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The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in *Arabidopsis thaliana* is seed-specific and related to dormancy

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Abstract

We have isolated a gene, *AtPer1*, from the dicotyledon *Arabidopsis thaliana*, which shows similarity to the 1-cysteine (1-Cys) peroxiredoxin family of antioxidants. In higher plants, members of this group of antioxidants have previously only been isolated from monocotyledons. It has been suggested that seed peroxiredoxins protect tissues from reactive oxygen species during desiccation and early imbibition and/or are involved in the maintenance of/protection during dormancy. *AtPer1* expression is restricted to seeds. Despite differences in seed development between monocots and dicots, *AtPer1* shows an expression pattern during seed development and germination similar to the dormancy-related transcript *Per1* in barley. *In situ* hybridization identifies *AtPer1* as the first aleurone-expressed transcript characterized in developing *Arabidopsis* seeds. The transcript is also expressed in the embryo. *AtPer1* expression in seeds is unaltered in an ABA-deficient mutant (*aba-1*) during seed development, while expression in seeds of an ABA-insensitive mutant (*abi3-1*) is reduced. The transcript is not induced in vegetative tissue in response to stress by ABA or drought. *AtPer1* transcript levels are correlated to germination frequencies of wildtype seeds, but *AtPer1* transcript abundance is not sufficient for expression of dormancy in non-dormant mutants. Hypotheses on peroxiredoxin function are discussed in view of the results presented here.

Introduction

To protect themselves against damage caused by free radicals, plants as other organisms possess an array of antioxidant systems. Peroxiredoxins are one of the most recently discovered types of enzymatic antioxidants [7, 9] and have been shown to be active upon substrates such as hydroperoxides and alkyl hydroperoxides [28, 40]. The removal of hydroxyl radicals has also been shown [40]. The first peroxiredoxin was isolated and characterized in yeast (TSA or thiol specific antioxidant [34]) and members of this family have since been shown to be present in organisms ranging from bacteria to mammals [7].

The peroxiredoxin family, members of which share considerable amino acid identity, can be divided into two main groups. One group (2-Cys), including the subgroups of thioredoxin peroxidase- and alkyl hydroperoxidase-like enzymes, contains two conserved cysteine residues (corresponding to Cys-47 and Cys-170 in yeast TSA), where the Cys-47 residue makes up the active site of the enzyme [8]. The second group (1-Cys), which has not been studied as extensively, possesses one conserved cysteine residue, corresponding to the Cys-47 active site in TSA. While different substrate specificities and mechanisms for regeneration of enzyme activity have been suggested for the different subgroups/groups [8], all peroxiredox-

The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y12089 (*AtPer1* gene).

ins are dependent upon the presence of thiol in one form or another for reactivation (reduction). Peroxiredoxins have been shown *in vitro* to protect DNA, membranes and certain enzymes against damage caused by thiyl or oxygen radicals. Stacy *et al.* [59] suggest that the members of the 1-Cys and 2-Cys groups play different roles in the organism, 2-Cys variants being general protectants and the 1-Cys variant performing more specific tasks.

A specific role in desiccation tolerance during the late stages of seed development has been suggested by our group for *Per1* [59], a barley 1-Cys peroxiredoxin whose expression is restricted to the aleurone layer and embryo of developing seeds [1]. An important aspect of desiccation tolerance is protection against free radicals [38]. And while several other antioxidant systems, including catalases, superoxide dismutases (SOD) and peroxidases, exist in the developing seed, most are not expected to play their main protective role in desiccated and resting seeds [6, 49, 53], but rather in the germinating seed. In contrast, the single-copy *Per1* gene is expressed only during mid/late seed development, in mature dry seeds and in the very earliest stages of imbibition before signs of germination are present. *Per1* is environmentally regulated in a manner similar to the *l*ate *e*mbryogenesis-*a*bundant (*Lea*) transcripts which encode putative dehydration protectants [3, 19]. In fact, *Per1* has been classified as an 'atypical' *Lea* gene [13].

The PER1 gene product is also implicated in either controlling dormancy or indicating conditions of dormancy [1, 59]. This is based on studies showing expression of the barley *Per1* peroxiredoxin to be positively correlated with dormancy, a correlation which has also been previously described for the *Per1* homologue in *Bromus secalinus*, pBS128 [22]. In addition to increased expression of *Per1* in imbibed dormant seeds, a transient upregulation is seen in imbibed nondormant seeds prior to germination [59], suggesting that the role of PER1 is not solely related to dormancy. We have previously shown PER1 to be functionally active, i.e. PER1 protects DNA against degradation, *in vitro* [59], in a thiol-MFO system [39, 40]. While PER1 may be performing a protective function in imbibed dormant seeds, a more speculative theory involves the removal of H_2O_2 *in planta*, thereby possibly preventing dormancy release [59].

To date plant members of the 2-Cys peroxiredoxin group have been isolated and characterized in barley and spinach (Hv-*bas1*, So-*bas1* [2]) as well as *Arabidopsis* (accession number X94218 [2]). Additionally, northern blot analyses have revealed transcripts hybridizing to *bas1* in numerous mono- and dicotyledons [2]. In higher plants, the 1-Cys gene has so far been isolated only from monocotyledons (barley [59]; brome grass [22]; rice, accession number D63917). We therefore undertook the task of isolating and characterizing the 1-Cys peroxiredoxin gene from the dicot *Arabidopsis thaliana*, in order to expand the studies begun on barley. *Arabidopsis*, an easily transformable model plant which exhibits seed dormancy, will provide an excellent system for functional studies, where the speculated roles in protection, dormancy and seed germination can be investigated further. This work describes sequence data and expression patterns of *AtPer1*, the *Per1* homologue. In addition, previously proposed hypotheses concerning the function of 1-Cys peroxiredoxins are discussed.

