Peroxiredoxin antioxidants in seed physiology

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Abstract

Peroxiredoxins are thiol-requiring antioxidants found in organisms ranging from bacteria to humans. They can be divided into two subgroups with either one or two conserved cysteine residues. In plants, 1-Cys peroxiredoxins have been identified in a number of grasses and cereals, and in the dicotyledonous species *Arabidopsis thaliana*. In contrast to other antioxidants, the 1-Cys peroxiredoxin genes are expressed solely in seeds, and only in the parts of the seeds surviving desiccation, i.e. the embryo and the aleurone layer. The expression pattern is characteristic of late embryogenesis-abundant genes. The PER1 protein of barley is present in high concentrations in the nucleus at the onset of desiccation. 1-Cys genes are expressed in a dormancy-related manner in mature seeds, in that transcript levels are high in imbibed dormant seeds, but disappear upon germination of their non-dormant counterparts. 1-Cys transcript levels can be up-regulated by ABA and osmotic stresses and suppressed by gibberellic acid. Two hypotheses have been put forward on the function of 1-Cys peroxiredoxins in seed physiology. First, these proteins might protect macromolecules of embryo and aleurone cells against damaging reactive oxygen species during seed desiccation and early imbibition. And second, seed peroxiredoxins might play a role in the maintenance of dormancy. These hypotheses are discussed, taking into account present knowledge of the biochemistry and molecular biology of peroxiredoxins.

Keywords: *AtPer1*, desiccation tolerance, peroxidoxin, thiol-specific antioxidant (TSA), thioredoxin peroxidase (TPx), subcellular localization

Introduction

Desiccation tolerance and dormancy are two crucial and remarkable features of seeds. In contrast to the vegetative tissue of the majority of vascular plants, seeds commonly survive massive water loss. They can persist in a dry state of metabolic quiescence for weeks, months or even years. Seeds of diverse species are dormant for a period after they have been shed from the mother plant. This state is characterized by the inability to germinate under otherwise supportive conditions (Bewley, 1997). Like non-dormant seeds, dormant seeds resume respiration when imbibed (phase I and II of germination), but they will not enter the third phase of germination characterized by elongation of the radicle and a second respiratory burst (see Bewley and Black, 1994).

Preservation of macromolecules and cell structures through desiccation, dormancy and rehydration is required for the development of a vital plant from the germinating seed. One important aspect of desiccation tolerance is the protection against potentially damaging reactive oxygen species (ROS, Leprince *et al.*, 1994). Both desiccation and resumption of respiration during hydration give rise to ROS, like hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{-\cdot}$) and hydroxyl radicals ($HO\cdot$). Such species can generate secondary products such as alkyl hydroperoxides and lipid peroxides. When not removed or neutralized, ROS may have deleterious effects on membranes, proteins and DNA. A central issue in understanding the molecular biology of desiccation tolerance is to identify its protective mechanisms.

Considering the importance of germination in a plant's life cycle, it is disconcerting that little is known about the molecular mechanisms involved in establishment and termination of dormancy (Bewley, 1997; Li and Foley, 1997). However, numerous lines of evidence indicate that abscisic acid (ABA) is involved in the control of dormancy in most seeds. ABA-deficient or –insensitive mutants are non-dormant (see Bewley, 1997). These mutants show changes in patterns of gene expression during seed development and can be desiccation intolerant. So far, genes that specifically control and regulate dormancy have not been cloned. However, reduced dormancy (*rdo*) mutants with normal ABA-synthesis and -response have been isolated in *Arabidopsis* (Léon-Kloosterziel *et al.*, 1996).

Identification and functional characterization of desiccation- and dormancy-related seed proteins can be

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expected to have a large impact on future selection for or modulation of important seed properties. A number of transcripts have been identified that have high steady state levels in dormant imbibed seeds, but rapidly disappear upon germination of non-dormant seeds. Many of these transcripts encode LEA (Late Embryogenesis Abundant) proteins speculated to play a protective role during seed desiccation (see Li and Foley, 1997). This paper will review seed peroxiredoxin antioxidants, which have a dormancy-related and *lea*-like expression pattern. Prior to a discussion of these seed enzymes, a review of the history and biochemistry of peroxiredoxin antioxidants is presented.

Peroxiredoxins

Peroxiredoxins (Prx) are thiol-dependent antioxidants, first identified in yeast (thiol-specific antioxidant, TSA, Kim *et al*., 1988) and later shown to be present in organisms ranging from bacteria to humans (Chae *et al*., 1994a). Prx enzymes do not contain any tightly bound metal ions. They have no amino acid sequence similarity with other enzymatic antioxidants like catalases, peroxidases or superoxide dismutases. The Prx family can be divided into two main groups, one with proteins containing two conserved cysteine residues (Cys-47 and Cys-170 in TSA), and another with only the Cys-47 conserved (Figs 1 and 2). There is sequence similarity along the whole polypeptide chain with the longest stretch of highly conserved residues around Cys-47 in both the 1-Cys proteins (DFT*P*V**C***T*TE) and in the 2-Cys proteins (DFT*F*V**C***P*TE). The amino acid similarity is higher amongst 1-Cys proteins from different species than between 1- and 2-Cys Prx of the same species (Fig. 2). This strongly suggests that a gene duplication event, leading to these two forms of Prx, took place prior to the evolution of eukaryotes.

