



RESEARCH PAPER

A germin-like protein with superoxide dismutase activity in pea nodules with high protein sequence identity to a putative rhicadhesin receptor

Sébastien Gucciardo, Jean-Pierre Wisniewski, Nicholas J. Brewin and Stephen Bornemann*

John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

Received 2 August 2006; Revised 5 November 2006; Accepted 27 November 2006

Abstract

The cDNAs encoding three germin-like proteins (PsGER1, PsGER2a, and PsGER2b) were isolated from *Pisum sativum*. The coding sequence of PsGER1 transiently expressed in tobacco leaves gave a protein with superoxide dismutase activity but no detectable oxalate oxidase activity according to in-gel activity stains. The transient expression of wheat germin gf-2.8 oxalate oxidase showed oxalate oxidase but no superoxide dismutase activity under the same conditions. The superoxide dismutase activity of PsGER1 was resistant to high temperature, denaturation by detergent, and high concentrations of hydrogen peroxide. In salt-stressed pea roots, a heat-resistant superoxide dismutase activity was observed with an electrophoretic mobility similar to that of the PsGER1 protein, but this activity was below the detection limit in non-stressed or H₂O₂-stressed pea roots. Oxalate oxidase activity was not detected in either pea roots or nodules. Following *in situ* hybridization in developing pea nodules, PsGER1 transcript was detected in expanding cells just proximal to the meristematic zone and also in the epidermis, but to a lesser extent. PsGER1 is the first known germin-like protein with superoxide dismutase activity to be associated with nodules. It shared protein sequence identity with the N-terminal sequence of a putative plant receptor for rhicadhesin, a bacterial attachment protein. However, its primary location in nodules suggests functional roles other than as a rhicadhesin receptor required for the first stage of bacterial attachment to root hairs.

Key words: Germin-like protein, nodule, oxalate oxidase, *Pisum*, rhicadhesin, superoxide dismutase.

Introduction

Germins and germin-like proteins (GLPs) are cell wall glycoproteins with robust quaternary structures that show an unusual resistance to detergent treatment, heat denaturation, and degradation by proteases (Carter and Thornburg, 1999; Bernier and Berna, 2001). It has been shown that germin and GLPs can sometimes play a structural role as targets for protein cross-linking to reinforce the cell wall during pathogen attack (Schweizer *et al.*, 1999; Christensen *et al.*, 2004). Some members of the GLP family are associated with activities such as oxalate oxidase (Dumas *et al.*, 1993; Lane *et al.*, 1993), superoxide dismutase (SOD) (Yamahara *et al.*, 1999; Carter and Thornburg, 2000; Nakata *et al.*, 2002, 2004; Segarra *et al.*, 2003; Tabuchi *et al.*, 2003; Christensen *et al.*, 2004; Zimmermann *et al.*, 2006), phosphodiesterase (Rodriguez-Lopez *et al.*, 2001), and possibly serine protease inhibition (Segarra *et al.*, 2003). Oxalate oxidase and SOD activities result in the production of hydrogen peroxide and, for this reason, germins and GLPs have been implicated in cell wall strengthening and resistance to pathogen attack and abiotic stresses (Vallelian-Bindschedler *et al.*, 1998; Schweizer *et al.*, 1999; Christensen *et al.*, 2004; Zimmermann *et al.*, 2006).

The accumulation of germin and GLP sequences has led to a classification within the context of the cupin superfamily of beta-barrel proteins (Dunwell *et al.*, 2000). The GLPs have, in turn, been classified into subfamilies (Carter *et al.*, 1998; Carter and Thornburg, 2000). For example, the true germin subfamily includes proteins with oxalate oxidase activity, such as wheat and barley germins. Both GLP subfamilies 1 and 2 contain examples of proteins with SOD activity. Subfamily 3 includes the phosphodiesterase described above. Further

* To whom correspondence should be addressed. E-mail: stephen.bornemann@bbsrc.ac.uk

subdivisions have been proposed recently (Nakata *et al.*, 2004). A key feature of the GLP-related subfamilies, including the germins (Carter and Thornburg, 2000), is the conservation of a motif derived from that of the cupin superfamily (Carter and Thornburg, 1999; Khuri *et al.*, 2001). As with the cupin motif, the motif of GLPs is composed of two submotifs intercalated with an intermotif region with a variable length, usually between 19 and 25 amino acids. The motif contains three highly conserved histidines and one glutamate residue involved in binding a metal ion. Sequences belonging to the GLP family also include at least one or two putative *N*-glycosylation sites at conserved positions. These glycosylations are thought to be responsible for the shift between the predicted size of the monomer and the molecular weight visualized by SDS-PAGE. Different extents of *N*-glycosylation are also responsible for the presence of two germin isoforms named G and G', seen as a doublet on SDS gels, derived from a single gene product. This difference in glycosylation could be the result of specific cellular localization (or compartmentation) of the protein (Jaikaran *et al.*, 1990; Lane, 1994; Lerouge *et al.*, 1998). GLPs are typically hexameric, being trimers of dimers, although in some cases different oligomerization states have been identified, as for renatured nectarin I from tobacco (Carter and Thornburg, 2000).

Antioxidants are essential in plants and rhizobia-legume symbioses, primarily because of the formation of superoxide as a side-product of respiration, photosynthesis, and nitrogen fixation (Matamoros *et al.*, 2003). Both plants and bacteroids possess SOD isoforms in various subcellular compartments; cytosolic and plastidial Cu/Zn-SOD, mitochondrial and bacteroidal Mn-SOD, and plastidial and cytosolic Fe-SOD. Bacterial infection also leads to the production of reactive oxygen species. Initial production is thought to be part of the oxidative burst, but subsequent production may be more related to cell wall formation and the cross-linking of glycoproteins, which are essential elements of successful infection (Brewin, 2004). Possible sources of hydrogen peroxide in cell walls include peroxidases, oxalate oxidase, and diamine oxidase (Wisniewski and Brewin, 2000). One paper describes the presence of an oxalate oxidase in bacteroids (Trinchant and Rigaud, 1996). There is evidence that a plant Cu/Zn-SOD is partially located in the cell wall and co-locates with hydrogen peroxide production in pea nodules (Rubio *et al.*, 2004). Alternatively, an apoplasmic oxalate oxidase or GLP SOD could be the source. However, the existence of a legume-derived GLP SOD or oxalate oxidase in nodules has not been reported previously.

In the present study, three pea GLP cDNAs, PsGER1, PsGER2a, and PsGER2b, were isolated. The PsGER1 protein was demonstrated to be associated with heat-stable SOD activity similar to that of the extracellular nectarin I

GLP (Carter and Thornburg, 2000) that is found in the nectar of tobacco. Interestingly, PsGER1 also shared sequence identity at its N-terminus with a putative plant receptor protein for the Rhizobiaceae attachment protein, rhicadhesin (Swart *et al.*, 1994). Furthermore, it was demonstrated that heat-stable soluble SOD activity with similar electrophoretic mobility to PsGER1 was detectable in pea roots subjected to salt stress. In tissue sections derived from pea nodules, *in situ* hybridization showed a specific localization of the PsGER1 mRNA in the nodule apex. The first known plant-derived GLP SOD of nodules is discussed in relation to the previously proposed role as a putative rhicadhesin receptor protein (Swart *et al.*, 1994) and to alternative roles in plant cell development and stress biology.