Materials and methods

Plant material

Arabidopsis thaliana ecotype C24 and Landsberg *erecta* wild-type and mutant lines *abi3-1* and *aba-1* [35, 36] were cultivated in growth chambers, 22 °C, 8 h dark/16 h light (100 $\mu E/m^2 \cdot s$). Staging of developing siliques was performed by tagging individual flowers at the day of pollination, which was set to the day the corollas appeared visible and the anthers were positioned above the stigma [48]. At different stages (DPA, days post anthesis), siliques were harvested for mRNA extraction. Seeds used for germination experiments were plated on dH_2O-soaked paper disks, and imbibed first at 4 °C for 24 h dark and then at 22 °C, 8 h dark/16 h light. Freshly harvested seeds were collected from the plants at 6 weeks after the onset of flowering. The afterripened seeds were kept at 4 °C for two weeks, then at room temperature for four weeks. Germination was scored when the root appeared visible. For northern experiments, 100 mg seeds, plated out in parallel with the germination experiments, were collected at regular intervals post imbibition. Seed germination tests on *abi3-1* and *aba-1* mutants [35, 36] were performed in order to confirm the lack of dormancy. The *aba-1* mutant is nondormant and germinates soon after the start of imbibition. The *abi3-1* mutant has reduced seed dormancy, and requires a longer imbibition time than *aba-1* to germinate. The mutants *aba-1* and *abi3-1* reached 100% and 97% seed germination respectively after 4 days. For freshly harvested Landsberg *erecta*

wild-type seeds, only 1.3% germination was measured. For cultivation of *Arabidopsis* plants, seeds were plated on and seedlings grown on paper discs soaked in MS2, i.e. MS [45] medium containing 20 g/l sucrose and 0.5 g/l 2-N-morpholino/etanesulfonic acid, KOH-regulated pH to 5.7. These seeds and seedlings were subjected to stress treatments with drought and 50 μM ABA. Exposure to water stress was achieved by removing the lids of the plates and allowing the plants to dry for the times indicated. ABA treatment was performed by adding 50 μM ABA (final concentration) to the media. Tissues for mRNA isolations were frozen in liquid N_2 and stored at -80 °C.

cDNA and genomic cloning

The *AtPer1* cDNA was cloned with RT-PCR on mRNA isolated from mature seeds of the C24 ecotype, using *AtPer1*-specific primers (see Figure 1). The primers (atper1D: 5′-GGA-ATTCGATCGGCGGTCTTGAAAC-3′ and atper1UB: 5′-CGGGATCCCTAGGAGACACGGTGCCG-3′) contain the cloning sites *Eco*RI and *Bam*HI, respectively, at their 5′ ends and were designed to fit two partial *Arabidopsis* cDNA sequences (accession numbers Z37278 and Z37279) found in the EST (expressed sequence tag) database, which show high similarity to the *Per1* sequence. A genomic *Arabidopsis* fragment, containing the *AtPer1* gene, was identified by screening a λ-GEM11 genomic *Arabidopsis* library as described [52], using as a probe a genomic *AtPer1* fragment amplified with the primers atper1D and atper1UB (see above). The nucleotide sequence of the isolated *AtPer1* gene (accession number Y12089) was determined by cyclic sequencing of both strands with a Thermo Sequenase core sequencing kit (Amersham), using a Vistra DNA Sequencer 725 and accompanying software.

DNA and RNA extraction

Genomic DNA was isolated from *Arabidopsis thaliana* rosette leaves by a modification of the method of [11]. Poly(A)$^+$ RNA was extracted from 100 mg N_2-frozen tissue using magnetic oligo d(T) dynabeads (Dynal AS) as described [29].

Southern and northern blot analyses

The Southern blot analysis was performed as described [51], on 10 μg DNA per lane, digested with different restriction enzymes. For the northern analyses, 0.5 μg purified mRNA was separated on a denaturing RNA gel, blotted and hybridized as described [20, 52].

DNA labeling and hybridization probes

Hybridization probes were labeled in a random priming reaction with biotinylated single-stranded template bound to magnetic streptavidin-coated beads (Dynabeads M280-streptavidin, Dynal AS) as described [14]. Probes include *AtPer1* (see above), *Rab18* (684 bp cDNA, pRABAT1 [42]) and DFR (1153 bp cDNA [56]). A CHS probe (1750 bp *Pst*I-fragment [15]) was generated in a standard random-priming reaction using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). A rDNA probe was used as a loading control, since we observe a constitutive rDNA expression pattern under the conditions used within each experiment (determined by comparing rDNA signal to hybridizations with a polyT oligonucleotide probe, not shown). In that rDNA shows high conservation between different organisms, we have used a 450 bp fragment from the 18S rDNA of *Pyropyxis rubra*, belonging to the Sclerotiniaceae, obtained by PCR according to [26], using specific primers (NS3: 5′-GCAAGTCTGGTGCCAGCAGCC-3′ and 18Sint3: 5′-CCGATCCCTAGTCGGCATAG-3′). The rDNA probe was generated using biotinylated single-stranded template bound to streptavidin coated beads in a specific priming reaction [58]. The *AtPer1*, *Rab18*, DFR and CHS probes were hybridized at 68 °C, the rDNA probe was hybridized at 57 °C. Quantification of expression levels was performed using a BioRad GS-250 Phosphor Imager and Molecular Analyst software (BioRad). Hybridization signals were scanned and *AtPer1* levels were adjusted for loading as determined by the rDNA expression level.

In situ *hybridization*

For *in situ* hybridization, digoxigenin-labeled sense and antisense RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim). Probes were transcribed from an *AtPer1* cDNA clone in the pBluescript SK− vector (Stratagene). Hybridization was performed as described [21] on tissue from plants of the Columbia ecotype.

