; *tAPX*, AY484492; *sAPX*, AY484493; *pAPX*, AY466858.

EPACE-1 could maintain cellular homeostasis at lower leaf water potentials compared with 1183 (Vasquez-Tello *et al*., 1990). This resulted from a lesser stimulation of lipolytic and proteolytic activities (Roy-Macauley *et al*., 1992; Sahsah *et al*., 1998) and from lower expression of the corresponding genes (El Maarouf *et al*., 1999; Cruz de Carvalho *et al*., 2001; Matos *et al*., 2001). Also, EPACE-1 was less prone to photooxidative damage (Ferrari-Iliou *et al*., 1994) and could accumulate more protective molecules (d'Arcy-Lameta *et al*., 1996).

In this study, first APX activities were measured in the leaves of cowpea plants submitted to drought, then four cowpea APX cDNAs corresponding to *Vu*1183 cytosolic (*cAPX*), peroxisomal (*pAPX*) and chloroplastic (thylakoid and stromatic, *tAPX* and *sAPX*, respectively) leaf compartments were isolated and characterized. The APX gene expression was studied in response to progressive drought and air desiccation. Exogenous abscisic acid (ABA) treatments were also carried out, as ABA is known to be involved in drought-induced signalling pathways leading to adaptive antioxidant processes (Zhu, 2002).

MATERIALS AND METHODS

Plant material and stress treatments

Two *Vigna unguiculata* (L.) Walp. (*Vu*) cultivars were used: 'EPACE-1' which is drought-tolerant originated from semi-arid north-eastern Brazil and '1183' which is drought-sensitive originated from humid areas of China. Plants were grown in pots (9 cm diameter), in a mixture of peat and vermiculite (50 : 50, v/v), in a greenhouse under conditions described previously (El Maarouf *et al*., 1999). They were watered daily with tap water and twice a week with a modified Hewitt's nutrient solution. At 5 weeks, second and third expanded leaves were harvested at 10 a.m. after 4 h of illumination, frozen in liquid nitrogen and stored at −80 °C until needed.

Different harmful conditions known to cause oxidative stress were applied to cowpea EPACE-1 and 1183 plants to characterize expression of the *VuAPX* cDNA isolated here: air desiccation, exogenous ABA treatment and progressive drought. Desiccated leaves were air-dried at 24 °C under dim light for 30 min, 2 h and 5 h. Exogenous ABA was applied by immersing the petioles of excised leaves in a 0.1 mM aqueous solution of mixed ABA isomers (Sigma, St Quentin Fallavier, France) for 30 min, 2 h and 24 h under ambient conditions (24 °C and 250 µmol s^{-1} m^{-2} light intensity). Progressive drought was applied by withholding irrigation from treated plants for 2–15 d. Leaf water potentials (ψ_w) were measured using a pressure bomb (PMS instrument, Corvallis, USA) (Scholander *et al*., 1964). In control leaves, water potentials were −0·3 to −0·5 MPa. Treated leaves were harvested at ψ_w = −1·0 ± 0·1 MPa (S1), ψ_w = −1·5 ± 0·2 MPa (S2) and ψ_w = −2·0 ± 0·2 MPa (S3).

Ascorbate peroxidase activity

To prepare APX extracts, 1 g of fresh leaf tissue was homogenized in 10 ml of 50 mM phosphate buffer pH 7·8, containing 2 mM EDTA, 0·5 mM phenylmethylsulfonyl fluoride and 2 mM ascorbate, in the presence of 1·2 % insoluble PVP and 0·1 % Triton X-100. Leaf homogenates were centrifuged at 30 000 *g* for 20 min at 4 °C, APX activity was measured in the supernatant as the decrease in A_{290} per mg of protein and per minute (Nakano and Asada, 1981). Protein contents were determined according to Bradford (1976) with the Bio-Rad protein assay reagent, using bovine serum albumin as a standard.

RNA extraction and cDNA identification

Total RNA was isolated from leaf samples using RNeasy plant midi or maxi Kit (Qiagen, Courtaboeuf, France). Poly $(A)^+$ RNA was isolated from total RNA using Oligotex columns (Qiagen, France), following manufacturer's instructions. Total and mRNA were quantified using a Nanodrop ND-1000 spectrophotometer (Starlab, Paris, France) at 260 nm.