Materials and methods

Isolation of cDNA

Germin oxalate oxidase cDNAs and GLP sequences exhibit a consensus motif (QDFCVA/G) near the N-terminal end of the mature protein sequence. Degenerate PCR primers (5'-TNCAR-GAYTTYTGYGTNGC) based on this conserved sequence were used to obtain 3'-RACE products from pea root RNA extracted using a QIAGEN[®] RNA mini-kit (QIAGEN, Poole, UK). This led to the cloning of partial cDNA sequences, PsGER1, PsGER2a, and PsGER2b. The coding sequence of PsGER1 was completed by 5'-RACE using reverse primers ROXO-1 (5'-GGTACATTGGAA-GCTGGTTTGCAT) and ROXO-2 (5'-TTGAGAAGAAATCTTC-TGCGGTTAC). Finally, the full-length clone was obtained by PCR on pea root cDNA using V2001f (5'-ACGCGTCCGACCACGAT-GAAGC) and V2002r (5'-CCATCGATGGCCAACAACACTACTTC) with pea root cDNA. The PsGER1, PsGER2a, and PsGER2b sequences were deposited in the NCBI database under the accession numbers AJ250832, AJ250833, and AJ250834, respectively.

Southern hybridization

Total DNA from leaves of pea plants was isolated according to Dellaporta *et al.* (1983) and digested to completion with *Eco*RI, *Hind*III, *Sph*I, and *Xba*I. Restricted DNA was then separated on a 0.8% agarose gel, denatured, and blotted onto a Hybond N⁺ membrane (Amersham). A 520 bp PCR-amplified DNA fragment of PsGER1 was labelled using the BrightStar psoralen-biotin kit (Ambion) and hybridized to the membrane overnight at 65 °C in 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 mg ml⁻¹ denatured herring sperm DNA. The filter was washed at 65 °C in 2× SSC, 0.1% SDS (2×10 min); 1× SSC, 0.1% SDS (15 min); and 0.5× SSC, 0.1% SDS (15 min). The hybridized biotinylated probes were detected using the BrightStar BioDetect kit (Ambion) according to the manufacturer's recommendations.

Transient assays in *Nicotiana benthamiana*

The cDNAs encoding GLPs were expressed *in planta* following the protocol for *Agrobacterium* infiltration into the abaxial air space of *N. benthamiana* leaves (Kapila *et al.*, 1997). *Agrobacterium* strains, harbouring wheat germin oxalate oxidase gf-2.8 or PsGER1 sequences cloned into the binary vector pBIN61 (Voinnet *et al.*, 2000), were co-inoculated with an *Agrobacterium* strain containing the sequence of the suppressor of silencing, p19, cloned behind the 35S promoter of pBIN61 (Voinnet *et al.*, 2003). As a negative

control, tobacco leaves were infiltrated with the empty binary vector pBIN61.

Protein extraction and SDS-PAGE

Soluble protein samples were extracted from plant leaves, which were previously ground in the frozen state, with 100 mM TRIS-HCl buffer, pH 8.0, separated from insoluble material by centrifugation, and quantified using Bradford reagent (Bio-Rad, Poole, UK) before heat treatment at 85 °C for 5–10 min. After centrifugation at 10 000 g for 10 min, supernatants corresponding to 30 µg were loaded on a 10% SDS-polyacrylamide gel in Laemmli buffer (Sambrook *et al.*, 1989). Samples contained SDS but did not contain a reductant and were not boiled; hence the gels were described as semi-native. For transient expression, protein was extracted 8 d post-*Agrobacterium* infiltration. Gels were stained for protein with Coomassie blue.

SOD assay

For positive staining, gels were soaked for 1 h at room temperature in 2 mM dianisidine, 0.1 mM riboflavin, 10 mM potassium phosphate at pH 7.2. After a brief rinse with potassium phosphate buffer, gels were illuminated for 5–50 min (Misra and Fridovich, 1977). A brown band, marking the localization of SOD activity, became visible after 5 min of illumination and reached maximum intensity after 50 min of exposure. Gels were digitally imaged, and the intensity of the reaction product, associated with the specific SOD activity, was quantified using the AIDA program version 3.11 (Raytest GmbH).

For negative SOD staining, the gels were incubated for 1 h in the dark in 80 ml of 0.1 M potassium phosphate, pH 7.8, containing 1 mg riboflavin, 16 mg nitro-blue tetrazolium, and 200 µl *N,N,N',N'*-tetramethylethylenediamine (Beauchamp and Fridovich, 1971). Gels were then incubated in 0.1 M potassium phosphate buffer, pH 7.8, on a light table for 30 min to 1 h. Nitro-blue tetrazolium is converted from a light yellow substrate to a dark blue product upon reduction by superoxide produced by riboflavin. Nitro-blue tetrazolium was not reduced within regions of the gel where SOD activity had consumed the superoxide and so a negatively stained band indicated SOD activity.

SOD inhibition

Extracted proteins were subjected to semi-native SDS-PAGE. Gels were then incubated for 1 h in phosphate buffer (10 mM) containing different concentrations of H₂O₂ up to 5 mM H₂O₂. Following this preincubation, gels were rinsed in phosphate buffer and negatively stained for SOD activity. The commercial enzymes, Fe-SOD from *Escherichia coli* and Cu/Zn-SOD from bovine erythrocytes, were used as controls.

Oxalate oxidase assay

All chemicals and biochemicals were obtained from Sigma (Poole, UK) unless otherwise stated. Oxalate oxidase activity in semi-native SDS-PAGE gels was detected by immersing the gel in a cocktail of 2 mM oxalic acid, 0.5 mg ml⁻¹ 4-chloro-1-naphthol, and 5 U ml⁻¹ horseradish peroxidase in 50 mM citrate, pH 4.0, containing 60% ethanol (Zhang *et al.*, 1996). Enzyme activity was visualized after 45 min at room temperature. A negative control, to rule out laccase activity, omitted oxalate from the reaction cocktail.

Pea root culture and stress conditions

Peas (variety Frisson), supplied by the John Innes Centre seed store, were grown in an aeroponic system for 5 d post-germination in Fahraeus medium (Fahraeus, 1957). Plants were then placed in a beaker containing different aqueous solutions such as water, NaCl

(250 mM), or H₂O₂ (5 mM) so that the roots were completely immersed in the liquid. Plants were left standing for 8 h in the different aqueous solutions under the same growth condition as in the aeroponic cultures. After this incubation, each batch of plants was divided into two groups; one destined for RNA extraction and the other destined for protein extraction.

