

A peroxiredoxin antioxidant is encoded by a dormancy-related gene, *Per1*, expressed during late development in the aleurone and embryo of barley grains

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Abstract

Antioxidants can remove damaging reactive oxygen species produced as by-products of desiccation and respiration during late embryogenesis, imbibition of dormant seeds and germination. We have expressed a protein, PER1, encoded by the *Balem* (barley aleurone and embryo) transcript previously called B15C, and show it to reduce oxidative damage *in vitro*. PER1 shares high similarity to a novel group of thiol-requiring antioxidants, named peroxiredoxins, and represents a subgroup with only one conserved cysteine residue (1-Cys). PER1 is the first antioxidant belonging to the 1-Cys subgroup shown to be functionally active, and the first peroxiredoxin of any kind to be functionally described in plants. The steady state level of the transcript, *Per1*, homologous to a dormancy-related transcript (pBS128) from bromegrass (*Bromus secalinus*), increases considerably in imbibed embryos from dormant barley (*Hordeum vulgare* L.) grains. Our investigations also indicate that *Per1* transcript levels are dormancy-related in the aleurone layer of whole grains. In contrast to most seed-expressed antioxidants *Per1* disappears in germinating embryos, and in the mature aleurone the transcript is down-regulated by the germinating embryo or by gibberellic acid (GA). Our data show that the barley seed peroxiredoxin is encoded by a single *Per1* gene. Possible roles of the PER1 peroxiredoxin in barley grains during desiccation, dormancy and imbibition are discussed.

Introduction

Seeds of many species are dormant for a shorter or longer time after maturation and do not germinate under seemingly favorable environmental conditions. Internal and external factors influencing the acquisition and breakage of dormancy have been investigated (reviewed in [3]), but the molecular mechanisms underlying dormancy are poorly understood. During desiccation and in the dry resting or dormant seed, protection of the cellular components of living tissues is required, such as protection against damage by water loss and free radical attack [30, 31]. Transcripts present dur-

ing late embryogenesis (late embryogenesis-abundant or *Lea* transcripts) have been implicated in providing a tolerance to desiccation, and some *Lea* transcripts have been shown to be maintained in hydrated dormant seeds, but disappear rapidly in non-dormant seeds [33, 39]. It is not clear whether their encoded proteins play a role in the maintenance of dormancy.

Water uptake and resumption of respiration, which produce reactive oxygen molecules as metabolic by-products, are similar in dormant and non-dormant seeds during early imbibition (phases I and II of germination), but dormant seeds do not enter the third phase of germination, which is associated with radicle elongation and a second burst of respiration (reviewed in [3]). One would thus expect germination ability or inability to be reflected through molecular differ-

The nucleotide sequence of the *Per1* gene will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X96551.

ences between dormant and non-dormant seeds during imbibition [23, 33, 39]. While some studies have focused on these molecular differences [9, 32, 33], only a few transcripts have been cloned which show differential expression patterns during imbibition. Using differential display, Johnsen *et al.* [23] identified two transcripts as dormancy-associated and three as non-dormancy-related in a recent study of wild oat embryos. To date, the only transcript shown not only to remain present, but also to increase considerably during imbibition of dormant embryos, is pBS128 from brome grass [16].

In looking for transcripts expressed in aleurone cells but not in starchy endosperm cells of developing grains, the barley homologue to pBS128, B15C, was isolated from an aleurone cDNA library [20], and shown to belong to a group of *Balem* (barley aleurone and embryo expressed) transcripts [1]. B15C expression is similar to *Lea* genes and is highest in the root and shoot apices of developing embryos [1]. The transcript is present in the only two cereal seed tissues which survive desiccation and are involved in dormancy. Degree of dormancy may be partly determined by the aleurone layer, and is characterized by a lower GA and a higher abscisic acid (ABA) responsiveness in dormant versus non-dormant seeds [50, 52].

Our interest in this *Balem* transcript has been promoted by a database search which revealed that the proteins encoded by pBS128 (homology published in [6]) and B15C belong to a recently discovered group of antioxidants called peroxiredoxins [8]. We have therefore renamed the B15C gene *Per1* (for peroxiredoxin). This kind of antioxidant was first isolated and characterized in yeast (thiol-specific antioxidant, TSA [25]) and has since been shown to be present in organisms ranging from bacteria to man [6]. Peroxiredoxins are, in contrast to previously described enzymatic antioxidants, dependent on the presence of thiol. Peroxiredoxins have been shown *in vitro* to protect DNA, membranes, and certain enzymes against damage by thiol or oxygen radicals, and to remove H₂O₂, alkyl hydroperoxides and hydroxyl radicals [19, 25, 34, 35, 54].

This paper presents results describing the antioxidant activity of the expressed B15C protein, PER1, the first peroxiredoxin to be characterized in plants. In an effort to elucidate possible *in vivo* role(s), we have studied expression patterns in barley lines with different degrees of dormancy, and have investigated expression in the aleurone layer under different developmental and hormonal influences. Additionally, we have examined whether *Per1* is a single-copy gene.

In view of the developmental and hormonal regulation of *Per1* expression, hypotheses on the role of the peroxiredoxin during seed desiccation, dormancy and germination are discussed.