Previously isolated APX sequences were aligned using the CLUSTALW program (Thompson *et al*., 1994). Degenerated oligonucleotides were designed from conserved regions and used as primers. Reverse-transcription PCR (RT–PCR) was performed from 100-ng 1183 mRNA template, using One Step RT–PCR kit (Qiagen, France). cDNA fragments were PCR amplified in 35 cycle reactions at 50 °C annealing temperature for *cAPX* and 60 °C for *pAPX*, *tAPX* and *sAPX*. Amplified APX cDNA fragments were purified by Wizard PCR Prep (Promega, Charbonnières, France) after electrophoretic separation on 1·2 % (w/v) agarose gels and cloned in pGEM-T Easy plasmid, according to the manufacturer's instructions (Promega, France). DNA sequencing was performed on both strands (ESGS, Lille, France).

The four putatively full-length APX cDNA (*cAPX*, *pAPX* and chloroplastic *t* and *sAPX*) were obtained, using 3′- and 5′-RACE method (5′/3′ RACE kit, Roche Diagnostics, Meylan, France), following the manufacturer's protocols. 1183 purified poly $(A)^+$ mRNA was used as a template.

RT–PCR and gene expression

APX gene expression analysis was carried out by RT–PCR reactions, using One-Step RT–PCR Kit (Qiagen, France). Fifty or 100 ng of total leaf RNA were used as templates. *Vu* APX specific primers were used to amplify cDNA fragments of 790 bp (*cAPX)*, 387 bp (*pAPX)*, 396 bp (*tAPX)* and 551 bp (*sAPX)*. To evaluate the amount of template RNA in each RT–PCR reaction, a region of *Vu* 18S gene encoding a ribosomal RNA with near constitutive expression under water deficit conditions was amplified. All PCR reactions included an initial denaturation step at 94 °C, followed by 30–35 cycles depending on the template with a denaturation step (40 s at 94 °C), an annealing step (40 s at 60 °C) and an extension step (40 s at 72 °C). A final extension was carried out for 7 min at 72 °C. Amplification products were visualized on 1·2 % (w/v) agarose gels, using a UV light transilluminator (Snapshot, Syngene, Ozyme, Saint Quentin en Yvelines, France). Densitometric evaluation of DNA bands were performed with the Imager 1D/2D software (Appligene, Strasbourg, France). Preliminary

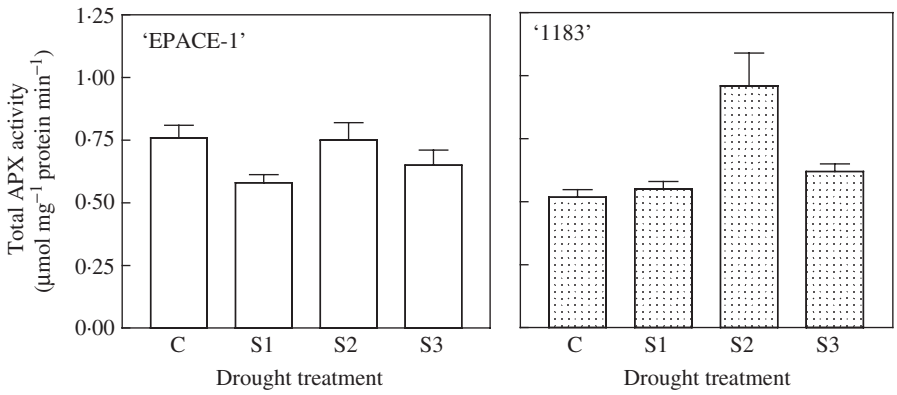

FIG. 1. Effect of drought treatment on total APX activity of extracts from cowpea (*Vigna unguiculata*) 'EPACE-1' and '1183' leaves. Control plants C, $\psi_w = -0.5 \pm 0.1$ Mpa; or droughted plants: S1, $\psi_w = -1.0 \pm 0.1$ MPa; S2, $\psi_w = -1.5 \pm 0.2$ MPa; S3, $\psi_w = -2.0 \pm 0.2$ MPa. APX activity was assayed by following oxidation of ascorbate to dehydroascorbate (decrease in A_{290}) and expressed in μmol mg^{-1} protein min^{-1}. ANOVA analysis was carried out on APX activity corresponding to five to nine independent experiments.