RT-PCR

Total RNAs were extracted using an RNA mini-kit from QIAGEN®. The concentration of the samples was measured by spectrophotometry and was also estimated on agarose gels. Reverse transcription was performed using the same amount of total RNA (2 µg), with oligo d(T)_n and with or without Superscript transcriptase (Invitrogen, Poole, UK). cDNA samples were normalized using primers based on ubiquitin (Schneider *et al.*, 1999). The primers used were: Ubif, 5'-ATGCAGATYTTTGTGAAGAC; and Ubir, 5'-ACCACCACGRAGACGGAG. The primers used for the PCR on PsGER1 were: V2003, 5'-GATGCCGATGCTCTTCAAGATC; and V2004, 5'-GCAGGTTCCCAACCATTGTC. The PCRs were usually performed with 4 µl of cDNA, 330 pmol of each primer, and commercial *Taq* polymerase (Perkin Elmer). The PCR programme used was 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 50 s.

In situ hybridization

The spatial expression of PsGER1 in the mature root nodule of pea was investigated by *in situ* hybridization. Pea nodules were harvested at 2 weeks post-inoculation with *Rhizobium leguminosarum* bv. *viciae* 3841, fixed in 4% formaldehyde in phosphate-buffered saline, and embedded in paraffin wax as described by Jackson (1991). Longitudinal nodule sections, 8 µm thick, were prepared with a Jung microtome (Heidelberg, Germany), and attached to γ -aminopropyltriethoxysilane-treated glass microscope slides. *In situ* hybridization was carried out as described by Gardner *et al.* (1996) with digoxigenin-labelled riboprobes prepared from linearized plasmids carrying sense and antisense PsGER1 inserts primed by the T7 promoter.

Results

Isolation of GLP cDNAs

A comparison of published germin oxalate oxidase and GLP sequences had revealed a consensus motif (QDFCVA/G) near the N-terminal end of the mature protein sequences (Membre *et al.*, 1997). This consensus motif was used to isolate pea GLP cDNA clones using degenerate PCR primers. Sequencing analysis revealed three GLP cDNAs, henceforth named PsGER1, PsGER2a (both 730 nucleotides), and PsGER2b (770 nucleotides). At the nucleotide sequence level, PsGER2a and PsGER2b shared 97% identity (594 out of 609 nucleotides aligned). At the protein sequence level, PsGER1 [GenInfo Identifier (GI) number 6689034] shared 52% identity and 65% similarity with PsGER2a (GI 6689036, 195 amino acids). PsGER2a shared 98% sequence identity with PsGER2b (GI 6689038, 195 amino acids), differing by only three amino acid substitutions. All three GLPs protein sequences contained glutamate and three histidines associated with the cupin motif.

A BLAST search (Altschul *et al.*, 1997) with the PsGER2a protein sequence showed that it was most similar to GLP3 from *Vitis vinifera* (GI 34304196, $5e^{-68}$, 70% identity and 79% similarity). It was also very similar to members of the GLP subgroup 1, as defined in the phylogenetic analysis conducted by Carter and Thornburg (1999), such as AtGLP2a from *Arabidopsis thaliana* (GI 15241662, $2e^{-65}$, 68% identity and 78% similarity). It was much less similar to the *Barbula unguiculata* GLP SOD (GI 27529869, $8e^{-44}$, 54% identity and 66% similarity), which is also a member of subfamily 1 according to Carter and Thornburg (1999), or of the bryophyte subfamily 2 according to Nakata *et al.* (2004). It was also much less similar to *Hordeum vulgare* germin (GI 83754952, $6e^{-39}$, 46% identity and 61% similarity). This protein has oxalate oxidase activity (Dumas *et al.*, 1993; Lane *et al.*, 1993) and is a defining member of the true germin subfamily (Carter and Thornburg, 1999) along with wheat germin oxalate oxidase gf-2.8 (GI 121129), which is 95% identical and 97% similar.

A BLAST search with the PsGER1 sequence identified the most similar protein to be the *A. thaliana* GLP, AtGLP10 (GI 15228673, $6e^{-71}$, 71% identity and 83% similarity). AtGLP10 belongs to the GLP subfamily 2. Nectarin I (GI 15341550) is also a member of this subfamily and has been demonstrated to be a SOD (Carter and Thornburg, 2000). The BLAST search revealed that nectarin I also shares very significant sequence identity with PsGER1 ($8e^{-70}$, 70% identity and 84% similarity). *Hordeum vulgare* germin oxalate oxidase was much less similar to PsGER1 ($9e^{-43}$, 50% identity and 65% similarity).

This bioinformatic analysis suggested that PsGER1 was likely to possess SOD activity but not oxalate oxidase activity. By contrast, PsGER2a and PsGER2b were the least likely to have either of these H_2O_2 -producing activities, with the caveat that the connectivity between catalytic activity and cupin subfamily membership is not absolute. Therefore, the next step was to test the activity of PsGER1. Prior to this, the coding sequence of PsGER1 was completed by 5'-RACE and the full-length clone was obtained with pea root cDNA. This yielded a 1160 nucleotide sequence coding for a 217 amino acid protein. The protein molecular mass was predicted to be 22 978 Da. A 20 amino acid secretion signal peptide was predicted (Nielsen *et al.*, 1997; Emanuelsson *et al.*, 2000), giving a predicted mature protein molecular mass of 20 982 Da. Since other GLPs are secreted, the presence of a secretion signal was to be expected (Bernier and Berna, 2001). Three putative glycosylation sites conforming to the NX(S/T) motif were present.

Southern hybridization

PsGER1 was shown to be a single copy gene using Southern hybridization with total pea leaf genomic DNA

(Fig. 1). The PsGER1 gene contained one *EcoRI* site resulting in two bands when this restriction enzyme was used (Fig. 1, lane A). All the remaining restriction enzymes used yielded a single band.

Transient expression of germin and GLP

PsGER1 and the wheat oxalate oxidase gf-2.8 were each transiently expressed in tobacco leaves together with p19, a suppressor of silencing (Voinnet *et al.*, 2003). The empty vector (pBIN61) used to clone the germin and GLP sequences was used as a negative control throughout. Proteins were extracted from disrupted plant tissue in TRIS-buffer and partially purified by heat treatment (see below) before being separated by semi-native SDS-PAGE. Expression of gf-2.8 showed that this heat-stable protein migrated as a doublet of 76 kDa and 67 kDa bands (Fig. 2), as reported previously (Jaikaran *et al.*, 1990). The doublet had been determined previously to be due to differential glycosylation giving the larger G and the smaller G' isoforms. No other proteins were observed at comparative levels in either this or the empty vector control samples, indicating the relative lack of any other soluble heat-stable proteins. Without heat treatment, the germin bands were less conspicuous due to the presence of numerous other protein bands. The unexpectedly higher mobility of active oxalate oxidase hexamers in semi-native SDS-PAGE (128 kDa predicted for the non-glycosylated protein) has been observed previously (Dumas *et al.*, 1993; Requena and Bornemann, 1999), presumably being due to the protein's unusually compact structure (Woo *et al.*, 2000).