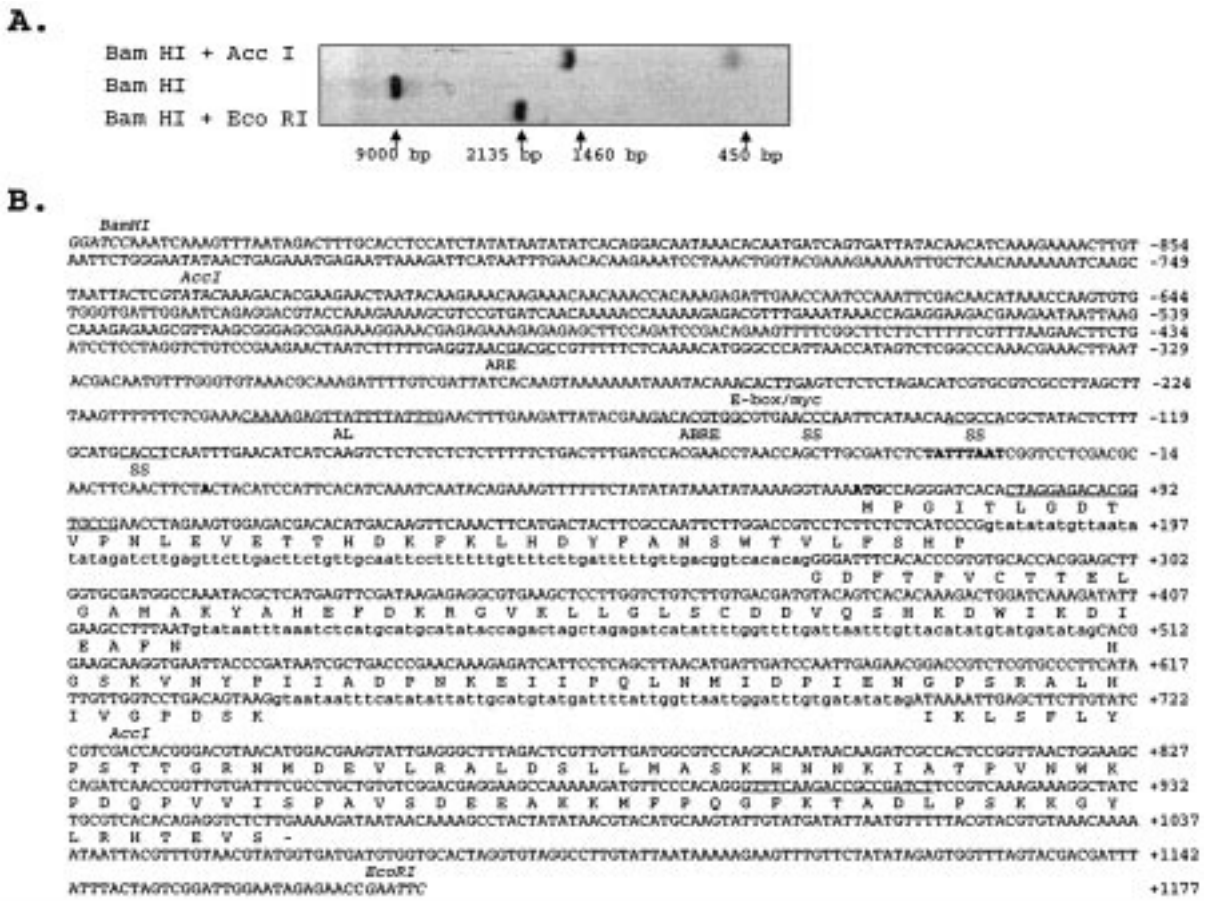

Figure 1. Southern analysis and *AtPer1* nucleotide sequence and alignment. A. Southern blot hybridization of genomic *Arabidopsis* DNA digested with *Bam*HI and double-digested with *Bam*HI-*Eco*RI and *Bam*HI-*Acc*I. The filter was probed with a PCR product from the coding region of *AtPer1*. Arrows indicate band sizes. A hybridization to genomic DNA digested with *Ava*I also gave a single band when probed with the *AtPer1* gene (not shown). Since the hybridization was carried out at high stringency, we cannot exclude the possibility that other bands could be detected at a lower stringency. B. Nucleotide and deduced amino acid sequence of *AtPer1*. Transcriptional initiation site and putative TATA-box are in bold. The ABRE element (−167), the antioxidant responsive element (ARE; −396), seed specific (E-box and SS) and aleurone elements (AL) are underlined. The sequences used as primers (nucleotides +80 to +97 and +894 to +912) to generate a gene specific probe are double underlined. Restriction sites for enzymes used in the Southern analysis are shown above the sequence, except for *Ava*I which cuts outside the borders of the subcloned fragment, and a second *Acc*I site which is mapped to just 3′ of the *Eco*RI site at +1177. The introns are shown in lower-case letters.

Results

The AtPer1 *sequence shows similarity to 1-Cys members of the peroxiredoxin antioxidant family*

The PER1 protein has been proposed to (1) take part in desiccation tolerance of developing seeds and/or (2) control or indicate conditions of dormancy [1, 59]. PER1 function is, however, not easily testable in barley, which so far is not routinely transformable. Since the seeds of the dicot *Arabidopsis* also exhibit dormancy as well as desiccation tolerance in late development, we chose to isolate the *Per1* homologue from this model plant. Screening of a genomic *Arabidopsis* library did, in fact, result in the isolation of a sequence showing high similarity to the barley *Per1* gene. This gene has been called *AtPer1*.