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sAPX  ATTGTGATAGATGAATCTCCAAATGCAGGGGCAGAGAAGTTCGTAGCAGCCAAATACTCC 1140
tAPX  ATTGTGATAGATGAATCTCCAAATGCAGGGGCAGAGAAGTTCGTAGCAGCCAAATACTCC
      ************************************************************
                    ⇓
sAPX  ACTGGAAAGGAATAAAACAAGTGTCATGTTTTCTTGTGCAAGTATCTTTTAGTAATACA 1200
tAPX  ACTGGAAAG---------------------------------------------------- 1149
      *********

sAPX  AGAAGAAGCTATTTTCAAATCATGTGATATCATGTAAAATATGGAAGTTTTAAATGAAGA 1260
tAPX  ------------------------------------------------------------

sAPX  AGAAGAATAAAATTCAATAGTGTTCCTATTTGAATTGCAGAGAGAGCTGTCAGAAGCAAT 1320
tAPX  ----------------------------------------AGAGAGCTGTCAGAAGCAAT 1169
                                               ********************

sAPX  GAAGCAGAAGATACGGGCTTATGAGGCCGTTGGTGGAAGCCCAGATAAGCCTCTACAGTC 1380
tAPX  GAAGCAGAAGATACGGGCTTATGAGGCCGTTGGTGGAAGCCCAGATAAGCCTCTACAGTC 1229
      ************************************************************

sAPX  AAACTATTTTCTAAATATCATGATTATTATTGCAGTTTTGGCACTTTTGACATCACTTCT 1440
tAPX  AAACTATTTTCTAAATATCATGATTATTATTGCAGTTTTGGCACTTTTGACATCACTTCT 1289
      ************************************************************
            ⇓                                  *
sAPX  TGGAAACTAGTTGGATTTTGTTGTACTGTACCATTTTTTCTATAATGGCATTGCTATTTG 1500
tAPX  TGGAAACTAGTTGGATTTTGTTGTACTGTACCATTTTTTCTATAATGGCATTGCTATTTG 1349
      ************************************************************

sAPX  CACAA 1505
tAPX  CACAA 1354
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FIG. 2. Nucleotide sequence alignment of the 3′ ends of *Vigna unguiculata* '1183' *sAPX* and *tAPX* cDNA (nucleotides 1081–1505). *sAPX* has an additional 151-bp nucleotide sequence with a stop codon TAA (⇓) at position 1153 bp. In *tAPX*, a stop codon TAG (⇓) is found at position 1297 bp. The asterisk indicates the putative polyadenylated signal.

experiments (not shown) with various PCR cycle numbers indicated that in the RT–PCR conditions in this study (50 ng RNA, 30 cycles for *cAPX* and *pAPX*, 250 ng RNA and 35 cycles for chloroplastic *APX*), amplifications were not in the plateau phase, and therefore allowed for semi-quantitative estimations of transcript levels.

RESULTS AND DISCUSSION

Effect of water withholding on APX activity

APX activity was 60 % higher in control EPACE-1 plants than in control 1183 plants (Fig. 1). In response to drought stress (S1, S2 and S3 plants), variations in APX activity were not significant in EPACE-1. Conversely, drought conditions induced significant changes in the levels of APX activity in 1183 since S2 plants showed a 78 % increase compared with control plants. In S3 plants, however, APX activity decreased back to control level (Fig. 1). Changes in APX activities in higher plants subjected to environmental stresses have been extensively studied (for a review see, Shigeoka *et al*., 2002). Results reported in the literature are contradictory and it is of no doubt that the plant response largely depends on its tolerance or susceptibility to stress and on the way the stress is applied to plants. The present