Materials and methods

Purification of the PER1 fusion protein and antioxidant assay

The plasmid B15C-A, where the B15C cDNA [1] is inserted in the *EcoRI* site of pBluescript SK⁻ (Stratagene) with the 3' end closest to the *EcoRV* site of the vector polylinker, was used to amplify a PCR product encompassing the B15C cDNA from nucleotide 54 (2 bp before the ATG start codon). A B15C-specific primer with an attached *Bam*HI site in the 5' end (5'-GGATCCATGCGGGGGCTCACC-3') and a vector-specific primer (5'-AAAGGGGATGTGCTGCAAGGCG-3') was used for the amplification. The PCR product was digested with *Bam*HI and *EcoRV* and cloned into the PinPoint-Xa-3 vector (Promega) behind the PinPoint tag, which encodes a 13 kDa polypeptide. Sequencing confirmed that in the resultant PP15C-12 plasmid, the coding sequence of B15C is in the same reading frame as the polypeptide tag. Seven amino acids (Glu-Lys-Pro-Gln-Leu-Gly-Ser) are generated from the PinPoint polylinker between the position of the Factor Xa cleavage site and the initial Met-residue of PER1.

The fusion protein was purified using the PinPoint batch purification protocol with 50 mM Hepes pH 7.4 and 5 mM biotin as elution buffer. The purity of the eluted protein was checked by SDS-polyacrylamide gel electrophoresis. The fusion protein was concentrated in a Centricon-10 concentrator (Amicon). The antioxidant activity of the fusion protein was tested in a DNA-cleavage assay modified after Lim *et al.* [34, 35]. Two μ g pUC19 plasmid was exposed to a mixed function oxidation (MFO) system containing 4 mM DTT and 0.3 or 3 μ M FeCl₃. The reaction was initiated 30 min before the addition of DNA, and contained varying amounts of concentrated fusion protein. Control reactions with the thiol-MFO system were performed on the last (fifth) wash before protein elution, on filtrate from the fusion protein concentrate, with elution buffer or with 50 mM Hepes pH 7.4 only. The degree of DNA degradation is similar in these solutions. Control reactions were also performed by adding up to 400 μ g/ml BSA to washes, filtrate or Hepes buffer.

Plant material

Barley plants (*Hordeum vulgare* L. var. *disticum* cvs. Bomi, Nordlys, Stange and Agneta) were grown and staged as previously described [20]. Aside from Bomi, varieties utilized for the experiments shown in Figs. 2, 3 and 4 were acquired from H. Skinnes (Dept. of Genetics and Plant Breeding, Agricultural University of Norway, Ås-NLH, Norway) and were as described in [4]. Seeds for the experiments presented in Figs. 2, 3 and 4 were harvested three weeks after yellow ripeness and stored for either a short period at 4 °C (1–3 weeks) or for longer periods at –80 °C at which point germination tests and incubations for mRNA isolations were performed.

Seeds used for all experiments were first surface sterilized (1 min 70% EtOH, 1 min. 0.2% AgNO₃, 3×1 min distilled H₂O). For each variety, germination tests were carried out on 50–100 whole seeds and embryos, from the same seed batches used for northern experiments (Figs. 3 and 4), by imbibing them sterile on paper discs soaked in distilled water. Germination was followed over a 6–8 day period. In order to break dormancy in the dormant Agneta variety, a subset of the dormant seeds was incubated at 37 °C for 3 weeks. A 100% germination frequency of the incubated Agneta seeds confirms that the low germination frequency prior to release reflects dormancy and not reduced viability.

Seeds for northern experiments shown in Fig. 5 were incubated in standard incubation solution (20 mM CaCl₂, 1 mM sodium acetate pH 4.8) for 3 days with or without supplements, unless otherwise stated in the figure legend. It is worth noting that visible signs of germination were observed for all whole-seed embryos utilized in these experiments.

mRNA extraction, northern blots and primer extension

Poly (A)⁺ mRNA was isolated using magnetic oligo d(T) Dynabeads (DynaL AS), as described by Jakobsen *et al.* [21]. Yield of purified mRNA from embryos was measured spectrophotometrically, as described in Espelund *et al.* [11]. For all aleurone isolations, additional control hybridizations were performed, since quantification by spectrophotometric measurements may be less accurate for high starch containing tissues. Probes for control hybridizations were chosen which give opposite expression patterns to *Per1* (α -amylase, B22E) under the examined incubation conditions. Hybridization probes include, B15C cDNA [1],

an 800 bp fragment from the rice Rab 21 (16A) cDNA [40], a 900 bp fragment from the barley α -amylase (clone E) cDNA [46], and B22E (clone e49) cDNA from barley [28], generated using a standard random priming reaction. However, the B15C and B22E probes were generated using biotinylated single-stranded templates bound to magnetic streptavidin-coated beads (Dynabeads M-280-Streptavidin, Dynal AS) in the standard random-primed reaction [10]. Hybridization conditions and washes are as described [1].

Primer extension was performed using AMV Reverse Transcriptase (Promega) under the conditions recommended by the manufacturer. Radioactive incorporation and sequencing reactions were performed on the same mRNA isolate [48], using an end-labeled primer complementary to a region of B15C lying ca. 150 bp downstream of the putative TATA-box (5'-GTAGTCGTGGATGCGGATCTTGCCA-3').