In preparation for gene isolation and characterization, a PCR product homologous to *Per1* was amplified from *Arabidopsis* genomic DNA. This product was generated with primers designed to fit an *Arabidopsis* EST clone (see Materials and methods), identified by a database search as the *Per1* homologue. The same PCR product was used as a probe in a Southern blot analysis

Figure 2. Alignment of some translated peroxiredoxin sequences which show similarity to ATPER1. Sequences are from *Arabidopsis* (atper1; atbas1, accession number X94218), rice (osrab24p, accession number D63917), barley (hvb15c, [1]), human (hsorf06, accession number D14662), *Onchocerca volvulus* (ov31052, accession number U31052), *Methanobacterium thermoautotrophicum* (orfk, accession number X74264) and yeast (ysctsao, [9]). A consensus is shown for the first six sequences, all belonging to the 1-Cys group. Below the consensus, examples from the 2-Cys group (atbas1 and ysctsao) are shown. ATBAS1 has an amino terminal extension of 34 residues (represented by *) also found in other plant 2-Cys genes [2]. Conserved residues and synonymous substitutions are in upper case.

(Figure 1A) in order to chose appropriate cloning sites and to examine gene copy number. Single hybridizing bands were observed for all enzymes used, except *Acc*I, which was later shown by sequence analysis to cut in the coding region. Screening of the genomic library using this *AtPer1* probe resulted in about 70 positives. PCR with specific *AtPer1* primers performed on 18 positive plaques from the secondary screening all gave products of the same size. Restriction mapping of eight positives produced a 2100 bp *Bam*HI-*Eco*RI hybridizing fragment in each. This fragment was subcloned from one of the positives into pBluescript for further analysis. Figure 1B shows the entire *AtPer1* sequence obtained from the subclone, including 958 bp of the promoter region. A primer extension analysis on seed mRNA, which produced only one major extension product (not shown), identified the initiation site of transcription to be at position −64 from start of translation. The promoter was examined for regions showing similarities to known promoter elements. The putative TATA box is positioned at −34 from transcription start. Sequences resembling the ARE (antioxidant response element, [23, 31, 51]), ABRE (ABA response element, [24]), seed-specific enhancer elements (E-box, see [33]; and AA/G/CCCCA [10]) and an element for aleurone-specific expression [37], are indicated in Figure 1B. Sequence comparison of an *AtPer1* cDNA clone (see Materials and methods) to the genomic clone allowed the identification of introns. The *AtPer1* gene possesses three introns of 80 bp, 89 bp and 60 bp (Figure 1B). The position of introns I

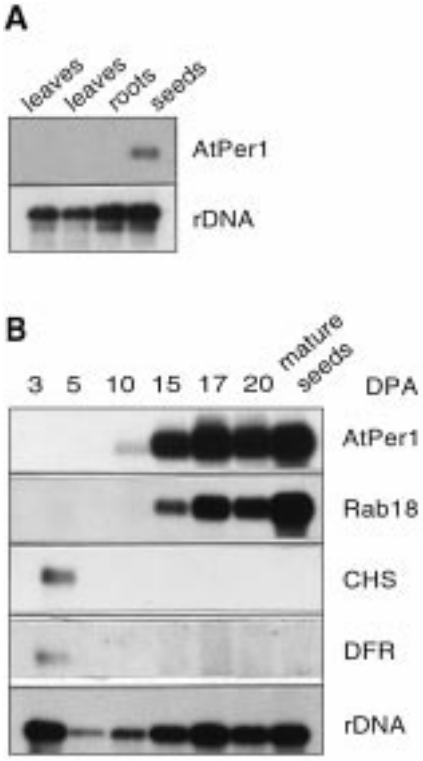

Figure 3. Expression pattern of the *AtPer1* transcript in different tissues and during seed development. A. Poly(A)$^+$ RNA was extracted from leaves, roots and dry seeds. B. Poly(A)$^+$ RNA was extracted from siliques harvested at different stages of development. Filters were hybridized to *AtPer1*, *Rab18*, CHS, DFR and to rDNA as a loading control (see Materials and methods). The rDNA probe indicates underloading at 5 and 10 DPA in B.

and II corresponds to those of barley (*Per1*; accession number X96551), intron III is absent in barley.

As expected, a comparison of the translated *AtPer1* sequence to other peroxiredoxins shows high amino acid identity to members of the 1-Cys group, with the highest identity, 74.2%, to the translated cDNA sequence PER1 (hvb15c in Figure 2). The highest identity to a non-plant sequence was 51.2%, to a translated cDNA from human (hsorf06). For comparison, the amino acid sequence of the *Arabidopsis* 2-Cys peroxiredoxin (atbas1) showed an identity of only 30.3% to ATPER1. The Pileup of ATPER1 and representatives from the 1-Cys group of the peroxiredoxin family (Figure 2) shows sequence conservation around the cysteine residue, Cys-46 in ATPER1, which corresponds to the catalytic site, Cys-47 in TSA (ysctsao [8]).

AtPer1 *expression is seed-specific and increases dramatically late in seed development*

Expression of *Per1* and its homologue in brome grass, pBS128, is restricted to seeds [1, 22]. To investigate the spatial expression pattern of *AtPer1* in *Arabidopsis*, mRNA from various parts of the plant, leaves, roots, and mature seeds were extracted. *AtPer1* transcripts were detected in seeds but not in leaves and roots (Figure 3A), revealing seed specific expression. This expression pattern was further confirmed using *in situ* hybridization (see below). To characterize the temporal expression pattern of *AtPer1*, transcript accumulation during seed development was examined (Figure 3B). Expression is first detected at 10 DPA. From 15 DPA and through the maturation and desiccation phases as well as in the mature quiescent seed, a high transcript level is maintained (Figure 3B). This expression pattern is in agreement with that shown for the *AtPer1* homologue in barley, *Per1* [1]. As a staging control, the blot shown in Figure 3B was hybridized to *Rab18*, chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR). Hybridizations with these control probes are in agreement with data from Landsberg *erecta*, *Rab18* is first detected at 15 DPA, while hybridizations of CHS and DFR, both expressed only in the first stages of seed development, show no expression as *AtPer1* accumulates.