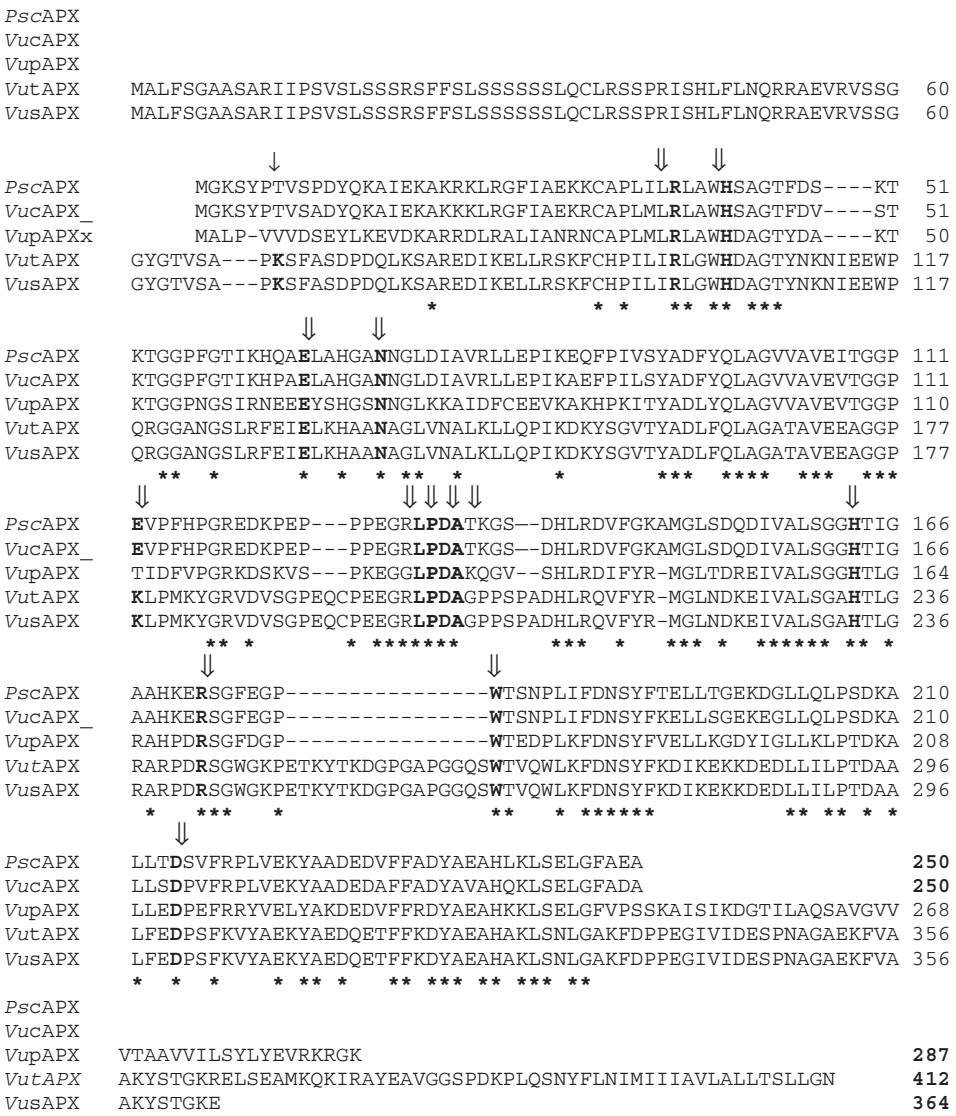

FIG. 3. Comparison of *Vu* APX-deduced amino acid sequences with *Pisum sativum* (*Ps*) cAPX (Mittler and Zilinskas, 1991). *Vuc*APX, cytosolic APX; *Vup*APX, peroxisomal APX; *Vut*APX, thylakoidal APX; *Vus*APX, stromatic APX. The asterisks at the bottom indicate conserved amino acids; dashes indicate gaps introduced to maximize alignment. The arrow at the top indicates the putative cleavage sites of transit peptides of the chloroplast isoforms. The arrows (⇓) at the top indicate essential amino acid residues: Arg38, Trp41, His42, Glu65, Asn71, Glu112 (cAPX) or Lys112 (chloroAPX), His143, His163, Arg172, Trp179, Asp208 (Shigeoka *et al*., 2002).

results on total APX activities showed different effects of water stress on the two cowpea cultivars. In response to mild water stress, APX activity was stimulated in the drought-susceptible plant and remained stable in the tolerant one. Several APX isoforms exist in plant cells (review by Shigeoka *et al*., 2002). Since drought induces damage to membranes leading to loss of cell compartmentation (Pham Thi and Vieira de Silva, 1975; Ferrari-Iliou *et al*., 1984), it was not possible to measure accurately APX activities in different cell compartments in plants submitted to water stress. Therefore, to understand better the respective role of each APX isoform, the corresponding genes were cloned and the effect of water deficit on their expression levels was studied.