Biochemical properties

In vitro Prx can protect enzymes, lipids and DNA against radical attack in oxidation systems containing thiol groups (Kim *et al*., 1988; Lim *et al*., 1993, 1994; Netto *et al*., 1996). For plants, Prx antioxidant activity has been demonstrated for the 2-Cys barley BAS1 (Baier and Dietz, 1997) and the 1-Cys barley PER1 (Stacy *et al*., 1996). Of the 1-Cys homologues, antioxidant activity has also been shown for some mammalian proteins (Shichi and Demar, 1990; Netto *et al*., 1996; Peshenko *et al*., 1996). Prx can reduce H_2O_2, alkyl hydroperoxides and hydroxyl radicals (e.g. Chae *et al*., 1994b; Lim *et al*., 1993; Netto *et al*., 1996; Baier and Dietz, 1997).

Mutational studies of TSA identified Cys-47 as essential for antioxidant activity (Chae *et al*., 1994b). Cys-170 makes dimer formation possible through Cys-47 to Cys-170 disulphide bridges (Chae *et al*., 1994c; Fig. 3A). There are no indications of dimer formation for the 1-Cys proteins. In a human 1-Cys protein, biochemical evidence exists for the creation of an intramolecular disulphide bridge between the conserved Cys-47 and an additional cysteine present (Cys-91; Kang *et al*., 1998; Fig. 1, dark grey circle). No cysteine is found in this latter position in the plant 1-Cys Prxs. However, these proteins and the nematode protein OVPRX1 (ac. no. U31052) do have a cysteine at position 76 (Fig. 1, horizontal hatched).

The 2-Cys Prxs can be divided into two subgroups according to their electron transfer regeneration systems. The *Salmonella typhimurium* and *Escherichia coli* homologues to TSA, AhpC, make up an alkyl hydroperoxide reductase when complexed with another component (AhpF), which contains FAD as a co-factor (Jacobson *et al*., 1989, Fig. 3A). AhpC is involved in reduction of alkyl hydroperoxide substrates to their corresponding alcohols, and re-reduction of AphC is exerted by AhpF coupled to NAD(P)H (Jacobson *et al*., 1989). In yeast, the antioxidant activity of TSA was shown to be supported by a thioredoxin system consisting of thioredoxin, thioredoxin reductase and NAD(P)H (Chae *et al*., 1994b; Kwon *et al*., 1994; Fig. 3A). TSA was consequently renamed thioredoxin peroxidase (TPx). Thus TSA is not thiol-specific as originally suggested (Yim *et al*., 1994), but rather dependent on reduction of disulphide by thiol.

Figure 1. Alignment of 1-Cys peroxiredoxin amino acid sequences using Pileup (Genetics Computer Group sequence analysis software). Dark grey – conserved cysteine-47; medium grey – residues identical or similar in more than 50% of the sequences; light grey – additional residues identical or similar at least in all higher plant sequences; cross hatched – the GXSXG lipase motif; horizontal hatched –second cysteine residue conserved in all higher plant sequences; dark grey circle – second cysteine residue possibly involved in intramolecular disulphide bridging in human 1-Cys protein; circle – cysteine-170 conserved in 2-Cys Prx. SmAHR, *Sulfolobus metallicus* alkyl hydroperoxide reductase, AF007757 and U32479; Mtorfk, open reading frame k in *Methanobacterium thermoautotrophicum*, X74264; Ca1Cys, *Candida albicans* cosmid clone AL033396; ScYBL, *Saccharomyces cerevisiae*, Z35825; PER1, barley, X76605 and X96551; pBS128, *Bromus secalinus*, X63202; OsRab24p, rice, D63917; AtPER1, *Arabidopsis thaliana*, Y12089; Tr155, *Tortula ruralis*, U40818; Bt1Cys, bovin, AF090194 non-selenium glutathione phospholipid hydroperoxide peroxidase, and AF080228 glutathione peroxidase; Hsorf06, human, D14662; Mm1Cys, mouse, AF004670, and Y12883 non-selenium glutathione peroxidase; Rn1Cys, rat, Y17295, and AF014009 acidic calcium-independent phospholipase A_2; Di1Cys, *Dirofilaria immitis*, AF027387; Ov1Cys, *Onchocerca volvulus*, U31052; Dm1Cys, *Drosophila melanogaster*, P1-clone, AC004154; and AtBAS1, *Arabidopsis thaliana* 2-Cys peroxiredoxin.