AtPer1 *is expressed only in the embryo and a thin tissue layer surrounding the embryo of developing seeds*

In barley, *Per1* is expressed only in the embryo and aleurone layer. In order to determine whether the *Arabidopsis AtPer1* gene is expressed in a similar tissue-specific manner, *in situ* hybridization was performed on developing and mature siliques and seeds. Results using an antisense probe hybridized to developing siliques measuring 10 mm in length show accumulation of *AtPer1* mRNA to be restricted to specific tissues (Figure 4A). While purple staining shows that expression is apparent throughout the embryo, the hybridization signal is much stronger in the vascular tissue of the hypocotyl and cotyledons and in the epidermal cells. No signal is observed in the maternal silique tissue (see arrows, Figure 4A), seeds of developing siliques measuring 5 mm in length (not shown) nor in the sense control (Figure 4D). The expression pattern for seeds from yellowing siliques (Figure 4B) is very similar to that of developing siliques. However,

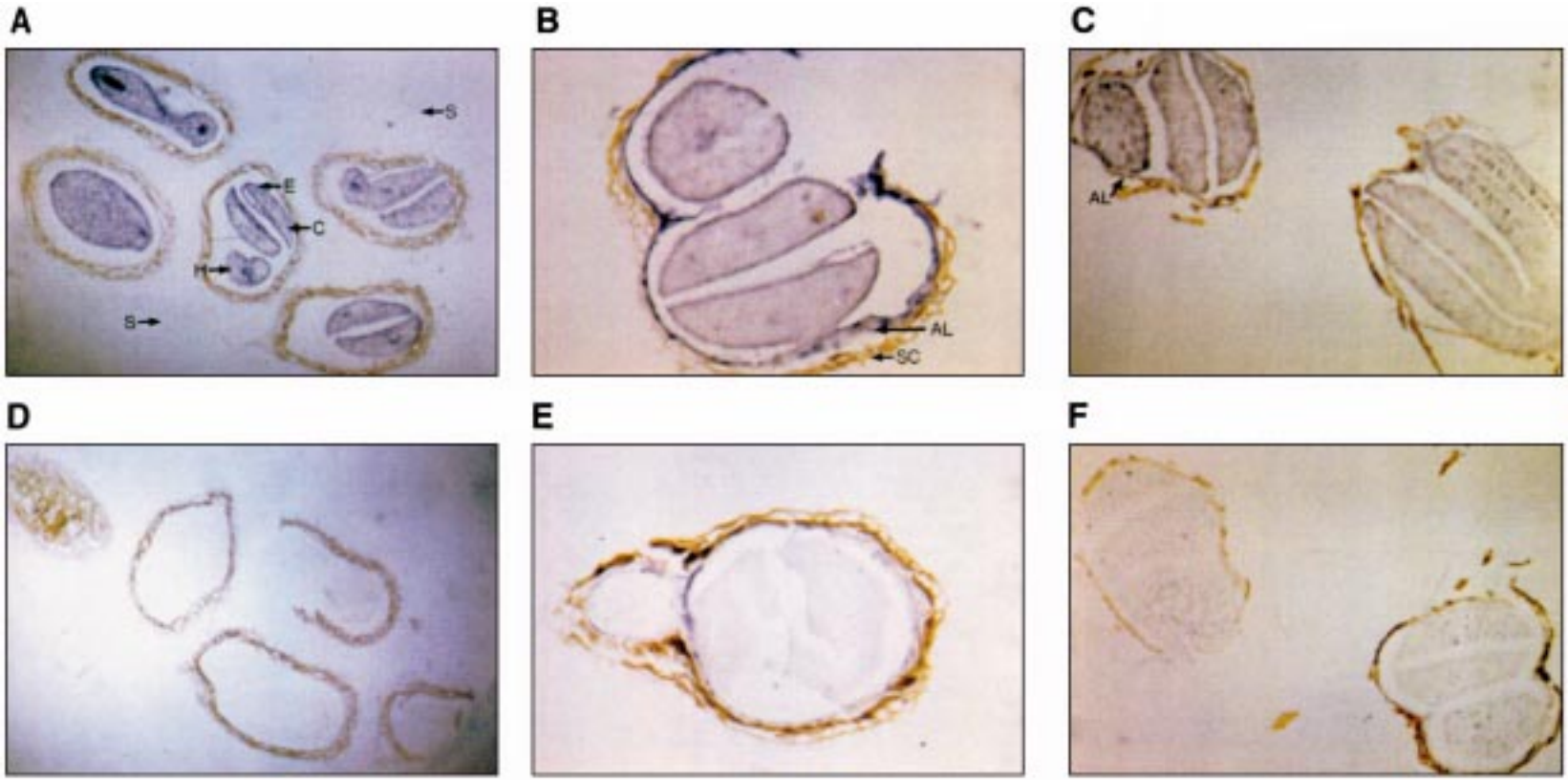

Figure 4. Localization of *AtPer1* mRNA in *Arabidopsis* seeds. A. *In situ* hybridization of longitudinal paraffin-embedded sections of 10 mm long immature silique, hybridized with digoxigenin-labeled antisense *AtPer1* and viewed under bright field, which gives a purple label. B. Localization in seeds from yellowing siliques. C. Mature dry seeds. Controls (D, E, F) were hybridized with an *AtPer1* sense probe. Abbreviations: S, silique; SC, seed coat; C, cotyledon; E, epidermis; H, hypocotyl; AL, aleurone. Magnification $\times 45$ (A, D) or $\times 90$ in (B, C, E and F).