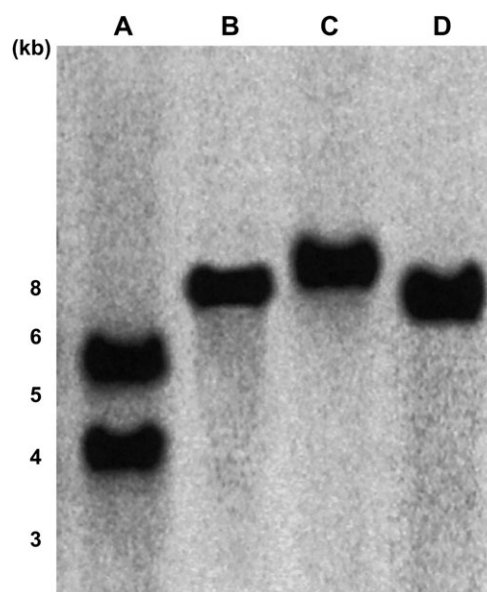


Fig. 1. Southern blot of pea genomic DNA hybridized with a partial probe of PsGER1. Genomic DNA (10 μ g) was restricted with *EcoRI* (lane A), *HindIII* (B), *SphI* (C), and *XbaI* (D). The mobility of molecular weight markers is indicated on the left.

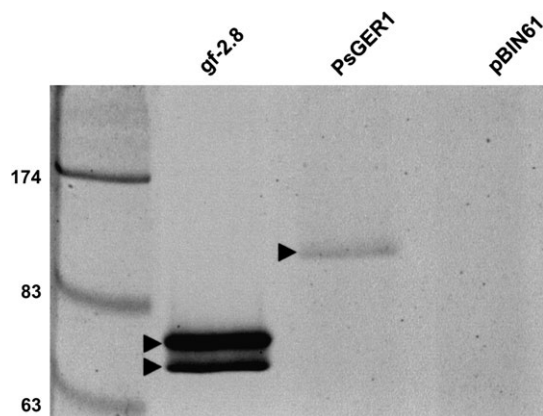


Fig. 2. Germin gf-2.8 and PsGER1 transiently expressed in tobacco leaves. A vector-only control (pBIN61) is also shown for comparison. The semi-native SDS-PAGE gel was stained with Coomassie blue. Arrowheads indicate bands of interest.

Extracts from leaves expressing the PsGER1 protein gave a band corresponding to a molecular weight of 106 kDa (Fig. 2). There appeared to be relatively less PsGER1 protein produced than gf-2.8 and this could have been due either to lower levels of expression or to greater relative association of PsGER1 with insoluble cell wall components, as has been observed previously with germin (Lane *et al.*, 1986). At higher concentrations of PsGER1, it was also possible to observe a smaller, but less abundant, band at 97 kDa (data not shown; see below). This doublet implies differential glycosylation of PsGER1 similar to that of gf-2.8, with the G isoform being predominant in both. Other GLPs give similar results (Bernier and Berna, 2001). As with gf-2.8, the mobility in the gel was higher than expected (126 kDa predicted for the non-glycosylated protein assuming homo-hexameric oligomerization as with other GLPs), but the effect was less marked with PsGER1.

SOD activity

SOD activity of PsGER1 and gf-2.8 was first tested using a positive gel stain. Following illumination for 15 min to develop the stain, a strong SOD signal appeared at a position corresponding to the electrophoretic mobility of the larger and more abundant PsGER1 isoform (Fig. 3A). This signal was detected with the same sample loading shown in Fig. 2, illustrating the ease of detecting this activity. The intensity of the stain reached saturation after about 50 min, when the minor PsGER1 G' isoform also gave a signal (data not shown; see below). The detection of SOD activity after heat treatment and semi-native SDS-PAGE showed that the enzyme activity was heat-stable (see below) and stable in the presence of the detergent SDS. No SOD signal could be detected for a sample of gf-2.8-transformed tobacco leaves (Fig. 3A) despite its relatively higher level of expression.

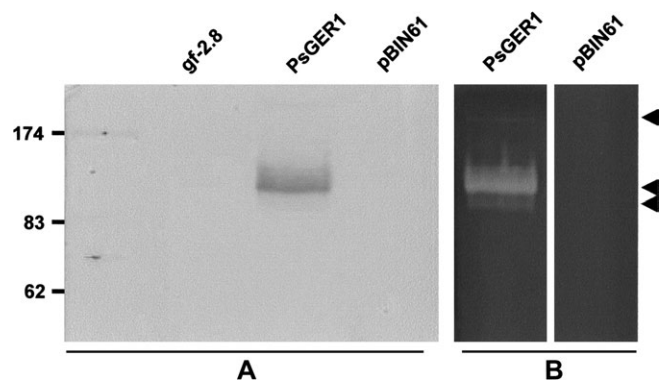


Fig. 3. Evidence for SOD activity following transient expression of PsGER1 in tobacco leaves. A semi-native SDS-PAGE gel was stained positively (A) or negatively (B) for SOD activity. Arrowheads indicate the position of SOD activity associated with PsGER1. Activity was not detected in tobacco leaves transformed with either the germin gf-2.8-expressing vector or the empty vector.

In order to confirm the SOD activity associated with PsGER1, a duplicate gel was negatively stained using nitro-blue tetrazolium. A doublet of bands was clearly visible with PsGER1 (Fig. 3B). The negative stain showed higher sensitivity than the positive stain, allowing the doublet to be more conspicuous. Furthermore, a faint band at high molecular weight (>200 kDa) was also observed in the PsGER1 samples. This indicated a minor association of possibly two hexamers of PsGER1. No SOD activity could be detected for any of the samples derived from tissues transformed with either the gf-2.8-expressing vector (data not shown) or the pBIN61 empty vector (Fig. 3B).

Thermostability of SOD activity

GLP family members are known to have stable quaternary structures that are not easily denatured at high temperature (Bernier and Berna, 2001) and the SOD activity of PsGER1 was detected after the sample was heat-treated. Therefore, the thermostability of PsGER1 SOD activity was investigated. After separation on semi-native SDS-PAGE, the gel was positively stained for SOD activity and the relative activity of each band was plotted as a function of time for each temperature of incubation (Fig. 4). SOD activity associated with the G isoform of PsGER1 showed strong thermostability. At both 80 °C and 85 °C, the enzyme activity was >95% stable for 15 min and >80% stable after 75 min. After 45 min and 60 min at 90 °C and 95°C, respectively, SOD activity was lowered to <10% of the initial activity.

Resistance to hydrogen peroxide

GLP SODs, such as nectarin I, have been demonstrated to be manganese-containing enzymes (Yamahara *et al.*, 1999; Carter and Thornburg, 2000; Christensen *et al.*,

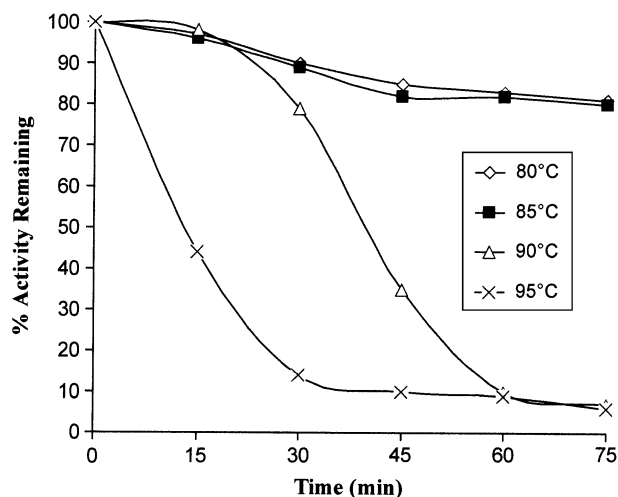


Fig. 4. Thermostability of SOD activity associated with PsGER1 transiently expressed in tobacco. PsGER1 samples were heated at different temperatures (80, 85, 90, and 95 °C) for periods of time up to 75 min and SOD activity was quantified after semi-native SDS-PAGE followed by SOD staining.