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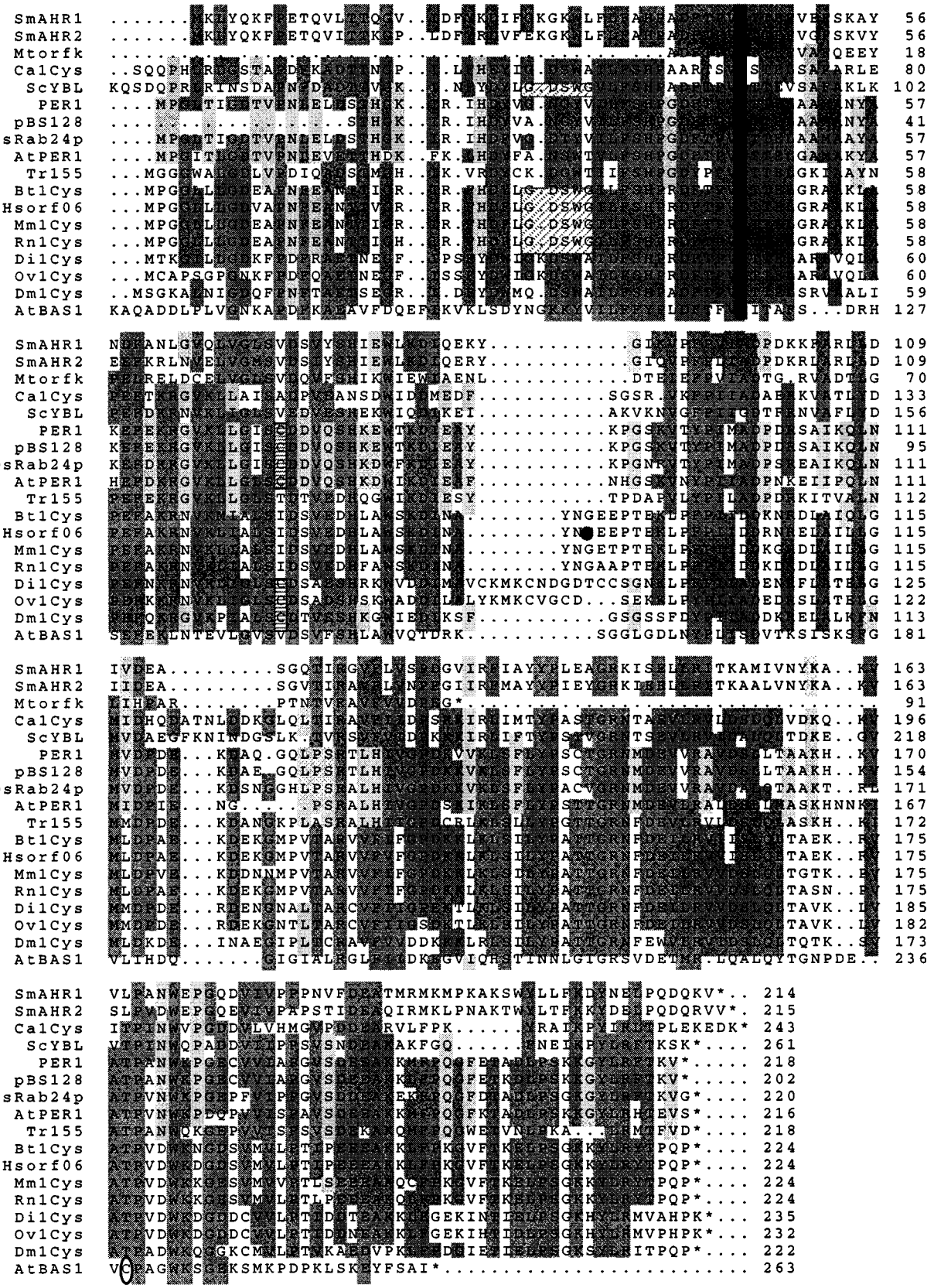

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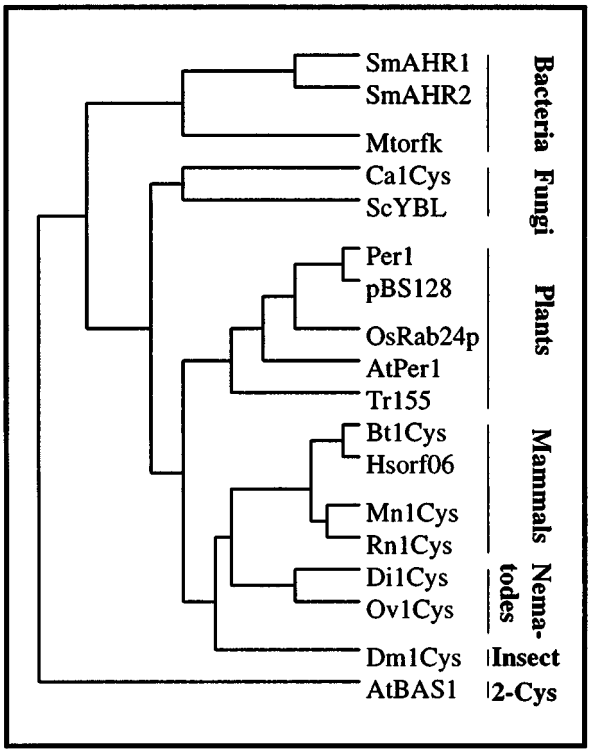

Figure 2. Dendrogram showing the relationship between 1-Cys peroxiredoxin proteins, with AtBAS1 as a 2-Cys outgroup, using Pileup. Protein depictions as in Fig.1A.