Microprojectile bombardment and histochemical assay

Construct DNA was precipitated onto gold particles (1.0 μ m, BioRad Laboratories) using a procedure modified from Klein *et al.* [26]. The precipitation mixture included 37.5 μ l gold particles (50 mg/ml), 20 μ l DNA (1 μ g/ μ l), 0.75 M CaCl₂ and 6.0 mM spermidine, in a total volume of 122.5 μ l. After precipitation, washing and resuspension in 60 μ l Abs EtOH, 10 μ l of the suspension was pipetted onto the macrocarrier. Bombardment was performed using a Biolistic PDS-1000/He Particle Delivery System (BioRad), as recommended by the manufacturer. Settings were as follows: gap distance, 6.4 mm; macrocarrier travel distance, 6 mm; sample position, 3 from the bottom; rupture disk, 9.3 MPa; chamber vacuum, 71 mm Hg (9.4 kPa). Samples were bombarded and incubated on 1/2 MS solid medium with or without supplements (as indicated in the figure legend). Embryos were dissected out of the seed and placed apex side up on the medium. For the aleurone bombardment, palea/lemma were removed and the pericarp/testa and nucellus were peeled away on the dorsal side to reveal the aleurone layer. Control bombardments were performed for each experiment with the construct pACT1-F [37]. After bombardment, samples were incubated in the dark at room temperature for two days, prior to staining for β -glucuronidase activity as described [22].

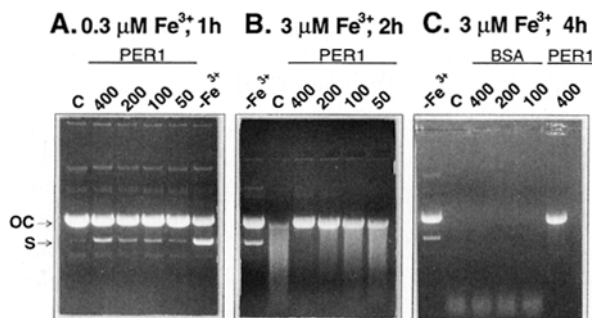


Figure 1. Protection of DNA against cleavage by free radical attack by the expressed B15C fusion protein, PER1. Supercoiled plasmid DNA (S) and open circular (OC) are indicated in the figure. A. pUC19 incubated one hour in a thiol-MFO-system with $0.3 \mu\text{M Fe}^{3+}$. Lane 1, (C) without protein shows maximum nicking under these assay conditions; lanes 2–5, 400–50 μg per ml fusion protein, show that protection against nicking is reduced with decreasing PER1 amounts in the reaction mixture; and lane 6, without protein and without Fe^{3+} , shows results of the assay when no free radicals are created and maximal amounts of supercoiled plasmid are retained. B. pUC19 incubated for 2 h with $3 \mu\text{M Fe}^{3+}$. Lane descriptions are the same as for A. C. pUC19 incubated for 4 h with $3 \mu\text{M Fe}^{3+}$. Lanes 3–5, 400–100 $\mu\text{g/ml}$ BSA. Other lane descriptions are the same as for A.

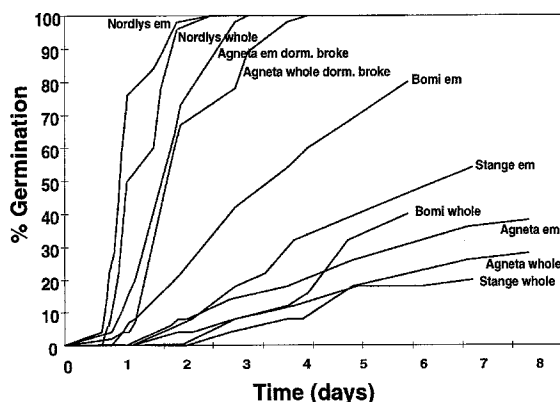


Figure 2. Test of embryo and whole seed germination frequencies for four barley varieties. Varieties are indicated in the figure series labels. 'em' indicates embryos imbibed alone and 'dorm. broke' indicates embryos or seeds which have first been incubated to release them from dormancy. See Materials and methods for germination test conditions.

Results

PER1, belonging to the recently described peroxiredoxin antioxidant family, protects against DNA cleavage by free radical attack

The *Per1* and pBS128-encoded proteins share 98% amino acid identity [1, 16], and show significant sequence similarity to members of the novel family

of peroxiredoxin antioxidants [6]. This family includes three main subgroups, two with two conserved cysteine residues (2-Cys), corresponding to amino acid positions 47 and 170 in the yeast peroxiredoxin, TSA, and one with only one conserved cysteine (1-Cys) at position 47 [6]. Experiments with the 2-Cys yeast peroxiredoxin TSA have confirmed the protein's catalytic center to consist of the cysteine residue at position 47 and suggest that the second conserved cysteine (Cys-170) may contribute to stable dimer formation [7]. PER1 is conserved at the position relative to Cys-47 (residue 46 of PER1) and shows most overall similarity to members of the 1-Cys subgroup. The highest identity, aside from pBS128, is found to the human clone hsof06 (69%, accession number D14662). Recently a barley cDNA (*Bas1*, Z34917), showing about 60% similarity to 2-Cys peroxiredoxins, was cloned from young leaves. Under the standard hybridization conditions used in our lab (68°C , $1 \times \text{SSC}$), the 2-Cys barley peroxiredoxin transcript will not cross-hybridize with *Per1* probes.