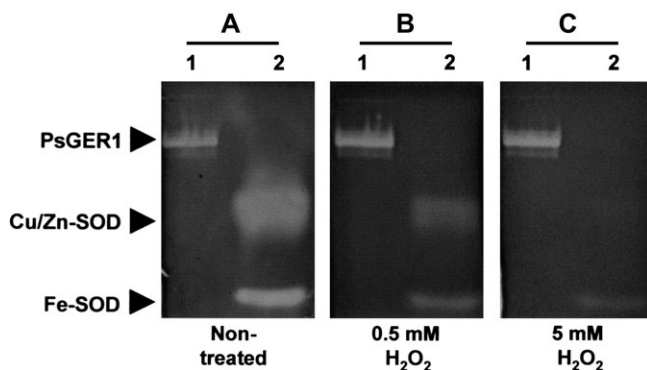


Fig. 5. Hydrogen peroxide resistance of SOD activity associated with PsGER1. Semi-native SDS-PAGE gels were pre-incubated in phosphate buffer (A), or in phosphate buffer containing either 0.5 mM H₂O₂ (B), or 5 mM H₂O₂ (C). Following preincubation, gels were rinsed in phosphate buffer and negatively stained for SOD activity. Lane 1, PsGER1; lane 2, a mixture of commercial SOD enzymes comprising a Cu/Zn-SOD and Fe-SOD (30 U each).

2004). It is known that Mn-SODs are resistant to treatment with 5 mM H₂O₂, unlike Fe- and Cu/Zn-SODs (Koster and Slee, 1980; Geller and Winge, 1983). It has been shown here that treatment with concentrations of H₂O₂ up to 5 mM had no effect on the SOD activity associated with PsGER1 (Fig. 5), suggesting that it is a Mn-SOD. In parallel experiments, a mixture of commercial Cu/Zn- and Fe-SODs was used as a control. As expected, a partial lowering of activity was observed with the Fe-SOD after treatment with 5 mM H₂O₂, while the activity of the Cu/Zn-SOD was dramatically decreased to below the detection limit.

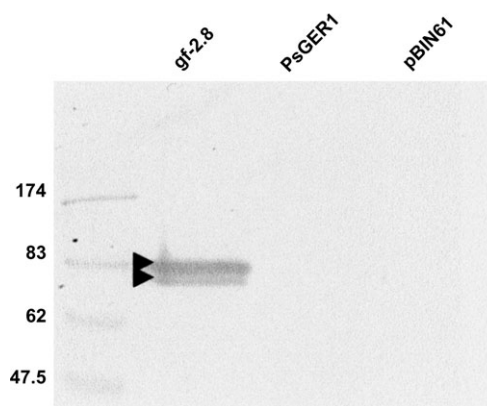


Fig. 6. Oxalate oxidase activity detected in a semi-native SDS-PAGE gel with germin gf-2.8 and PsGER1 proteins transiently expressed in tobacco leaves. A vector-only control (pBIN61) is also shown for comparison. Arrowheads indicate the position of the doublet bands associated with germin gf-2.8 oxalate oxidase activity, G being the upper band and G' being the lower.

Oxalate oxidase activity

Extracts from leaves expressing PsGER1 and wheat germin gf-2.8 were tested for oxalate oxidase activity using a specific gel stain (Fig. 6). In a control experiment with the gf-2.8 sequence transiently expressed in leaves of *N. benthamiana*, both protein isoforms showed oxalate oxidase activity within 45 min, with stronger staining for the G isoform, as expected from the protein levels observed in Fig. 2. Loading one-fifth of this sample still gave a detectable signal. Similar results were obtained with a stable transgenic line in tobacco (data not shown). However, following transient expression of PsGER1, no oxalate oxidase activity could be detected under the conditions tested, even after 24 h of incubation. PsGER1 transient expression was lower than that of gf-2.8, but applying several-fold more of the PsGER1 sample to a gel still did not give any detectable activity, even after sample concentration using centrifugal ultrafiltration devices. Given that PsGER1 exhibited SOD activity following heat treatment and semi-native PAGE, it seems unlikely that heat denaturation could have masked any intrinsic PsGER1 oxalate oxidase activity.

Tissue-specific expression of PsGER1

In order to investigate the pattern of expression of PsGER1 from different pea tissues, total RNAs were extracted and cDNAs were obtained by using RT-PCR. Ubiquitin primers were used as a control to normalize the concentration of cDNA in each sample. The expression of *PsGER1* mRNAs was detected strongly in roots, nodules, stems, and leaves, but more weakly in flowers (Fig. 7). It must be noted that the PsGER1 primers used for the RT-PCR were designed to be as specific as possible. Searches with the primer sequences against all available databases gave only single matches, even

allowing for nearly exact matches (bearing in mind that not all of the pea genome is currently known). Furthermore, RT-PCR with these primers yielded only PsGER1 with no PsGER2a, PsGER2b, or other transcripts.

SOD activity in pea roots grown under stress conditions

Germin and GLPs have been shown to be regulated during abiotic stress responses of plants (Hurkman and Tanaka, 1996; Nowakowska, 1998; Berna and Bernier, 1999; Nakata *et al.*, 2002; de los Reyes and McGrath, 2003; Tabuchi *et al.*, 2003). Therefore, the SOD activity in pea roots was monitored after abiotic stresses (250 mM NaCl or 5 mM H₂O₂). Following protein extraction, it was observed that pea roots treated with salt showed SOD activity on a semi-native gel (Fig. 8A). This SOD enzyme

activity, which was also heat resistant up to 85 °C like that of PsGER1, co-migrated with the G' isoform of PsGER1 transiently expressed in tobacco. No corresponding protein band could be detected with Coomassie staining and the level of activity was lower than that observed when transiently expressed in tobacco, reflecting the low endogenous level of soluble protein in the native tissue. Non-stressed and H₂O₂-stressed pea roots did not give a detectable SOD signal. The presence of PsGER1 transcript was detected using RT-PCR in both stressed and non-stressed roots (data not shown). It has not been possible to detect oxalate oxidase activity in either pea roots or nodules (data not shown).