AtPer1 expression is also apparent in a thin tissue layer, which presumably is the aleurone layer, surrounding the embryo and attached to the brown seed coat (see arrow, AL). While a slight dark purple stain is observed in the sense control (Figure 3E), the lighter purple stain indicating *AtPer1* expression in this layer is present only in the antisense hybridization. In mature dry *Arabidopsis* seeds, expression is more evenly distributed throughout the embryo, with only a slightly stronger signal in the outermost epidermal cell layer (Figure 4C). Again, staining is observed in the aleurone layer attached to the seed coat (arrow, AL). No staining is seen for the sense control (Figure 4F). *In situ* hybridization was simultaneously performed on preparations of tissue from leaves, roots, stems, and flowers. No *AtPer1* signal was observed in these tissues (not shown).

AtPer1 *is not induced in vegetative tissue*

Since *AtPer1* shows similar expression patterns during seed development as *Lea* genes, we tested whether *AtPer1* is stress-induced in vegetative tissue, as are many of the *Lea* genes [12, 57]. Seeds and seedlings were exposed to ABA and drought. As expected, northern analysis showed *AtPer1* expression at high levels in quiescent seeds, while the transcript disappears in germinating seeds and is not detected in seedlings. Seeds plated on MS2 medium containing 50 μM ABA did not germinate due to ABA repression [36], and the *AtPer1* transcript remained at a high steady state level. In seedlings, however, neither ABA nor drought could up-regulate *AtPer1* expression (Figure 5). The efficiency of the stress treatments were controlled using the *Lea* gene *Rab18*, which is normally expressed in seeds but can be induced in vegetative tissue by ABA or drought [42]. This control showed the expected expression pattern, being induced by the stress treatments in the vegetative tissue but not expressed in unstressed tissue.

AtPer1 *expression correlates with germination frequencies of freshly harvested (dormant) and afterripened (non-dormant) seeds*

Expression of the *Per1* peroxiredoxin and its homologue in brome grass (pBS128) have been correlated with degree of dormancy [22, 59]. To investigate if the *AtPer1* transcript levels can be correlated to germination frequencies as well, the expression levels have been examined in afterripened (nondormant) and

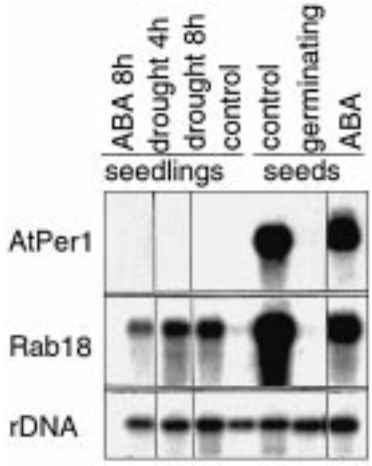

Figure 5. Effect of drought and ABA stress on the expression of *AtPer1* and *Rab18* genes in seeds and seedlings. Poly(A)$^+$ RNA was extracted from untreated seedlings (control) and seedlings treated with 50 μM ABA for 8 h, or drought 4 h and 8 h, as well as from seeds incubated with 50 μM ABA, germinating seeds and dry seeds (control). The blot was hybridized to *AtPer1* and *Rab18*. Hybridization to rDNA was used as a loading control.

freshly harvested (dormant) seeds. This study was run in parallel with seed germination tests. All seeds (fresh and afterripened) were first imbibed for 24 h at 4 °C, and thereafter imbibed at 22 °C. Afterripened C24 seeds began showing signs of germination after 54 h of imbibition and over 90% germination was measured at 196 h imbibition (Figure 6A). A germination frequency of only 5% was measured for freshly harvested seeds after 196 h imbibition. This low germination frequency indicates that the C24 ecotype possesses a strong degree of dormancy when the seeds are harvested fresh from the plant.

A northern analysis (Figure 6B) of imbibed samples harvested at appropriate intervals shows a reduction in *AtPer1* expression in afterripened seeds at the onset of germination (48–60 h), and expression decreases dramatically with length of imbibition. The *AtPer1* steady-state level in freshly harvested seeds remains relatively stable even after 120 h imbibition, and correlates with a low germination frequency.

Arabidopsis abi3-1 *and* aba-1 *mutants are not dormant but express* AtPer1

Comparisons of freshly harvested and afterripened C24 seeds show that *AtPer1* expression is dormancy-related. Therefore we wanted to extend our investigation of *AtPer1* expression and dormancy to *Arabidop-*

sis mutants, *aba-1* [35] and *abi3-1* [36], with reduced dormancy. Expression levels of *AtPer1* were examined in mature freshly harvested seeds from Landsberg *erecta* wild-type, and Landsberg *erecta abi3-1* and *aba-1* plants (Figure 7). The *AtPer1* expression level in wild-type and *aba-1* mutant seeds is approximately similar (only a 3% difference), as determined by quantification of *AtPer1* levels compared to rDNA levels (see Materials and methods). The *abi3-1* mutant, on the other hand, shows a significantly lower expression level, only about 10% of wild type. This is in contrast to *Rab18* (Figure 7), for which an expression level comparable to wild type is seen for both mutants.

Discussion

AtPer1 *homology to 1-Cys peroxiredoxins*

We have isolated and characterized a gene, *AtPer1*, from the dicotyledon *Arabidopsis thaliana* which shows high homology to the 1-Cys subgroup of the peroxiredoxin family of antioxidants. We have previously shown the closely related barley PER1 peroxiredoxin to possess antioxidant activity [59] and recently *in vitro* antioxidant activity has been demonstrated for the 1-Cys human homologue, HSORF06 [46]. Antioxidant activity has also been shown for several 2-Cys peroxiredoxins (TSA from yeast [34]; AHPC from *Salmonella* [28]; HRPRP from human red blood cells [39]). Based on the antioxidant activity described for both 1-Cys and 2-Cys peroxiredoxins from organisms ranging from plants to humans to yeast, we feel it reasonable to assume that the *Arabidopsis* protein possesses antioxidant activity as well. Primer extension (not shown), library screening, sequencing, and genomic Southern data indicate that *AtPer1* is a single-copy gene, as was shown for the homologous barley gene *Per1*. This indicates a difference between 1-Cys peroxiredoxins and most other antioxidants, in that these are usually encoded by gene families [18, 50, 53].