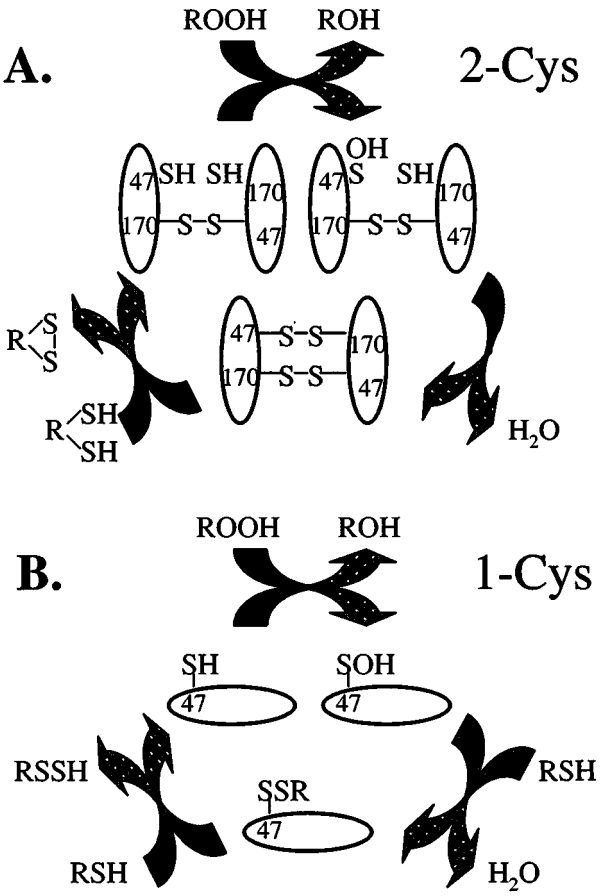

Figure 3. Peroxide reduction and regeneration of peroxiredoxin proteins, adapted after Chae *et al.* (1994b). **A.** 2-Cys Prxs are thought to be active as dimers connected through a disulfide bridge between the Cys-47 and Cys-170 residues. $R(SH)_2$ is thioredoxin in eukaryotes, and oxidoreductase AhpF in bacteria. **B.** 1-Cys Prxs are thought to be active as monomers, with Cys-47 as the active residue. 1-Cys Prx may be regenerated by a small thiol-containing compound (RSH), e.g. glutathione.

The regeneration mechanisms for 1-Cys Prx are less clear. When Cys-170 is mutated in TSA, the mutated protein is still able to protect proteins against inactivation in a mixed-function oxidation system containing dithiothreitol (DTT), Fe^{3+} and O_2 (Lim *et al.*, 1993; Chae *et al.*, 1994b). However, the Cys-170 mutant and the human 1-Cys Prx encoded by the human hsorf06 will not function in the presence of the thioredoxin system (Chae *et al.*, 1994c; Netto *et al.*, 1996). Therefore, it has been suggested that 1-Cys Prxs are regenerated by a small thiol-containing compound like reduced glutathione (GSH) (Fig. 3B). Experimental evidence for involvement of GSH is not clear. Kang *et al.* (1998) report that the GSH system – GSH, glutathione reductase and NAD(P)H – can not substitute for DTT in an *in vitro* assay system. In contrast, a protein isolated from bovine ciliary body was shown to decompose H_2O_2 and organic hydroperoxides in the GSH system (Shichi and Demar, 1990; Singh and Shichi, 1998). The bovine protein, named glutathione peroxidase, has 95 percent identity to human 1-Cys proteins (Frank *et al.*, 1997). The activity of a rat homologue, described as a non-selenium glutathione peroxidase, has also been shown to be supported by the GSH system (Peshenko *et al.*, 1996).

In addition to antioxidant activity, there is evidence for Ca^{2+}-independent phospholipase A_2 (PLA_2) activity of mammalian and rat 1-Cys Prx (Kim *et al.*, 1997). However, the antioxidant activity of these enzymes is at least two orders of magnitude higher than the PLA_2-activity (Kang *et al.*, 1998). One might, however, speculate that Prx could protect against lipid peroxidation in a dual fashion – first, by removing peroxidized fatty acyl moieties of phospholipids, and second by reducing the peroxides of the removed moieties.

The mammalian Prxs contain a putative "lipase" motif, GDSWG (see Fig. 1, cross-hatched), proposed to be important for PLA_2 function (Kim *et al.*, 1997). The

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motif matches the consensus sequence GXSXG found in serine proteases and some lipases. However, mutation of the serine in this motif or the cysteines in the human protein does not abolish phospholipase activity (Kang *et al*., 1998). The GXSXG motif is not present in plant 1-Cys Prxs (Fig. 1).