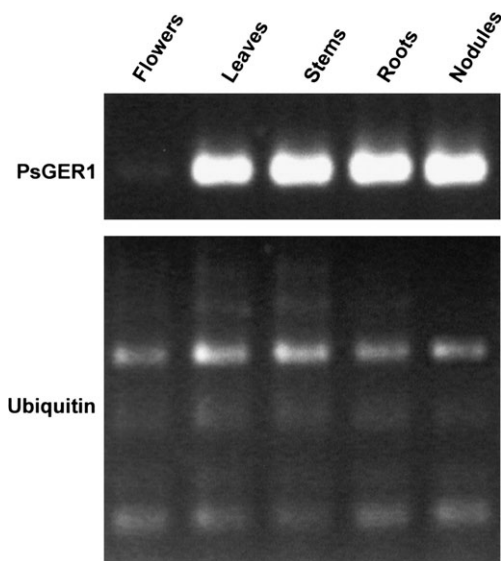


Fig. 7. Expression pattern of PsGER1 in different pea tissues after production of cDNA by RT-PCR. Ubiquitin primers were used as a control, and V2003 and V2004 primers were used to amplify PsGER1 transcripts.

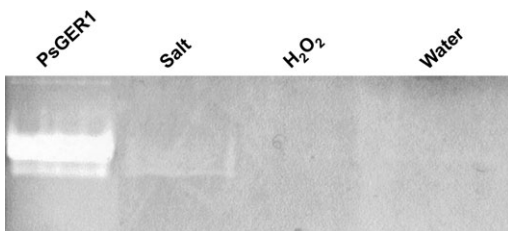


Fig. 8. Detection of SOD activity in pea roots subjected to abiotic stresses. Proteins were extracted from pea roots after incubation in either NaCl (250 mM), H₂O₂ (5 mM), or water. The semi-native SDS-PAGE gel was negatively stained for SOD activity. Protein extract from tobacco transiently transformed with PsGER1 was used as the control.

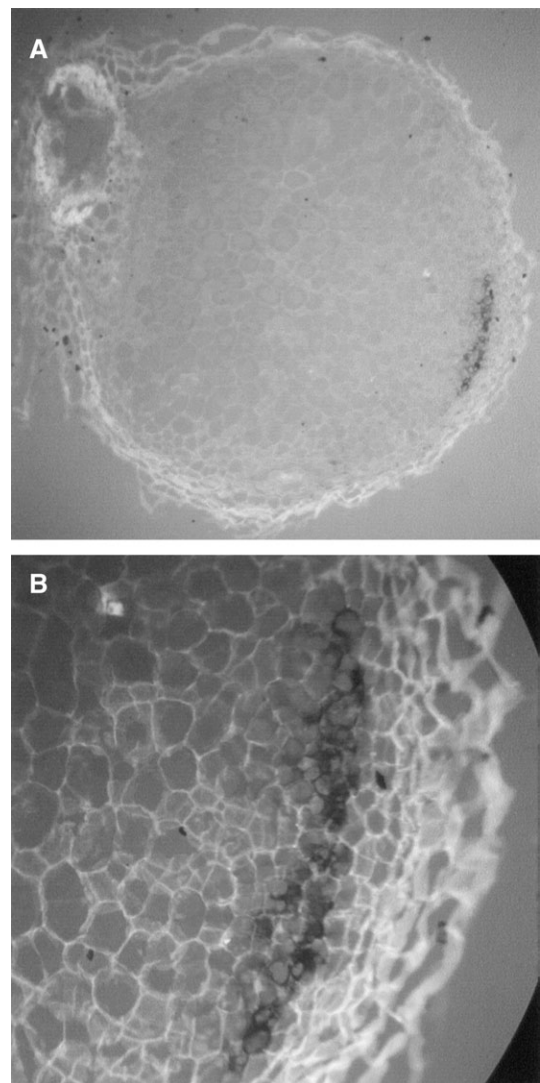


Fig. 9. *In situ* hybridization of longitudinal sections of young pea root nodules induced by *Rhizobium* spp. and probed with PsGER1 riboprobes. (A) The antisense PsGER1 probe showed a strong dark signal in the region of expanding cells just proximal to the apical meristem. A weaker signal could be observed in the epidermis. (B) Enlargement of the nodule apex.

In situ hybridization

In order to investigate the role of PsGER1 in nodulation, the PsGER1 transcript was detected by *in situ* hybridization in tissue sections from developing pea root nodules. The PsGER1 transcript was clearly expressed in the apex of young nodules in a region of expanding cells just proximal to the apical meristem (Fig. 9). In addition, PsGER1 transcript was more weakly expressed in the epidermis of the nodule.

Discussion

Three pea GLP cDNAs, of which PsGER1 was most closely related to hydrogen peroxide-forming GLPs at the protein sequence level, were isolated. Transient expression of GLPs in tobacco by leaf infiltration has not been reported before. This technique was successfully used to demonstrate that expression of the PsGER1 coding sequence from pea was correlated with expression of SOD activity (Figs 1, 2) but not with oxalate oxidase activity (Fig. 5). The amino acid residues associated with metal ion binding in cupin proteins (Khuri *et al.*, 2001) are conserved in PsGER1. It is known that the cupin superfamily can bind a number of metal ions including manganese, iron, zinc, and copper. However, the cupin proteins are all known to bind only mononuclear metal ions, making it very unlikely that PsGER1 is a Cu/Zn-enzyme. PsGER1 is most likely to be a manganese-containing protein since no iron-requiring GLP SODs have been described so far. The SOD activity of PsGER1 was resistant to high concentrations of H₂O₂ (Fig. 5) in a manner similar to other manganese-containing GLP SODs (Yamahara *et al.*, 1999; Carter and Thornburg, 2000) and mitochondrial Mn-SODs, but unlike Fe- or Cu/Zn-SODs. This provides experimental evidence that PsGER1-associated SOD activity requires a manganese cofactor. The sequence of PsGER1 is quite distinct from that of the pea mitochondrial Mn-SOD sequence, indicating independent evolutionary origins of these two enzymes. The SOD activity associated with PsGER1 was very resistant to high temperatures (Fig. 4) and the presence of SDS. This feature is presumably due to the stability of its quaternary structure, like that of other GLPs (Bernier and Berna, 2001).

It was also shown that a heat-stable soluble SOD activity in pea that co-migrated electrophoretically with PsGER1 was detectable in root tissues after treatment for 8 h with 250 mM NaCl (Fig. 8). It is interesting to note that the mitochondrial Mn-SOD protein was also up-regulated by salt stress in a recent study (Kav *et al.*, 2004). The report of Kav *et al.* (2004) was consistent with the current understanding that antioxidants are an element of the salt stress response in pea roots in a manner similar to general stress responses in plants. The PsGER1

transcript was detected in pea roots, whether stressed or not (data not shown). Further studies will be required to determine how PsGER1 SOD activity is up-regulated by salt stress. Nevertheless, the presence of the PsGER1 transcript and a heat-stable and SDS-resistant SOD activity with the same electrophoretic mobility as PsGER1 has been clearly demonstrated in pea roots.

It was also observed that two PsGER1 isoforms, equivalent to germin G and G', were present. In protein extracted from tobacco leaves expressing PsGER1, the G isoform was predominant, whereas in protein extracted from salt-treated pea roots the G' isoform appeared to be predominant. It must be borne in mind that the host plant is different in these two cases and that the relative level of glycosylation could differ for this reason. Although it is not expected that glycosylation regulates activity, it cannot be ruled out. The two isoforms of PsGER1 probably differ with respect to their level of *N*-glycosylation, as for gf-2.8 (Jaikaran *et al.*, 1990).