To test whether PER1 exerts antioxidant activity, a PER1 fusion protein was produced and purified by the PinPoint system (Promega). The theoretical molecular mass of PER1 is 23 kDa. In PAGE gels the molecular mass of the fusion protein was estimated at 39–40 kDa (not shown), close to the expected 36.7 kDa. Antioxidant activity of concentrated fusion protein was tested in a DNA-protection assay [34, 35] (Fig. 1). DNA was exposed to a mixed function oxidation (MFO) system (containing a sulfhydryl compound, Fe^{3+} , O_2 and DTT: thiol-MFO system, see Materials and methods). Both with a low Fe^{3+} concentration ($0.3 \mu\text{M}$) and short reaction time (1 h; Fig. 1A) as well as with a higher Fe^{3+} concentration ($3 \mu\text{M}$) and longer reaction time (2 h; Fig. 1B), protection of DNA against cleavage by radicals produced in the thiol-MFO system increases with increasing PER1 concentration (50 to 400 μg per ml fusion protein). Full protection, i.e. no nicking of the supercoiled pUC19 DNA could, however, not be shown. Addition of a non-antioxidant protein (BSA) at the same concentrations to the assay mixture, did not give protection against nicking, while still after 4 hours PER1 gives substantial protection (Fig. 1C).

The Per1 transcript is dormancy related in barley

To determine whether the *Per1* transcript is dormancy-related in barley, as in brome grass [16], expression levels were examined in four *Hordeum* varieties; one (Nordlys) which shows almost no dormancy, and two

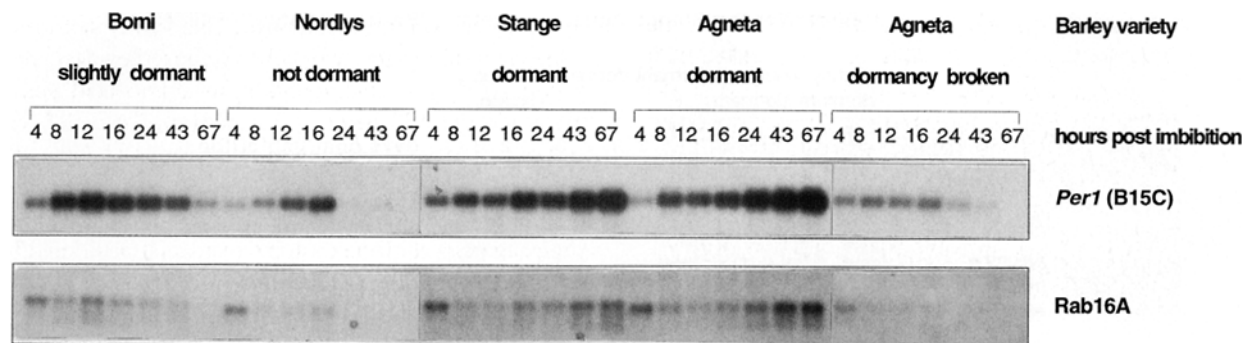


Figure 3. *Per1* transcript levels in the isolated embryo of barley varieties possessing varying degrees of dormancy. 350 ng poly(A)⁺ mRNA was applied in each lane. The same blot was hybridized to B15C and Rab16A.

(Stange, Agneta) which show considerable degrees of dormancy [4], in addition to Bomi, which has not been examined with respect to dormancy previously but was used for all other experiments. Levels of whole-seed dormancy for Nordlys, Stange and Agneta (Fig. 2) correlate well with those found by Buraas and Skinnies [4] under similar growth conditions. While Buraas and Skinnies did not examine degree of dormancy in isolated embryos [4], our results show both Stange and Agneta to possess a degree of embryo dormancy almost comparable to dormant bromegrass embryos [16]. Isolated embryos from Bomi were found to possess a degree of dormancy intermediate between the non-dormant and dormant varieties, while Bomi whole grains show a more dormant germination profile.

In isolated embryos from the above varieties, imbibed 4–67 h, *Per1* transcript levels increase during approximately the first 16 h of imbibition (Fig. 3). This increase is, however, only transient in non-dormant embryos or in embryos which have been released from dormancy. Steady-state levels in the Bomi variety correlate well with its germination profile. In Fig. 3, Bomi is thus termed 'slightly dormant'. In the dormant varieties, the *Per1* steady-state levels continue to increase, to a degree comparable to that found for bromegrass [16]. Transcript levels of the group II *Lea* transcript Rab16A, included for comparison, [40] decrease steadily during the first 12 h of imbibition in relation to *Per1* and only then begin to increase in the dormant varieties. Although *Per1* and Rab16A show similar increases after 16–67 h of imbibition, *Per1* transcript levels clearly increase more rapidly in imbibed dormant seeds compared to Rab16A. This same difference has been observed between *Per1* and *Ole1* [2], a barley oleosin (not shown).

An examination of expression in the aleurone layer (Fig. 4A) shows a correlation between dormancy and *Per1* expression in whole seeds imbibed 1–3 days; for example, the transcript is present in dormant seeds, but disappears in non-dormant, germinating seeds. A similar pattern of regulation is observed for Rab16A. A control hybridization with the germination-specific probe α -amylase reflects the germination of the non-dormant and dormancy broken varieties. The reduction in *Per1*/Rab16A signal for the dormant Stange variety at 3 days of imbibition (lane 9) is not accompanied with an α -amylase signal, thus indicating underloading of this lane. Contrary to germinating whole seeds, when mature seeds are imbibed with the embryo removed (embryoless), *Per1* and Rab16A transcripts are present in all cultivars for at least three days of imbibition (Fig. 4B).

Per1 is down-regulated by GA in the mature aleurone

Rab16A mRNA has been shown to disappear rapidly upon incubations of isolated aleurone layers [52]. The maintenance of the *Per1* transcript in the aleurone layer of embryoless imbibed seeds is, however, not due to the presence of the starchy endosperm, since the transcript level is stably maintained in isolated aleurone layers for at least 2 days of incubation (Fig. 5A, compare lanes 3 and 4).