Subcellular localisation

Antioxidants are needed in all compartments of a cell to protect cellular macromolecules against damage caused by ROS. ROS are generated as natural by-products of respiration and photosynthesis and also by biochemical reactions in peroxisomes and glyoxysomes (see for instance Scandalios, 1987). Catalases are found in glyoxysomes and peroxisomes, while different isoenzymes of superoxide dismutase are found in these organelles as well as in the cytoplasm. Subcellular localization has been studied for few Prxs. Among the 2-Cys proteins, TSA is found in the cytoplasm (Chae *et al*., 1993). However, a bovine Prx was found to have an N-terminal extension localizing it to mitochondria (Hiroi *et al*., 1996), while the barley 2-Cys Prx, BAS1, is localized to the chloroplast (Baier and Dietz, 1997).

As for the 2-cys proteins, the subcellular localization of 1-Cys Prxs does not seem to be identical in all organisms. Investigations of 1-Cys human Prxs indicate a cytosolic localization (Kang *et al*., 1998; Frank *et al*., 1997). The 1-Cys yeast protein has an N-terminal extension of about 45 residues compared to other 1-Cys Prxs (not shown), and preliminary evidence indicates mitochondrial localization (Alseth, Aalen, Seeberg unpublished results). PER1 is, on the other hand, found by confocal fluorescence immunolocalization to accumulate in the nucleus of barley embryo and aleurone cells at the onset of desiccation (Stacy *et al*., 1999). An especially high signal is observed over nucleoli. In imbibed mature dormant seeds, the PER1 protein is detected at high levels both in the nucleus and the cytosol (Stacy *et al*., 1999). A putative nuclear localization signal is found in the C-terminal end of PER1.

Seed peroxiredoxins

The first cDNA from plants, turning out to encode a seed peroxiredoxin, was isolated from *Bromus secalinus* (pBS128, Goldmark *et al*., 1992). This grass species produces seeds with high levels of embryogenic dormancy. The clone was identified in a cDNA library made from dormant *B. secalinus* embryos after differential screening with total ^{32}P-labelled cDNA from dormant and non-dormant embryos imbibed for 10 h in water.

The homologous barley cDNA (B15C, Aalen *et al*., 1994; later renamed *Per1* by Stacy *et al*., 1996) was identified in an effort to elucidate genes involved in aleurone cell development. cDNAs representing transcripts expressed in aleurone cells, but not in starchy endosperm cells, were identified in an aleurone cDNA library by differential screening (Jakobsen *et al*., 1989). In this collection, one group was truly aleurone specific (*Lpt1* and *Lpt2*, Linnestad *et al*., 1991; Kalla *et al*., 1994), while the majority of the clones were also expressed in the embryo. B15C was one of these *Barley aleurone and embryo expressed* (*Balem*) cDNAs. At the amino acid level it turned out to be identical to the pBS128 clone except for five residues (Aalen *et al*., 1994; see Fig. 1).

During the same year that the B15C sequence was published, Chae *et al*. (1994a, 1994b) defined for the first time the family of antioxidant enzymes later to be known as peroxiredoxins (or sometimes peroxidoxins, see Chae *et al*., 1994a; McGonigle *et al*., 1998). Among the more than 20 sequences from diverse species aligned by Chae *et al*., the protein encoded by the *B. secalinus* clone was included. By northern hybridization Goldmark *et al*. (1992) had shown the pBS128 transcript to be present in other cereal embryos as well (*B. tectorum*, *A. cylindrica*, *S. cereale* and *A. fatua*). The transcript was also present in anther-derived embryos from ryegrass (*Lollium perenne*, Aalen *et al*., 1994). The homologous rice sequence was submitted to the EMBL database in 1995 (D63917), described as an "ABA-responsive 24kDa polypeptide from rice calli related to the thiol-specific antioxidant family". Using the pBS128 clone as a probe, the wheat gene was mapped to the short arm of chromosome 2 (Cadle *et al*., 1994). This is also the position of the gene in barley (Kleinhofs, http://barleygenomics.wsu.edu/). The first seed peroxiredoxin gene identified in a dicotyledonous species, *AtPer1*, was cloned from *Arabidopsis thaliana* (Haslekås *et al*., 1998). Several lines of evidence suggest that seed peroxiredoxin genes are present as a single copy in the genomes of the species investigated (Cadle *et al*., 1994; Stacy *et al*., 1996; Haslekås *et al*., 1998).

Expression of 1-Cys Prx in the aleurone layer and the embryo

The barley studies have identified aleurone expression in addition to the embryo expression found in *B. secalinus* (Goldmark *et al*., 1992; Aalen *et al*., 1994). No expression is found in the parts of the seed not surviving desiccation, the maternal tissue and the starchy endosperm. In barley, *in situ* and northern hybridization showed the transcript to be present in the aleurone and embryo from 18 days post anthesis (dpa). Steady state levels increased until about 25 dpa and remained high during seed maturation and

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desiccation (Aalen *et al*., 1994). Also in *Arabidopsis*, expression is found in the aleurone and the embryo only. The temporal expression pattern was comparable to that of barley (Haslekås *et al*., 1998). *Per1* expression was first detectable in shoot and root apices, and at later stages (40 dpa) also in the scutellum (Aalen *et al*., 1994). The *Arabidopsis AtPer1* gene is expressed throughout the embryo. The strongest *in situ* hybridization signal is found in the vascular tissue and epidermal cells (Haslekås *et al*., 1998).