Tissue-specific expression of AtPer1 *in the seed*

Our northern data and *in situ* results show that *AtPer1* expression is restricted to the seed in a manner similar to *Per1* in barley [1, 59]. Expression is first detected in the embryo at approximately the bent cotyledon stage, and the transition to the maturation phase [5]. Embryo expression persists throughout seed development and *AtPer1* transcripts are detectable in mature embryos as well.

In situ experiments show expression of *AtPer1* in the embryo to be most prominent in the vascular and epidermal tissues. It is conceivable that a greater protection against desiccation-induced free-radical damage is necessary in these tissues. Interestingly, *in situ* hybridizations performed on barley embryos show *Per1* to be expressed strongest in shoot and root primordia [1]. Shoot and root primordia are however absent in the developing and mature *Arabidopsis* embryo [41], in contrast to the monocot barley embryo, where several true leaf primordia and roots are already formed during seed development [44].

In the later stages of development, *AtPer1* expression is not only observed in the embryo, but also in a layer attached to the inside of the seed coat. This layer has been described by others [4, 43, 60] as the aleurone layer, but a debate continues concerning the origin of this layer in crucifers. Vaughan and Whitehouse [60] argue that the aleurone is a thin, well-formed layer of remaining endosperm cells. On the other hand, investigations of *Sinapis alba* indicate that this layer of storage cells is derived from a particular cell layer within the inner integument of the seed coat, and therefore is of maternal origin [4]. *AtPer1* is the first transcript shown to accumulate in the aleurone layer of developing *Arabidopsis* seeds. The lipoxygenase transcript *LOX1* has, however, been shown previously to display a transient accumulation in the aleurone layer of germinating seeds [43]. The temporal accumulation pattern for *AtPer1* in the aleurone compared to the embryo is slightly different. Accumulation of *AtPer1* in the aleurone layer is not apparent until after expression in the embryo is detected (e.g. yellowing siliques compared to 10 mm immature siliques, Figure 4). The *AtPer1* transcript is maintained in the mature quiescent seed during storage, and disappears during germination, similar to the *Per1* expression pattern in barley [1].

LeaA-*like expression of* AtPer1

LeaA transcripts are characterized by their accumulation in the maturation and postabcission phases of development, and their presence in the mature quiescent seed [27]. The accumulation of *AtPer1* during seed development is similar to the pattern characteristic of the *LeaA* class of transcripts in *Arabidopsis*, which has been examined in detail for the ecotype Landsberg *erecta* [48]. Hybridizations we have performed with

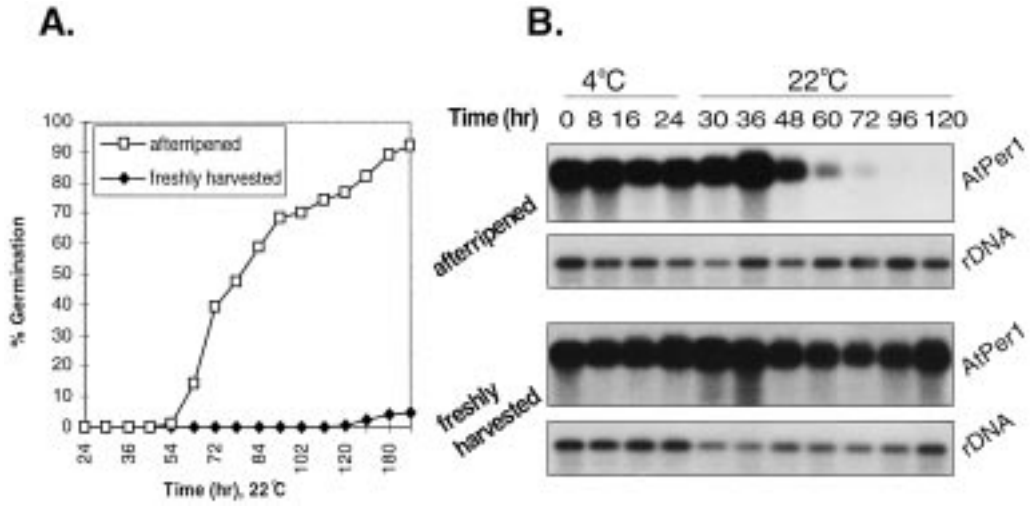

Figure 6. Germination frequencies and *AtPer1* expression in afterripened and freshly harvested C24 seeds. A. Seed germination frequencies for freshly harvested and afterripened *Arabidopsis thaliana* C24 seeds. The seeds were first imbibed at 4 °C for 24 h and thereafter at 22 °C for the times indicated. B. Northern analysis of poly $(A)^+$ mRNA extracted from afterripened and freshly harvested seeds which have been initially imbibed at 4 °C for the times indicated (0–24 h) and subsequently at 22 °C. Probes used were *AtPer1* and rDNA as a loading control.

markers used for developmental staging of the C24 ecotype (CHS, DFR, *Rab18*, Figure 3) show the same time course of expression for these genes in C24 as for Landsberg *erecta*. The first detectable expression of *LeaA* genes, e.g. *Rab 18*, in *Arabidopsis* occurs at approximately 13 DPA [48]. Based on our Northern and *in situ* data, we predict the onset of *AtPer1* expression to be between 6–10 DPA.

While *AtPer1* is maintained in mature imbibed seeds treated with ABA, the transcript cannot be induced by ABA in vegetative tissue. This is consistent with the expression patterns for the *AtPer1* homologous transcripts *Per1* and pBS128 [1, 22, 59], yet is in contrast to results for *Rab18*. Also, our studies show that *AtPer1* is maintained in the *aba-1* mutant but has a reduced expression level in the *abi3-1* mutant. This is divergent from the transcript accumulation pattern of *Rab18* which has a similar expression level in both mutants compared to wild-type plants.