Isolation and characterization of four APX cDNAs

Regarding cytosolic APX, a 1117-bp-long cDNA denoted *VucAPX* (Genbank, accession number U61379) was obtained. The open reading frame (ORF) started with an ATG codon at position 89 and was interrupted by a stop codon (TAA) at position 839. It was flanked by an 89-bp 5′ untranslated region (UTR) and a 278-bp 3′ UTR. A putative polyadenylation signal 'AAATAA' was found at position 984. The deduced protein had 250 amino acid residues and a calculated mol. wt of 27 kDa. At the amino acid level, it shared 92 % and 80·8 % identity with pea (X62077; Mittler and Zilinskas, 1991) and radish (X78452; Lopez *et al*., 1994) cAPX, respectively.

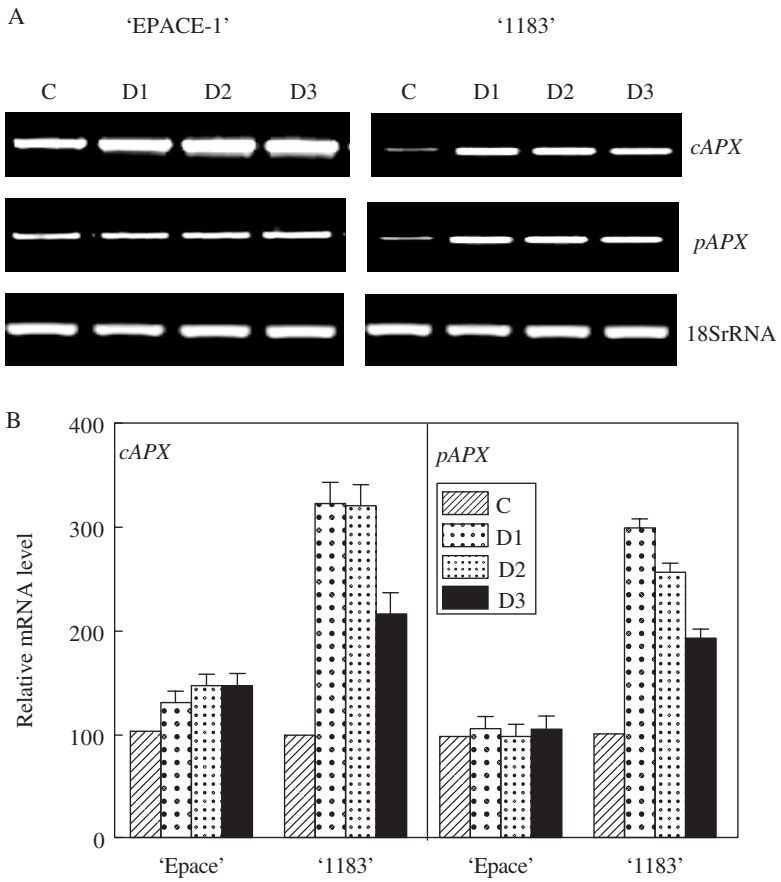

FIG. 4. Effect of desiccation (D) treatment on mRNA abundance of *cAPX* and *pAPX* and *18S rRNA* in *Vigna unguiculata* 'EPACE-1' and '1183' leaves. Desiccated leaves were air-dried at 24 °C for 30 min (D1), 2 h (D2) and 5 h (D3). Control leaf samples (C) were taken prior to treatments. (A) Gel analysis of APX isoenzyme transcripts. After RT–PCR carried out on 50 ng of total RNA with 30 cycles, samples were visualized using UV light transilluminator. (B) Relative mRNA levels. The mRNA level of each sample was quantified with the Imager 1D/2D software and normalized to the respective 18S ribosomal RNA. The values represent the mean ± s.d. of three experiments.

Regarding peroxisomal APX, a full-length cDNA of 1169 bp was obtained and denoted *VupAPX* (AY466858). Its ORF started with an ATG codon at position 130 and terminated with a stop codon (TAA) at position 1094. It was flanked by 172 additional nucleotides on the 3′ end. A putative polyadenylation signal 'AAATAA' was found at position 1109. The ORF encoded a deduced polypeptide of 320 amino acid residues (calculated mol. wt 31·7 kDa) that shared 84·7 % and 75·7 % sequence identity with *Cucurbita* (AB070626) and *Hordeum* (AB063117) pAPX, respectively.