The temporal expression patterns of *Per1* and *AtPer1* are similar to that of *late embryogenesis abundant (lea)* transcripts. *Lea* cDNAs were first identified as transcripts encoding highly hydrophilic proteins (Baker *et al*., 1988; Dure, 1993a, b; Close, 1996). They were divided into different groups according to amino acid sequence similarities. Since these proteins are hydrophilic, they were speculated to protect the embryo during desiccation. However, in recent years a number of transcripts with *lea*-like expression patterns have been identified which are not particularly hydrophilic (e.g. Galau *et al*., 1993). The seed peroxiredoxins can be said to belong to this group of atypical LEA proteins (Espelund *et al*., 1995). Group 1 *lea* genes are expressed only in embryos (Espelund *et al*., 1992), while most other *lea* genes are also expressed in the aleurone layer and can be induced in vegetative tissue by dehydration or exogenous ABA. The seed Prxs are atypical also from this perspective. Neither the bromegrass nor the *Arabidopsis* transcripts are expressed in ABA-treated or dehydration-stressed seedlings (Goldmark *et al*., 1992; Haslekås *et al*., 1998). This indicates a stringent regulation by tissue-specific transcription factors.

The potential of *Per1* (B15C) as a marker of embryogenesis has been tested in barley, wheat, maize and ryegrass. In maize suspension cultures, the B15C probe recognizes two mRNAs of different sizes both in embryogenic and non-embryogenic cultures (Stirn *et al*., 1995). Whether these bands represent seed Prx transcripts or other cross-hybridizing transcripts is not known, since maize 1-Cys Prxs sequences have not been published. However, in suspension cultures initiated from immature barley and wheat embryos the transcript is expressed in embryogenic, but not in non-embryogenic cultures (Aalen *et al*., 1994; Stirn *et al*., 1995). In anther- or microspore-derived embryos of barley and ryegrass, *Per1* is also expressed (Aalen *et al*., 1994; Stirn *et al*., 1995). *Per1* seems to be expressed earlier during somatic than zygotic embryogenesis. This has also been shown for other *lea* transcripts (Holbrook *et al*., 1991; Reinbothe *et al*., 1992; Aalen *et al*., 1994), indicating a less stringent developmental gene control in somatic embryos. Interestingly, the *Per1* probe can not identify somatic embryos capable of regenerating plants. In fact, *Per1* expression was higher in non-regenerable cultures (Stirn *et al*., 1995). These results support the notion that embryogenesis and plant regeneration are separate processes. 1-Cys Prx transcript levels are also high in non-germinating zygotic embryos (see below).

Effect of ABA and osmotic stresses on seed Prx gene expression

Both in dormant and non-dormant imbibed mature embryos of *B. secalinus*, pBS128 transcript levels increase when exogenous ABA is added (Goldmark *et al*., 1992). This is also true for barley embryos (Aalen *et al*., 1994). In immature embryos, *Per1* can be induced by osmotic stress, both salt and mannitol (Aalen *et al*., 1994; Espelund *et al*., 1995). In mature embryos, salt has a minor effect. Ionic stress is generated by low salt concentrations (200 mM). In immature embryos, *Per1* induction by ionic stress can be repressed by the ABA-biosynthesis inhibitor norflurazon, which lowers endogenous ABA concentrations (Espelund *et al*., 1995). The lack of low salt induction in mature non-dormant embryos may indicate a lower endogenous ABA level. Induction of *Per1* by salt concentrations higher that 600 mM and by 20% mannitol is independent of endogenous ABA concentrations. This indicates that non-ionic osmotic stress is transduced by an ABA-independent signal transduction pathway (Espelund *et al*., 1995).

Endogenous ABA rises during seed development and peaks around 10 dpa in *Arabidopsis* (Karssen *et al*., 1983). This rise correlates with the onset of *AtPer1* expression. In an ABA-insensitive mutant of *Arabidopsis* (*abi3-1*, Koornneef *et al*., 1982), the expression level of *AtPer1* is only 1/10 of the wild type seeds. In contrast, in the ABA-deficient mutant *aba-1* (Koornneef *et al*., 1984) the expression level is comparable to wild type. These results (Haslekås *et al*., 1998) taken together indicate that ABA is not an essential signal for *AtPer1* developmental expression. Instead, *AtPer1* expression may be governed by an ABA-independent developmental pathway involving the embryo specific ABI3 transcription factor (Parcy *et al*., 1994). In fact, in transgenic *Arabidopsis* plants expressing the ABI3 in vegetative tissue (Parcy and Giraudat, 1997), the *AtPer1* transcripts can be detected in leaves (Thorstensen and Aalen, unpublished results).

The *Per1* and *AtPer1* genes (acc. no. X96551 and Y12089) have been investigated for putative regulatory promoter elements. As shown in Fig. 4, both genes contain sequences resembling an ABA-responsive element (ABRE, Guiltinan *et al*., 1990), an antioxidant responsive element (ARE, Rushmore *et al*., 1991), seed-specific enhancer elements (AA/G/CCCCA, Chen *et al*., 1986), and elements for aleurone expression (Leah *et al*., 1994).