The disappearance of the *Per1* transcript in the aleurone layer of germinating mature seeds (Fig. 4A, Fig. 5A) suggests a signal from the germinating embryo to be down-regulating *Per1* expression in this tissue. In contrast to mature seeds, *Per1* is unresponsive to the germinating embryo in immature aleurone tissue, since incubations of immature whole seeds and embryoless seeds give similar *Per1* transcript levels in

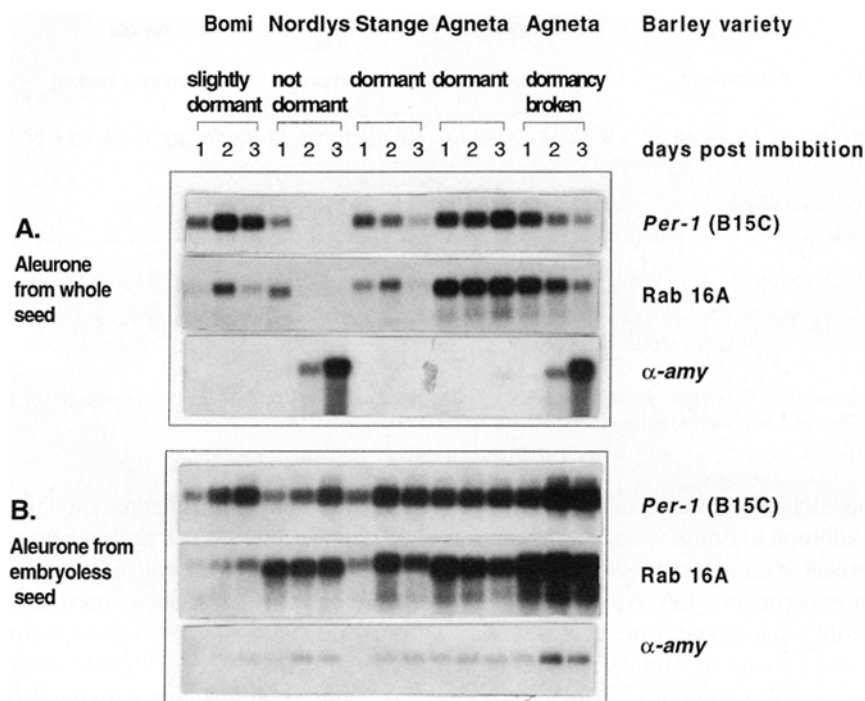


Figure 4. *Per1* transcript levels in the aleurone layer of whole seeds (A) and embryoless seeds, (B) from barley varieties possessing varying degrees of dormancy. 500 ng of poly(A)⁺ mRNA was applied in each lane. The same blot was hybridized to B15C and Rab16A and low-pI α -amylase, a germination-specific clone.

the aleurone layer (not shown). These facts suggest gibberellic acid (GA) to be the most likely signal candidate based on previous work which shows the immature aleurone layer to be insensitive to GA [12]. To test whether GA is responsible for down-regulating *Per1* levels in the aleurone of mature germinating whole seeds, transcript levels were examined in mature aleurone from embryoless seeds and isolated aleurone layers incubated in the presence of 10 μ M GA₃ for varying lengths of time (Fig. 5B). Incubating embryoless seeds for 3 days maintained transcript levels (lane 1, Fig. 5B), as shown previously (Fig. 4B, 5A). Inclusion of 10 μ M GA₃ to the incubation solution, however, led to the disappearance of *Per1* and an induction of α -amylase (lane 2). In order to examine how quickly GA₃ can reduce *Per1* transcript levels, embryoless seeds were incubated 3 days and then incubated for an additional 0.5, 1 or 3 h (with, (+) or without (–) the starchy endosperm left intact; lanes 3–7) with GA₃ added to the incubation medium. The results from this experiment show *Per1* transcripts to decrease in the aleurone cells already after 3 h of adding GA to the incubation medium, while a stabilizing effect is observed when the

starchy endosperm is left intact (compare lanes +/–, 1 h incubation for ex.).

Upon examination of immature seeds, little or no difference in *Per1* levels from immature aleurone (21–46 days after anthesis, dpa) was observed when the embryoless seeds were incubated with or without 10 μ M GA₃ (not shown), as expected for immature aleurone being unresponsive to GA [12]. At 56 dpa when the seed water content is estimated to be below 30%, where upon the aleurone layer acquires a sensitivity to this growth hormone, the *Per1* transcript level was lowered by GA₃ in the incubation medium, and an induction of α -amylase was observed (not shown).