Regarding chloroplastic APX, two cDNA were obtained. The longest thylakoidal cDNA sequence, designated *VutAPX* (AY484492), was 1354 bp long. The ORF started with an ATG codon at position 61 and terminated with a TAG stop codon at position 1299. A putative polyadenylation signal 'ATAAT' was found at position 1329. The ORF encoded a deduced protein of 413 amino acid residues (calculated mol. wt 45·2 kDa) that shared 77·5 % and 79·3 % sequence identity with tAPX from spinach (D77997) and pumpkin (D83656), respectively. The longest stromatic cDNA sequence, denoted *VusAPX (*AY484493), was 1505 bp long. Its ORF started with an ATG codon at position 61 and terminated with a TAA stop codon at position 1153, the ORF encoded a deduced protein of 364 amino acid residues identical to that of VutAPX (calculated mol. wt 39·8 kDa). It was followed by 350 additional nucleotides. Among these, a 151-nucleotide sequence corresponded to an intron (Ishikawa *et al*., 1996; Mano *et al*., 1997) and a 195-bp span was identical to the end of the 3′-end region of the *VutAPX* sequence (Fig. 2).

The four cowpea APX deduced proteins have been aligned and compared with a pea cytosolic APX (Mittler and Zilinskas, 1991) (Fig. 3). Amino acid residues essential for enzymatic activity (Jespersen *et al*., 1997; Shigeoka *et al*., 2002) were conserved in the cowpea sequences and were referenced according to the pea cAPX (Y62077).

The thylakoidal and stromal APX sequences described herein were identical, except for the presence of a 49 amino acid region at the C-terminal end of the thylakoidal sequence with a very hydrophobic profile which could correspond to a membrane-spanning region (Shigeoka *et al*., 2002). As is the case in spinach (Ishikawa *et al*., 1996), pumpkin (Mano *et al*., 1997), *Mesembryanthemum crystallinum* and tobacco (Shigeoka *et al*., 2002), cowpea tAPX and sAPX could be coded by a single gene alternatively spliced. In *Vu* chloroplastic APX, Phe 175 (as referred to pea cAPX) was changed in Trp, as is

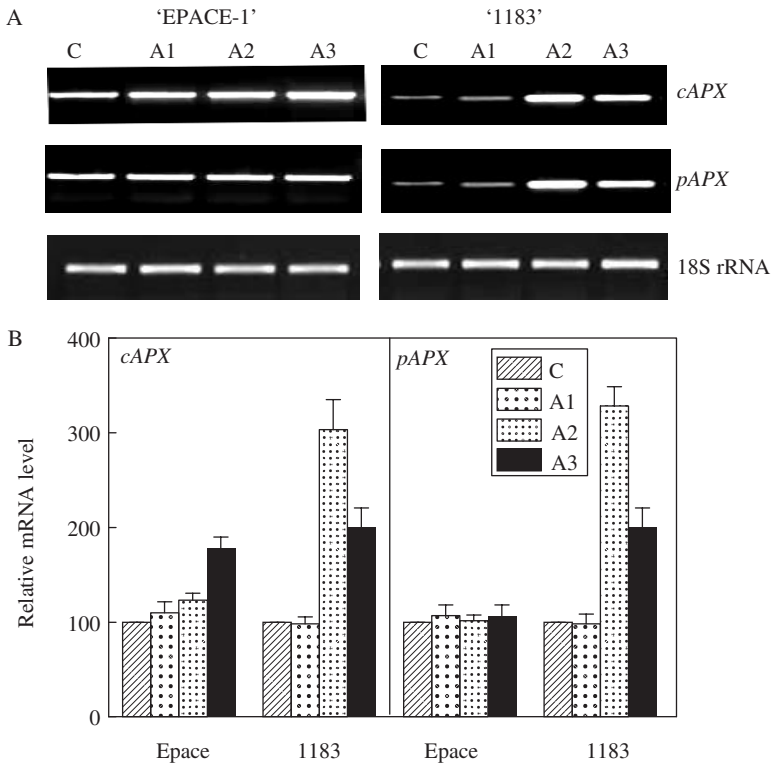

FIG. 5. Effect of exogenous abscisic acid (ABA) treatment on mRNA abundance of *cAPX* and *pAPX* and *18S rRNA* in *Vigna unguiculata* 'EPACE-1' and '1183' leaves. Exogenous ABA (0.1 mM) was applied by immersing petioles of excised leaves for 30 min (A1), 2 h (A2) and 24 h (A3) at 24 °C and 250 µmol s^{-1} m^{-2} light intensity. (A) Gel analysis of APX isoenzyme transcripts. After RT–PCR carried out on 50 ng of total RNA with 30 cycles, samples were visualized using a UV light transilluminator. (B) Relative mRNA levels. The mRNA level of each sample was quantified with the Imager 1D/2D software and normalized to the respective 18S ribosomal RNA. The values represent the mean ± s.d. of three experiments.