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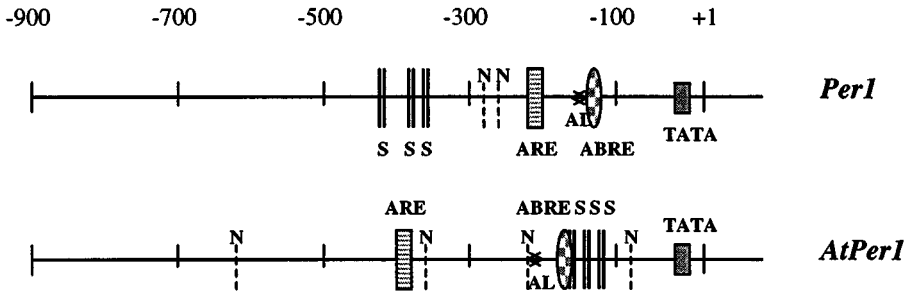

Figure 4. Organization of putative regulatory sequences in the *Per1* and *AtPer1* promoters. TATA – TATA-box; ABRE – ABA-responsive element; ARE – antioxidant responsive element; S – seed specific element; AL – aleurone specific element; N – negative regulatory element.

Seed Prx expression in dormant seeds

In dormant bromegrass embryos, the pBS128 transcript level increased four-fold during 48 h of imbibition, while in non-dormant embryos the transcript had disappeared after 6 h (Goldmark *et al.*, 1992). Visible signs of germination were apparent after 10 hours. In barley, transcript levels have been investigated in embryos from cultivars with different degrees of dormancy (Stacy *et al.*, 1996). In the most dormant cultivars, the increase in transcript levels is similar to that of bromegrass. A transient increase in transcript levels was also noted in non-dormant or slightly dormant cultivars. Transcript levels were found to decline, however, before visible signs of germination were evident. In *Arabidopsis*, *AtPer1* expression levels can be correlated to germination frequencies (Haslekås *et al.*, 1998). In freshly harvested seeds, which will not germinate, transcript levels are maintained, while in after-ripened seeds *AtPer1* levels decline at the onset of germination.

Gibberellic acid (GA) can act as an ABA antagonist and will also promote germination. In dormant *B. secalinus* embryos, imbibition of dormant embryos with GA_3 resulted in the disappearance of the pBS128 transcript by day four, while visible signs of germination were seen at day six (Goldmark *et al.*, 1992). In the aleurone layer of mature barley grains, the *Per1* transcript declines only in germinating seeds. In aleurone of mature, dormant or embryoless grains, transcript levels are maintained, indicating that a signal from the embryo is involved in transcript suppression during germination (Stacy *et al.*, 1996). In immature aleurone layers, transcript levels are maintained even if the seed germinates precociously. Since immature aleurone layers are insensitive to GA (Evans *et al.*, 1975), these results suggest GA to be the down-regulating signal from the embryo. And in fact, in mature aleurone layers exogenous GA_3 can substitute for the effect of the germinating embryo by suppressing the *Per1* transcript level (Stacy *et al.*, 1996).

Does 1-Cys Prx play a role in dormancy?

Based on the expression pattern of pBS128, Goldmark *et al.* (1992) suggested a role for this transcript in maintaining seed dormancy. This was also hypothesized by Stacy *et al.* (1996). The *AtPer1* transcript can also be said to be dormancy-related (Haslekås *et al.*, 1998).

However, other results cast doubt on the validity of this hypothesis. First, the normal expression level of the *AtPer1* transcript in the non-dormant *Arabidopsis* mutant *aba*-1 clearly demonstrates that the presence of the transcript is not sufficient for expression of dormancy. Second, in non-dormant barley embryos the PER1 protein levels decline between 5 and 8 days post-imbibition, while signs of germination are visible already after one day (Stacy *et al.*, 1999). Still, we cannot exclude that 1-Cys Prxs, although not sufficient for the expression of dormancy, partake together with other factors in the maintenance of dormancy.

Does 1-Cys Prx act as a protectant against ROS during desiccation and imbibition?

Several factors are important for desiccation protection (see Leprince *et al.*, 1993). First, the presence of high amounts of non-reducing sugars is necessary to enable lipid and protein stabilization. Second, the expression of LEA proteins is needed. Some of these proteins may play a structural role as desiccation protectants, for instance by providing a matrix of bound water to stabilize cytoplasmic structures. And thirdly, prevention of and repair following free radical attack is needed to prevent crucial damage to membranes, DNA and proteins.

Based on the special features of the 1-Cys Prx genes, we have proposed that the seed Prx plays a specific role in prevention of radical attack during desiccation (Stacy *et al.*, 1996). Other antioxidants, like catalases, superoxide dismutases and peroxidases, are

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encoded by gene families. In contrast, seed Prxs seem to be encoded by a single gene in each species. This indicates, and allows for, a stringent selective pressure ensuring the maintenance of highly specific functional tasks.