The antioxidant function for AtPer1 *in seeds*

During the desiccation stage of seed development and in the dry resting seed, protection of living tissue against oxidative damage is required [38]. Due to the antioxidant nature of *Per1*, and under the assumption that the accumulating mRNA is translated, Stacy *et al.* [59] hypothesized that one possible role for the *Per1* peroxiredoxin in barley could be to protect against free

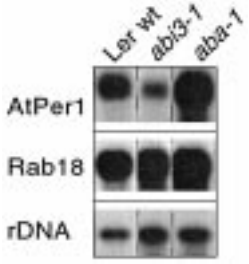

Figure 7. *AtPer1* expression in Landsberg *erecta* wild type (wt) and mutants *abi3-1* and *aba-1*. Poly$(A)^+$ RNA was extracted from mature freshly harvested seeds. Probes used were *AtPer1* and *Rab18* as well as the rDNA control.

radical damage in the two tissues which survive desiccation, the embryo and the aleurone. Since the *Arabidopsis AtPer1* gene is expressed to high levels during seed desiccation and maturation in the embryo and aleurone layer, we assume that *AtPer1* could perform this same antioxidant function as *Per1*. This theory is supported by the identification of a transcript from star moss (rehydrin, accession number U40818), which shows high homology to 1-Cys peroxiredoxins and is upregulated by rehydration of rapidly dried gametophyte tissue [55].

The seed specificity shown by 1-Cys peroxiredoxins is in striking contrast to what is seen for other antioxidants, which are most often not seed specific [18, 47, 50, 54, 63]. While catalases (mainly CAT-1) are also present in seeds, they are expressed to high levels

during germination (CAT-1 and CAT-2) and are present in some mature tissues [54]. SOD activity and isozyme patterns are found to be similar in all tissues and organs of *Arabidopsis* examined and the activity is stable during development [47], a pattern of expression consistent with a general protective function. While these antioxidants are more or less constitutively expressed or increase after signs of germination are evident, respiration in mature seeds is resumed already at the onset of imbibition. At this time the peroxiredoxin *AtPer1* is already expressed to a high level and could protect against activated oxygen species in this situation as well as during the desiccation stage.

During imbibition of dormant barley and brome grass seeds, transcript levels of the *AtPer1* homologues *Per1* and pBS128 increase dramatically [22, 59]. While this same dramatic increase in not observed for *AtPer1*, high *AtPer1* transcript levels are maintained during imbibition, significantly longer than in non-dormant seeds. We feel this expression pattern is still in agreement with the role suggested for the 1-Cys peroxiredoxins in dormancy [59], namely protection against reactive oxygen species arising as by-products of respiration during imbibition of dormant seeds. In this situation, the non-germinating seeds are unable to gain protection from germination-specific antioxidants. It is also interesting to note that at the onset of imbibition, approximately similar *AtPer1* transcript levels are present in afterripened and freshly harvested seeds in relation to rDNA levels. This implies that the transcript is stable during afterripening.

AtPer1 *is dormancy-related but not sufficient for expression of dormancy*

A second, more speculative role proposed for the *Per1* peroxiredoxin is involvement in maintenance of dormancy [59]. Our investigations of *AtPer1* in non-dormant *abi3-1* and *aba-1* mutants show the same expression pattern as the *Lea* gene *D19h* [16] and the seed storage protein cruciferin [17]. Seeds from *abi3-1* plants have reduced accumulation of the *AtPer1* transcript, about 1/10 of that measured in wild-type seeds. The *aba-1* mutant seeds accumulate about the same level of the *AtPer1* transcript as wild-type seeds. The endogenous ABA level rises during seed development and peaks at about 10 DPA [32]. This rise correlates well with the onset of *AtPer1* expression in wild-type seeds, making it a plausible signal for regulation of the *AtPer1* gene. However, equivalent *AtPer1* transcript levels in the ABA-defective mutant and wild-type plants, reveals that endogenous ABA is not the essential signal for expression of *AtPer1*, as has been seen for other *Lea* transcripts [48].

It is possible that *AtPer1* expression is mainly controlled by an endogenous ABA-independent stage-specific pathway, but may also be induced by an ABA-dependent pathway. It has been argued that ABI3 interacts with both ABA-dependent and independent pathways, regulating gene expression during seed development [48]. Since the *AtPer1* transcript level is not affected in the ABA-deficient mutant, it is likely that the reduced level in the *abi3-1* mutant reflects the involvement of ABI3 in a regulatory pathway governed by ABA-independent developmental signals.

Earlier studies using ABA-deficient or ABA-insensitive mutants of *Arabidopsis* and other plant species have shown that ABA is required for induction of seed dormancy [25, 32, 61]. ABA levels have not been investigated in dormant and non-dormant *Arabidopsis* seeds, but investigations in barley have shown that inability of dormant grains to germinate was due to both a higher endogenous ABA level, caused by higher *de novo* ABA synthesis, and a higher ABA sensitivity as compared to non-dormant grains [62]. Results with the *aba-1* mutant show that a high *AtPer1* level is not sufficient for expression of dormancy, and supports the proposal that dormancy expression is ABA-dependent. Presently, analyses of transgenic *Arabidopsis* plants which overexpress PER1 and express the antisensed *AtPer1* gene are under investigation. This work, and studies of peroxiredoxin protein levels in seeds could help determine the ultimate function of this gene during desiccation and dormancy.

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Note added in proof

Recently it has been shown that *E. coli* over-expressing the 2-Cys perixiredoxin BAS1 exhibit increased tolerance for alkyl hydroperoxides *in vivo* (Baier M. and Dietz K.-J., 1997, Plant J. 12(1): 179–190).

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