the case in spinach (Shigeoka *et al.,* 2002). Sequence alignment revealed the presence of 61 additional amino acid residues at the N-terminal end of the two chloroplastic APX compared with the other *Vu* APX. These regions were identified as chloroplastic targeting sequences (ChloroP program, //psort.nibb.ac.jp) with a putative cleavage site at Lys69 (Shigeoka *et al*., 2002) leading to putative mature polypeptides of 38·2 kDa and 32·8 kDa, for tAPX and sAPX, respectively.

Compared with VucAPX, VupAPX presented an additional C-terminal region of 39 amino acid residues that shared high sequence similarity with *Cucurbita* and arabidopsis pAPX C-terminal regions. In these putative membrane-spanning regions, the YEVKRXK-COOH motif seemed characteristic of peroxisomal (microbody) proteins (Mullen and Trelease, 2000). According to Bunkelman and Trelease (1996) the pAPX is bound to the external side of the membrane of peroxisomes.

Effect of stress conditions on APX gene expression

In EPACE-1 air-desiccated leaves, no significant changes were observed in steady-state levels of *VucAPX* and *VupAPX* transcripts in response to rapid water loss (Fig. 4) and exogenous ABA treatment (Fig. 5). In the case of 1183, important increases in steady-state transcript levels of *cAPX* and *pAPX* were observed after 2 h of ABA treatment and after 30 min of desiccation. It is interesting to note that *VupAPX,* which codes for a protein facing the cytosolic side of peroxisomes, showed an expression profile similar to that of *VucAPX*. The present results concerning EPACE-1 are equivalent to those obtained by Yoshimura *et al*. (2000) on spinach. However, results on the sensitive cowpea cultivar are different and indicate an up-regulation of APX gene expression by rapid water loss and ABA. ABA is implicated in signalling pathways induced by water stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Chinnusamy *et al*., 2004) and APX gene expression was stimulated by ABA (Jiang and Zhang, 2002). However, since APX gene expression induced by ABA occurred later than drought-induced transcript accumulation a role for ABA in drought-induced APX transcript accumulation is not supported. The role of other mechanisms, such as changes to photosynthesis, have to be considered (Chang *et al*., 2004).

In response to progressive drought (Fig. 6), as in the case of rapid water loss, variations in *VucAPX* and *VupAPX* transcript levels were more important in the drought-sensitive cultivar than in the drought-tolerant one. Concerning chloroplastic *APX*, two signals were visualized on the agarose gel, since *Vu* chloroplastic APX primers were chosen so as to include the 151-bp insertion found at the 3′ end of the stromatic isoform (Fig. 2). In EPACE-1, slight water stress strongly stimulated both chloroplastic APX gene expression (Fig. 6), in 1183, stimulation of the stromal isoform occurred much later, at severe water deficits. Although chloroAPX gene expression was weak compared with those of *VucAPX* and *VupAPX,* their role may be