Expression of the Per1 transcript in the aleurone and the embryo is exerted through the same gene

Similar transcript accumulation patterns have been observed for *Per1* in the aleurone and embryo during seed development [1]. To test whether the same *Per1* gene is expressed in both the aleurone and the embryo, several studies were undertaken. First, the previously described aleurone cDNA sequence [1] was compared to that in the embryo by sequencing complements of

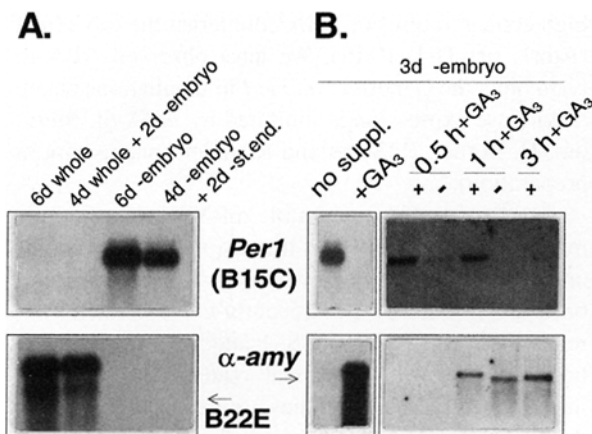


Figure 5. The effects of the germinating embryo and GA on *Per1* transcript levels in the mature non-dormant aleurone. **A.** Mature aleurone tissue isolated after 6 days, 4 days of imbibition, then 2 days of incubation as indicated (-embryo = embryoless; -st.end. minus starchy endosperm). mRNA isolated from 25 mg aleurone tissue was applied per lane. The blot was hybridized to B15C and as a control, B22E, a cDNA for a transcript up-regulated by germination [42]. **B.** Mature aleurone isolated from embryoless seeds imbibed for 3 days with or without 10 μ M GA₃ added to the incubation medium (lanes 1 and 2), and from embryoless seeds (palea/lemma intact) incubated first for 3 days in incubation medium, and then for the indicated times with added 10 μ M GA₃ (lanes 3–7). (+) indicates that the embryoless seeds were incubated intact while (–) indicates that the starchy endosperm was dissected away from the aleurone layer prior to the incubation with GA₃. 300 ng of polyA(+) mRNA was applied in each lane. Hybridized to B15C and low-pI α -amylase (a germination-specific cDNA).

the embryo transcript. The coding region of the embryo sequence was found to be identical to that of the aleurone with the help of RT-PCR performed on embryo mRNA. Also, sequencing of 8 clones from an embryo cDNA library showed no divergence between *Per1* in the embryo and that of the aleurone sequence within leader and trailer regions. These sequences were compared to the exon sequences of a *Per1* gene (accession number X96551) isolated from a genomic barley library, and found to be identical. Secondly, genomic hybridizations show a single hybridizing band for most restriction endonucleases tested (not shown). Third, primer extension studies performed on mRNA from aleurone and embryos of developing barley grains further substantiate that the same gene is expressed in both tissues since the extension products have exactly the same length (Fig. 6A). Finally, transient expression studies using a *Per1* promoter-GUS reporter-gene construct (Fig. 6B), transferred via particle bombardment into both the aleurone and the embryo, confirm that the same *Per1* promoter is active in both tissues

(Fig. 6B). Embryos incubated on 1/2 MS medium alone germinate rapidly and transcription of *Per1* is terminated (panel I). However, expression is maintained in the embryo when incubated on 1/2 MS medium containing the germination-inhibiting osmotic agent 20% mannitol (panel II). In agreement with *in situ* data [1], GUS expression units are most concentrated in the embryo apices although an even distribution of bombarded gold particles is evident over the entire embryo. Panel III shows that active transcription from the same *Per1* promoter is observed in the aleurone layer when incubated on 1/2 MS medium. Panel IV shows a control bombardment using the actin-GUS construct pACT1-F [37].

Discussion

Per1 is a single-copy gene which displays strict seed-specific expression patterns

Our sequencing, genomic Southern, primer extension and particle bombardment data clearly show that the temporally and spatially highly regulated peroxiredoxin in *Per1* transcript is encoded by one gene. This is supported by mapping data which has localized the *Per1* gene to the short arm of chromosome 2 [27]. Exerting expression of the same *Per1* gene in the aleurone layer and the embryo exemplifies one possible mode of coordinating similar temporal expression patterns in two parts of the seed. In contrast to Group II and III *Lea* genes, *Per1* is not induced by desiccation stress in leaf or root tissue [16] nor is it induced by ABA in these vegetative tissues (not shown). *Per1*'s presence in embryogenic cell suspensions, but not in non-embryogenic cultures [1, 38], further substantiates the association of *Per1* to seed development. Therefore, in many respects, the seed peroxiredoxin, PER1, differs from most described seed expressed antioxidants, such as catalases (Cat), superoxide dismutases (Sod), and peroxidases since they are encoded by gene families, are often induced to high levels during germination and are in most cases not seed-specific [5, 44, 49].

Per1 is down-regulated by GA in the aleurone

Previous data show the *Per1* transcript in the embryo to disappear upon germination [1]. Data presented here show *Per1* to disappear in the aleurone layer of non-dormant seeds upon germination as well, and that this disappearance is controlled by the embryo. We have