A particular function of the seed Prx is also indicated by the strict temporal and spatial control of gene expression. In the gene families of other antioxidants, one or more members are expressed in most cells at any point in time. In contrast, seed Prx expression is restricted only to the tissues surviving desiccation and cannot even be induced in vegetative tissue by drought stress or ABA. The temporal expression pattern of the PER1 protein follows the mRNA pattern closely in both the aleurone and the embryo of barley (Stacy *et al.*, 1999). Interestingly, PER1 accumulates in the nucleus at the onset of desiccation (Stacy *et al.*, 1999). This raises the exciting possibility that PER1 is involved in the protection of the intactness of nuclei containing the genetic information of the plant to be. So far, very few other LEA proteins or enzymatic plant antioxidants have been shown to have nuclear localization (see Stacy *et al.*, 1999).

Another specific feature of 1-Cys Prx expression is the rise in transcript levels during imbibition of mature, dormant grass embryos and the transient up-regulation in their non-dormant counterparts. This is not the case for most other antioxidant transcripts, which are first induced to high levels during germination (see Leprince *et al.*, 1993). Their encoded proteins are assumed to protect the growing embryo from ROS arising as by-products of respiration. However, respiration is resumed both in dormant and non-dormant seeds already at the onset of imbibition. Therefore, 1-Cys Prx could be an important protector against ROS in imbibed seeds, which are (still) not germinating and consequently are not protected by germination-associated antioxidants.

The expression pattern of *Per1* in aleurone layers of imbibed seeds can also support this hypothesis. Transcript levels increase or are maintained in aleurone of dormant seeds (Stacy *et al.*, 1996). It is important that the intactness of aleurone cells is preserved for their future role in mobilization of storage compounds, exerted when germination eventually takes place. In fact, the suppression of the 1-Cys Prx transcripts seem to be specifically controlled by a signal from the germinating embryo, possibly GA. In aleurone and embryo cells of dormant seeds imbibed for 20 h, the PER1 protein is amply present both in the nucleus and the cytoplasm (Stacy *et al.*, 1999). This indicates either increased translational activity or a more important role of the seed Prx in the cytoplasm during rehydration.

The expression pattern of the 1-Cys Prx in the moss *Tortula ruralis* (Tr155, Oliver, 1996, see Figs 1 and 2) is supportive of the suggested protective role of the seed Prx during rehydration. *T. ruralis* is a fully desiccation-tolerant plant able to withstand total loss of water. During desiccation of the gametophytes, mRNAs preferentially translated during rehydration are sequestered in to the polysomal fraction of the cells. This can presumably enable a faster repair of desiccation- and rehydration-induced damage (Oliver, 1996). Among mRNAs sequestered into polysomes, Tr155 is found (Oliver, 1996).

Approaches to unsolved questions

Although the expression patterns of 1-Cys Prx give clues to the function of this protein in seed physiology, a number of questions remain unanswered. Definite experimental evidence is needed to verify or refute the hypothesized involvement of seed peroxiredoxins in desiccation tolerance and dormancy. As a first attempt to provide such evidence, we have tested and found *in vitro* antioxidant activity of the PER1 protein (Stacy *et al.*, 1996). Other biochemical information on seed Prx is lacking. PLA_2 activity has so far not been proven or disproven for plant Prxs, and the regeneration system for seed Prx has not been identified.

To study 1-Cys Prx function *in vivo*, PER1 over-expressing and *AtPer1* antisensed transgenic *Arabidopsis* plants have been generated (Nygaard, Haslekås, Meza and Aalen, unpublished). A changed requirement for after-ripening prior to germination in such plants compared to wild-type *Arabidopsis*, would support a role of the enzyme in dormancy. Experiments are also underway to investigate whether the PER1 protein can provide antioxidant protection at the whole plant level or during seed germination. Over-expression of glutathione reductase and superoxide dismutase has to some extent given increased protection against oxidative damage (Foyer *et al.*, 1994). It has, however, been suggested that more than one antioxidant gene must be manipulated to achieve considerable stress tolerance. Therefore, we have also turned to the simpler yeast system, where strains mutagenized in Prx genes are under construction (Alseth, Aalen and Seeberg, unpublished). TSA knockout mutants of yeast show reduced growth rates when subjected to oxidative stress (Chae *et al.*, 1993). This may also turn out to be the case for yeast mutated in the 1-Cys YBL-gene, and plant Prx can then be examined for the ability to complement mutants with lower stress tolerance.

The presence of a putative ARE, in both the *Per1* and the *AtPer1* promoters (Fig. 4), calls for an investigation of induction of Prx genes by ROS-inducing agents. For this reason *Arabidopsis* transgenes transformed with *AtPer1* promoter/marker-gene constructs have been generated (Thorstensen, Haslekås and Aalen, unpublished). These plants

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might also be used to study other aspects of seed Prx gene regulation, e.g. control of tissue-specific gene expression and induction by ABA and osmotic stress.

Hopefully, these and other research efforts will soon provide a fuller understanding of the role of peroxiredoxin antioxidants in seed physiology.

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