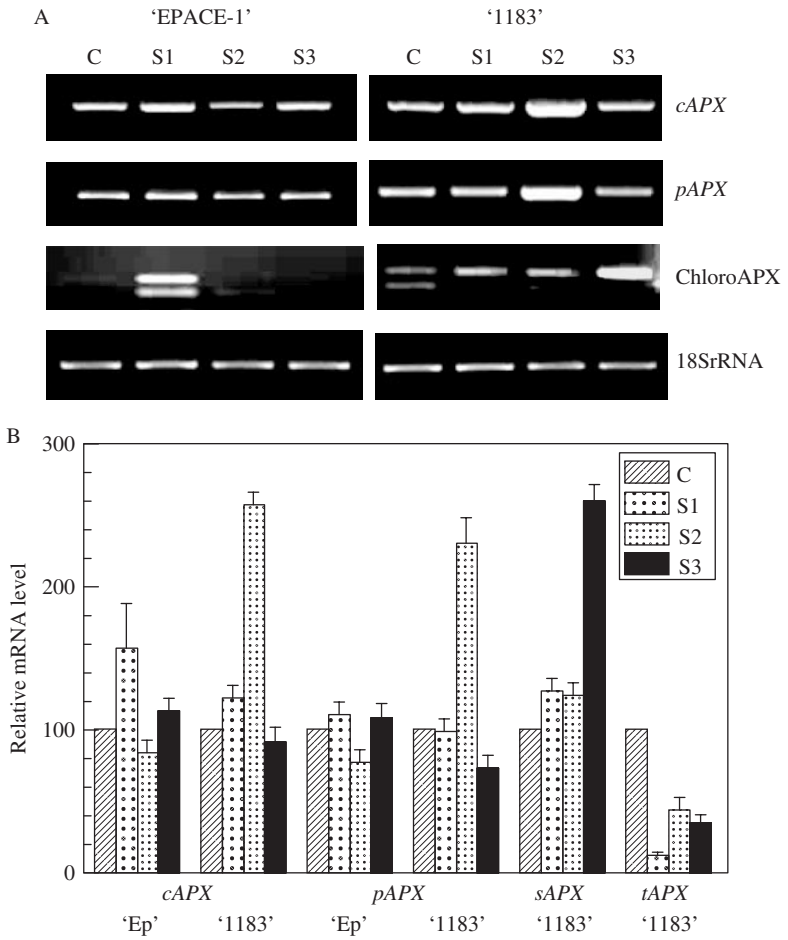

FIG. 6. Effect of progressive drought on mRNA abundance of *cAPX*, *pAPX*, chloroplastic APX (upper signals *sAPX*, lower signals *tAPX*), 18S rRNA in *Vigna unguiculata* 'EPACE-1' and '1183' leaves. Control plants C, $\psi_w = -0.5 \pm 0.1$ MPa; or droughted plants S1: $\psi_w = -1.0 \pm 0.1$ MPa; S2, $\psi_w = -1.5 \pm 0.2$MPa; S3, $\psi_w = -2.0 \pm 0.2$ MPa. (A) Gel analysis of APX isoenzyme transcripts. After RT–PCR carried out on 50 ng of total RNA with 30 cycles, in the case of *cAPX* and *pAPX* and 150 ng of tRNA and 35 cycles in the case of *tAPX* and *sAPX*, results were visualized using a UV light transilluminator. (B) Relative mRNA levels. The mRNA level of each sample was quantified with the Imager 1D/2D software and normalized to the respective 18S ribosomal RNA. The values represent the mean ± s.d. of three experiments.

important since they detoxify AOS at their production site. In transgenic tobacco plants, Yabuta *et al*. (2002) have shown that chloroAPX played an important role as the primary target of the AOS scavenging system and enabled leaf tissues to maintain the capacity of the water–water cycle and photosynthetic activity. However, cytosolic antioxidant proteins are also extremely important (Foyer and Noctor, 2003) as central components of AOS-scavenging gene network (Davletova *et al*., 2005). In the less-tolerant cowpea cultivar 1183, the stimulation of chloroplastic APX genes occurred later than for EPACE-1, but the plant was able to activate early the expression of genes coding for cytosolic isoforms. In fact, cowpea is a drought-tolerant species, compared with other cutivated plants, and even most sensitive cultivars are able to resist water deficits relatively well.

Regarding protective molecules against AOS, lipophilic extracts from EPACE-1 resisted photoperoxidation better than those from 1183 (Ferrari-Iliou *et al*., 1994). This phenomenon could be due partly to higher intrinsic levels of protective processes and/or to a better ability to accumulate protective molecules (d'Arcy-Lameta *et al.,* 1996; Chaudière and Ferrari-Iliou, 1999). This metabolic stability is important for plant survival.

CONCLUSIONS

When comparing gene expression patterns (Fig. 6) and enzymatic activity (Fig. 1), the present results suggest that subtle changes in the intracellular distribution of protective enzymes and different sensitivities to AOS exhibited by APX isozyme forms might be more important for protection than an overall increase in total enzyme activity. Adjustments of isozyme form affinities for their substrates may be the main strategy to increase the efficiency and the fine-tuning of the antioxidant system (Foyer *et al*., 1994).

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