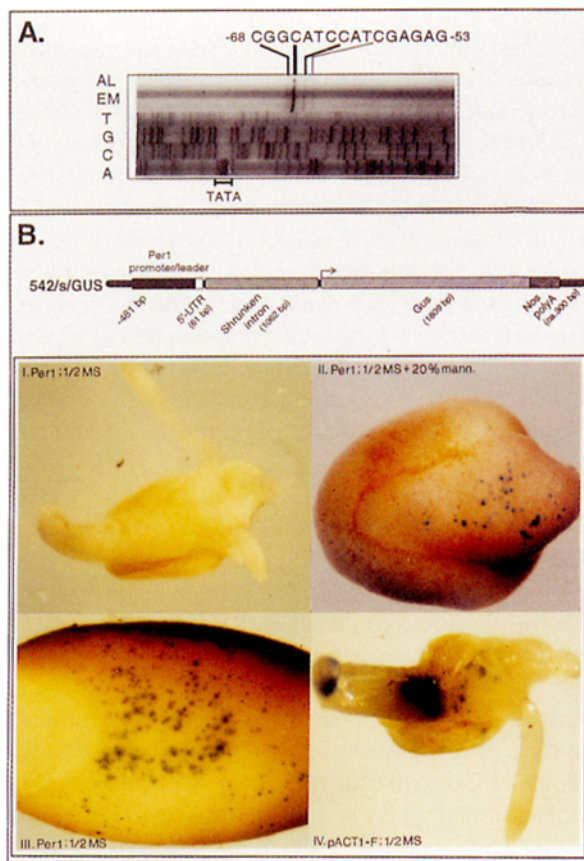


Figure 6. Primer extension on *Per1* mRNA (A) and transient expression of the *Per1* promoter (B) in aleurone tissue and the embryo. A. AL and EM indicate extension products performed upon mRNA isolated from 26 dpa aleurone (from 150 mg tissue) and embryo (50 mg tissue), respectively. Lanes adjacent show the sequence of the *Per1* gene using the same end-labeled primer. Position of transcription start sites are indicated next to the extension products, while relative amounts of extension products are indicated by thickness of lines. The main extension product shows transcription of *Per1* to be initiated from a C nucleotide at position -65 from start of translation. TATA indicates the putative TATA box. B. Map of the *Per1* promoter/GUS construct 542/s/GUS, which includes the Shrunken-1 intron [36]. Representative results of particle bombardment with 542/s/GUS are shown below the map. Text within each panel indicates the construct and type of incubation medium used for each bombardment. Panel IV shows representative results of bombardment with a strongly expressed control plasmid pACT1-F [37], which may be used for comparison to panel I.

further shown *Per1* to be down-regulated in the mature aleurone by GA₃. Many genes have been identified which are up-regulated in the aleurone by GA, such as α -amylases, β -glucanases and proteases (reviewed in [13]). Down-regulation by GA, as seen for *Per1*, has, however, been described for very few genes. Nolan and Ho [41] identified, in a detailed examination, three genes which are down-regulated by GA₃, while

high concentrations of ABA counteract the GA effect (Adh1, pHvU-1, PAPI). We have observed ABA to counteract the GA effect on *Per1* in incubations where α -amylase expression is inhibited by ABA (T. Steinum, H. Berner, R. Stacy and R. Aalen, manuscript in preparation).

For the few GA repressible mRNAs identified the mechanism of decrease in transcript levels by GA is unknown, but likely to involve lowered mRNA stability [41]. Data on two recently characterized GA-repressible cDNAs (pHvGS-1 and pCHth3) indicate, however, that transcriptional control may also be involved [17]. Further studies are needed to elucidate the mechanism of GA repression for *Per1*.

Per1 is dormancy-related in barley

Investigations of *Hordeum* varieties possessing varying degrees of dormancy show *Per1* to be dormancy-related in the isolated embryo and in the aleurone layer of whole barley seeds. Interestingly, also during *in vitro* embryogenesis in barley, where the *Per1* transcript can function as an embryogenic marker [1, 38], the transcript disappears only when microspore-derived embryos are cultured in media allowing germination [38]. This is in agreement with our findings (Agneta dormancy broken, Figs. 2 and 3) and those of Goldmark *et al.* [16], which show *Per1*/pBS128 levels to decline in embryos before visible signs of germination are evident. Taken together, these facts suggest that the presence of PER1 is either directly inhibiting germination or indicating conditions which inhibit germination, assuming that protein levels correlate with mRNA levels. Future studies will be directed towards production of antibodies against PER1 in order to confirm a dormancy-related protein accumulation pattern.

Clearly, both with respect to *Per1* and Rab16A transcripts, the accumulation pattern in the embryoless seeds differs from the situation in the intact seed. There does not seem to be a correlation between the levels of whole-seed dormancy of the different varieties, and transcript accumulation in the aleurone of their embryoless grains. This is in contrast to results of van Beckum *et al.* who used short-day grown dormant and long-day grown non-dormant barley seeds [52]. Their and our results underline that both environmental conditions and genetic background contribute to the degree of dormancy. Similar expression patterns in aleurone layers of whole and embryoless seeds for *Per1* and Rab16A are seen only for the dormant Agneta seeds (over three days of imbibition). For the less dormant

cultivars and dormancy broken Agneta, *Per1* transcript levels are stable in embryoless seeds and decrease in whole seeds, reflecting their sensitivity to germination signal(s). Without germination, a high *Per1* transcript level is found in the aleurone layer, as in the embryo.

The peroxiredoxin transcript is highly conserved between barley and brome grass and is present in several other grasses and cereals [1, 16, 53]. This suggests a relationship to dormancy in other monocotyledonous species as well. Work in our lab is also directed towards the study of this peroxiredoxin in the dicot *Arabidopsis thaliana*.

PER1 is a functionally active tissue-specific antioxidant

Our results are the first to confirm antioxidant activity *in vitro* of a plant peroxiredoxin, and the first to show that a member of the 1-Cys peroxiredoxin subgroup possesses such activity. 2-Cys enzymes have been shown to give full protection of DNA in a thiol-MFO system with 50–60 μ g purified protein per ml reaction mixture [34, 35]. Increasing amounts of PER1 fusion protein gives increasing protection in this system, although even with the highest concentration of fusion protein, which corresponds to about 270 μ g purified enzyme per ml, full protection could not be shown.