Wheat germin oxalate oxidase gf-2.8 was used as a control for oxalate oxidase activity and no SOD activity for this protein was detected under conditions in which PsGER1 SOD activity was easily detected. This is consistent with results obtained with the 95% identical recombinant barley germin oxalate oxidase (Whittaker and Whittaker, 2002). These data contrast with one report that barley germin oxalate oxidase possesses substantial SOD activity (Woo *et al.*, 2000).

In the majority of GLP members (but not in the germin subfamily) the tripeptide RGD, KGD, or KGE is conserved at a particular position within their sequences. In animal cells, this tripeptide domain is found in cell adhesion proteins from the extracellular matrix (such as vitronectin and fibronectin) that interact with transmembrane proteins called integrins. In the context of the *Rhizobium*-legume symbiosis, it has previously been proposed that a pea root GLP could mediate cell surface interactions with a bacterial component, rhicadhesin, thought to be involved in the first step of attachment of bacteria to legume root hair cells (Swart *et al.*, 1994). The plant protein was purified on the basis that it apparently interfered with the bacterial attachment process, and N-terminal sequence analysis subsequently revealed that it encoded a GLP protein. The reported sequence was ADADALQDL(C?)VADYASVILVNGFAS(K/Q)(P/Q)(L?) (I?) and the present N-terminal sequence translated from the PsGER1 nucleic acid sequence was, after removal of the predicted signal peptide, ADADALQDLCVADYASVILVNGFACKPAS (deviations are underlined). The only significant deviation from the putative receptor sequence was a serine to cysteine conservative substitution, the last two residues of the peptide sequence being only tentatively assigned. It is possible that the assignment of the serine in the peptide sequence was incorrect because it was followed immediately by several ambiguously

assigned amino acids. In addition, Edman sequencing without alkylation would have had difficulty in distinguishing between serine and cysteine on the 25th cycle because both would have yielded dehydroalanine. Swart *et al.* (1994) also reported an amino acid analysis of the putative receptor. However, the theoretical amino acid analysis for PsGER1 with or without its signal peptide was very different for reasons that are not clear (for example, 7.0% Glx in PsGER1 with its signal peptide rather than the reported 20.5%). The Edman sequencing data implied that this was carried out on a pure protein but perhaps the amino acid analysis was not. Nevertheless the fact that the first 24 N-terminal amino acids were identical to these two proteins makes it almost certain that they are from the same protein.

In the present study, *in situ* hybridization of pea nodule sections revealed that some PsGER1 transcript was localized in the epidermis of the nodule (Fig. 9), which is consistent with a possible role for PsGER1 in plant-bacterial surface interaction. However, the strongest expression was in the zone of cell expansion just proximal to the nodule apical meristem. Perhaps the stability of the PsGER1 protein would allow it to persist long enough to play some role in bacterial attachment later in the process of nodule invasion rather than at the first obligatory step on the root hair cell. In both the epidermis and the zone of cell expansion, the presence of H₂O₂ could serve as a regulator of cell extension or cell wall plasticity (Gucciardo *et al.*, 2005) as has been described for the defining member of the cupin superfamily, germin (Lane, 1994). PsGER1 also encodes a KGE peptide but its specific roles in cell surface attachment processes or cell plasticity have yet to be established.

Alternatively, PsGER1 could be involved in the regulation of superoxide and H₂O₂ production in the cell wall in tissue regions involved in active growth or active defence. These regions have been identified to be rich in hydrogen peroxide by Rubio *et al.* (2004). It is possible that PsGER1 is partially responsible for hydrogen peroxide production in this region. Indeed, it seems very likely that it is a secreted enzyme on the basis of sequence homology with nectarin I and its putative signal peptide. However, Rubio *et al.* (2004) have argued that the inhibition pattern of SOD activities in this region favours the Cu/Zn-SOD as the main SOD responsible for hydrogen peroxide formation rather than any Mn-dependent SOD activities. It is interesting to note that another GLP has recently been identified as a highly conserved mycorrhiza-specific induced gene (Doll *et al.*, 2003). Enzyme activity has yet to be established for the protein encoded by this gene, but it does not appear to be a member of the GLP subfamilies associated with either SOD or oxalate oxidase activities.

Described here is the first known GLP with SOD activity to be associated with nodules and it has been shown that it also shares sequence identity with a putative

bacterial attachment protein receptor. *Agrobacterium* infiltration has been successfully used to transiently express a GLP in tobacco leaves to allow the detection of enzyme activity. In order to understand the exact function of legume GLPs, it would be very useful to undertake future studies in the model legume *Medicago truncatula* which is more amenable to molecular genetic analysis. Blasts of PsGER1 on *M. truncatula* BAC clones gave a single significant hit on the BAC AC146554 which was associated with the linkage group I. By screening the *M. truncatula* tentative consensus sequences of the Institute for Genomic Research (TIGR) (<http://www.tigr.org>), the orthologue of PsGER2a (NCBI accession number AJ250833) was also isolated. Following transient expression in tobacco, biochemical assays indicated that the PsGER2 orthologue was neither a SOD nor an oxalate oxidase enzyme (data not shown), supporting the present bioinformatic analysis of the corresponding pea proteins. Additional studies of the GLP family from pea and *Medicago* will help to elucidate further the role of these proteins in plant cell growth and stress biology in the root and nodule.

Acknowledgements

This work was supported by the John Innes Foundation (SG) and by a Core Strategic Grant from the Biotechnology and Biological Science Research Council to the John Innes Centre. We thank Olivier Voinnet for the *Agrobacterium* strains and vectors used for transformation, Byron Lane for the coding sequence of wheat germin gf-2.8, and Cathie Martin for the transformed tobacco line carrying gf-2.8.

References

- Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.
- Beauchamp C, Fridovich I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276–287.
- Berna A, Bernier F. 1999. Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H₂O₂-producing enzyme. *Plant Molecular Biology* **39**, 539–549.
- Bernier F, Berna A. 2001. Germins and germin-like proteins: plant do-all proteins. But what do they do exactly? *Plant Physiology and Biochemistry* **39**, 545–554.
- Brewin NJ. 2004. Plant cell wall remodelling in the *Rhizobium*-legume symbiosis. *Critical Reviews in Plant Sciences* **23**, 293–316.
- Carter C, Graham RA, Thornburg RW. 1998. *Arabidopsis thaliana* contains a large family of germin-like proteins: characterization of cDNA and genomic sequences encoding 12 unique family members. *Plant Molecular Biology* **38**, 929–943.
- Carter C, Thornburg RW. 1999. Germin-like proteins: structure, phylogeny, and function. *Journal of Plant Biology* **42**, 97–108.