Different substrate specificities have been observed for the 2-Cys subgroups TPx (thioredoxin peroxidase or TSA) and AhpC, which preferentially remove H₂O₂ and alkyl hydroperoxide, respectively [8]. Some differences in substrate specificity have also been observed between the 2-Cys protein TSA and a mutant where Cys-170 is replaced with a serine residue (RC170S; [54]). Differences in the mode of regeneration for 1-Cys and 2-Cys enzymes have also been observed. *In vivo* reactivation of 2-Cys enzyme activity (reduction) has been shown to most likely occur by two mechanisms, where regeneration of the TPx subgroup involves the thioredoxin system (consisting of NADPH, thioredoxin and thioredoxin reductase) [8, 29], and reactivation of the AhpC subgroup occurs with the help of NAD(P)H-dehydrogenase and NAD(P)H [19, 51]. Some evidence exists which suggests that members of the 1-Cys subgroup may be reactivated by a third mechanism, in which participation of a small thiol, like GSH, is required [8]. We find it of great interest to test the activity of PER1 more extensively, also without an N-terminal protein tag, with regard to substrate specificity and mode of regeneration.

In mammals, transcripts hybridizing to 2-Cys peroxiredoxin probes are present in a number of tissues and cell lines [24, 43]. And in barley, the 2-Cys *Bas1* transcript has been detected in all analyzed organs of the plant while highest levels of expression are observed in the youngest tissues of the shoot (M. Baier and K.-J. Dietz, pers. comm.). The 1-Cys *Per1* transcript is, as shown, restricted solely to the aleurone and embryo of developing barley seeds. It seems, therefore, not unlikely that 1-Cys and 2-Cys peroxiredoxins may play different roles in the same organism; 2-Cys variants being general protectants and the 1-Cys variant performing more specific tasks.

Two proposed roles for the PER1 peroxiredoxin during development, early imbibition and dormancy

Due to the antioxidant nature and expression patterns of *Per1*, we postulate two possible roles for the peroxiredoxin in the barley seed, under the assumption that the transcript is translated into protein. First, the restriction of *Per1* expression to the developing and mature embryo and aleurone layer, suggests that the PER1 protein is performing a protective function in the only two tissues which survive desiccation. Several studies indicate that an important aspect of desiccation tolerance is protection against free radicals (reviewed in [30, 31]), and such a role has earlier been suggested for the manganese-containing dismutase *Sod3.3* [55].

Most antioxidants are not expected to play their main protective role in desiccated and resting seeds, since they are first induced to high levels during germination, where they are assumed to protect against activated oxygen by-products of respiration [5, 44]. Respiration is, however, resumed already at the onset of imbibition, days before most antioxidant transcripts reach appreciable levels in germinating seeds. The transient up-regulation of *Per1* in the embryo during imbibition of grains with low dormancy implies that the peroxiredoxin protects against oxidative damage in this situation as well as during late seed development. Respiration is likewise resumed in hydrated dormant seeds [3], and in imbibed embryos from such grains the *Per1* transcript level increases substantially. The role of the peroxiredoxin in dormancy could therefore simply be to protect against reactive oxygen species, since non-germinating grains are not able to gain protection from germination-specific antioxidants.

Although speculative, we cannot rule out a second and not necessarily contradictory role for the peroxiredoxin in seeds, namely actual involvement in

maintenance of dormancy. Peroxiredoxins are able to remove H_2O_2 , a compound shown to break dormancy in barley as well as other cereals [14]. Endogenous H_2O_2 , which arises as a by-product of respiration, has been implicated to play a role in dormancy breakage *in planta* by initiating a cascade of oxido-reductions [14]. Previously, catalase has been proposed to play a regulatory role in dormant seeds, by holding H_2O_2 levels to a minimum. However, several studies have shown that there is no correlation between reduced catalase activity and dormancy release (see [18]). The aleurone/embryo specific *Per1*, rapidly induced during imbibition of dormant embryos, could be envisioned to play the regulatory role previously proposed for catalase.

The importance of H_2O_2 levels for dormancy release has been linked to the highly debated hypothesis that the activation of the oxidative pentose phosphate (OPP) pathway is the main feature leading to germination [14, 45, 47]. Several experimental studies, e.g. in wild oat and nectarine seeds have, however, failed to show increased activity of OPP prior to germination [15, 18]. On the other hand, it is interesting that a non-dormancy-related transcript identified in oat encodes glutathione peroxidase [23], one of the enzymes suggested to link H_2O_2 to the OPP pathway [14]. Additionally NO_3^- , which in contrast to reduced nitrogen (NH_4) can break dormancy and induce the OPP pathway [45], is the prominent nitrogen source in media promoting germination during barley microspore derived *in vitro* embryogenesis [38].

Conclusions

The *Per1* transcript is encoded by one gene expressed in the embryo and aleurone cells. The embryo, most likely through GA, controls the disappearance of this transcript from the aleurone layer during germination. In considering its antioxidant function, the PER1 protein could play a role in protection against oxidative stress during seed desiccation and during imbibition of mature seeds. *Per1* expression is dormancy-related, but whether PER1 is involved in the control of dormancy needs to be investigated. Therefore, future analyses of PER1 protein levels in developing, dormant and germinating barley seeds will be of great interest.

Note added in proof

During the reviewing process of our manuscript a paper on the barley 2-Cys peroxiredoxin, *bas1*, has been accepted for publication (Baier and Dietz, Plant Mol Biol, in press).

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