- Carter C, Thornburg RW.** 2000. Tobacco nectarin I – purification and characterization as a germin-like, manganese superoxide dismutase implicated in the defense of floral reproductive tissues. *Journal of Biological Chemistry* **275**, 36726–36733.
- Christensen AB, Thordal-Christensen H, Zimmermann G, Gjetting T, Lyngkjaer MF, Dudler R, Schweizer P.** 2004. The germin-like protein GLP4 exhibits superoxide dismutase activity and is an important component of quantitative resistance in wheat and barley. *Molecular Plant–Microbe Interactions* **17**, 109–117.
- de los Reyes BG, McGrath JM.** 2003. Cultivar-specific seedling vigor and expression of a putative oxalate oxidase germin-like protein in sugar beet (*Beta vulgaris* L.). *Theoretical and Applied Genetics* **107**, 54–61.
- Dellaporta SL, Wood J, Hicks JB.** 1983. A plant DNA miniprep: version II. *Plant Molecular Biology Reporter* **1**, 19–21.
- Doll J, Hause B, Demchenko K, Pawlowski K, Krajinski F.** 2003. A member of the germin-like protein family is a highly conserved mycorrhiza-specific induced gene. *Plant and Cell Physiology* **44**, 1208–1214.
- Dumas B, Sailland A, Cheviet JP, Freyssinet G, Pallett K.** 1993. Identification of barley oxalate oxidase as a germin-like protein. *Comptes Rendus de l'Academie des Sciences Serie III-Sciences de la Vie* **316**, 793–798.
- Dunwell JM, Khuri S, Gane PJ.** 2000. Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiology and Molecular Biology Reviews* **64**, 153–179.
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G.** 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300**, 1005–1016.
- Fahraeus A.** 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Journal of General Microbiology* **16**, 374–381.
- Gardner CD, Sherrier DJ, Kardailsky IV, Brewin NJ.** 1996. Localization of lipoxygenase proteins and mRNA in pea nodules: identification of lipoxygenase in the lumen of infection threads. *Molecular Plant–Microbe Interactions* **9**, 282–289.
- Geller BL, Winge DR.** 1983. A method for distinguishing Cu, Zn-containing and Mn-containing superoxide dismutases. *Analytical Biochemistry* **128**, 86–92.
- Gucciardo S, Rathbun EA, Shanks M, Jenkyns S, Mak L, Durrant MC, Brewin NJ.** 2005. Epitope tagging of legume root nodule extensin modifies protein structure and crosslinking in cell walls of transformed tobacco leaves. *Molecular Plant–Microbe Interactions* **18**, 24–32.
- Hurkman WJ, Tanaka CK.** 1996. Effect of salt stress on germin gene-expression in barley roots. *Plant Physiology* **110**, 971–977.
- Jackson DP.** 1991. *In situ* hybridization in plants. In: Bowles DJ, Gurr SJ, McPherson M, eds. *Molecular plant pathology: a practical approach*. Oxford: Oxford University Press, 157–181.
- Jaikaran ASI, Kennedy TD, Dratewka-Kos E, Land BG.** 1990. Covalently bonded and adventitious glycans in germin. *Journal of Biological Chemistry* **265**, 12503–12512.
- Kapila J, De Rycke R, Van Montagu M, Angenon G.** 1997. An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Science* **122**, 101–108.
- Kav NNV, Srivastava S, Goonewardene L, Blade SF.** 2004. Proteome-level changes in the roots of *Pisum sativum* in response to salinity. *Annals of Applied Biology* **145**, 217–230.
- Khuri S, Bakker FT, Dunwell JM.** 2001. Phylogeny, function, and evolution of the cupins, a structurally conserved, functionally diverse superfamily of proteins. *Molecular Biology and Evolution* **18**, 593–605.
- Koster JF, Slee RG.** 1980. Lipid-peroxidation of rat-liver microsomes. *Biochimica et Biophysica Acta* **620**, 489–499.
- Lane BG.** 1994. Oxalate, germin, and the extracellular matrix of higher plants. *FASEB Journal* **8**, 294–301.
- Lane BG, Dunwell JM, Ray JA, Schmitt MR, Cuming AC.** 1993. Germin, a protein marker of early plant development, is an oxalate oxidase. *Journal of Biological Chemistry* **268**, 12239–12242.
- Lane BG, Grzelczak ZF, Kennedy TD, Kajioka R, Orr J, Dagostino S, Jaikaran A.** 1986. Germin – compartmentation of 2 forms of the protein by washing growing wheat embryos. *Biochemistry and Cell Biology* **64**, 1025–1037.
- Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Laine AC, Gomord V, Faye L.** 1998. N-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Molecular Biology* **38**, 31–48.
- Matamoros MA, Dalton DA, Ramos J, Clemente MR, Rubio MC, Becana M.** 2003. Biochemistry and molecular biology of antioxidants in the rhizobia–legume symbiosis. *Plant Physiology* **133**, 499–509.
- Membre N, Berna A, Neutelings G, David A, David H, Staiger D, Vasquez JS, Raynal M, Delseny M, Bernier F.** 1997. cDNA sequence, genomic organization and differential expression of three Arabidopsis genes for germin oxalate oxidase-like proteins. *Plant Molecular Biology* **35**, 459–469.
- Misra HP, Fridovich I.** 1977. Superoxide dismutase and peroxidase: a positive stain applicable to polyacrylamide gel electropherograms. *Archives of Biochemistry and Biophysics* **183**, 511–515.
- Nakata M, Shiono T, Watanabe Y, Satoh T.** 2002. Salt stress-induced dissociation from cells of a germin-like protein with Mn-SOD activity and an increase in its mRNA in a moss, *Barbula unguiculata*. *Plant and Cell Physiology* **43**, 1568–1574.
- Nakata M, Watanabe Y, Sakurai Y, Hashimoto Y, Matsuzaki M, Takahashi Y, Satoh T.** 2004. Germin-like protein gene family of a moss, *Physcomitrella patens*, phylogenetically falls into two characteristic new clades. *Plant Molecular Biology* **56**, 381–395.
- Nielsen H, Engelbrecht J, Brunak S, von Heijne G.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**, 1–6.
- Nowakowska J.** 1998. Gene expression and oxalate oxidase activity of two germin isoforms induced by stress. *Acta Physiologiae Plantarum* **20**, 19–33.
- Requena L, Bornemann S.** 1999. Barley (*Hordeum vulgare*) oxalate oxidase is a manganese-containing enzyme. *Biochemical Journal* **343**, 185–190.
- Rodriguez-Lopez M, Baroja-Fernandez E, Zanduetta-Criado A, Moreno-Bruna B, Munoz FJ, Akazawa T, Pozueta-Romero J.** 2001. Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (*Hordeum vulgare* L.) are distinct oligomers of HvGLP1, a germin-like protein. *FEBS Letters* **490**, 44–48.
- Rubio MC, James EK, Clemente MR, Bucciarelli B, Fedorova M, Vance CP, Becana M.** 2004. Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. *Molecular Plant–Microbe Interactions* **17**, 1294–1305.
- Sambrook J, Fritsch EF, Maniatis T.** 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schneider A, Walker SA, Poyser S, Sagan M, Ellis THN, Downie JA.** 1999. Genetic mapping and functional analysis of a nodulation-defective mutant (sym19) of pea (*Pisum sativum* L.). *Molecular and General Genetics* **262**, 1–11.

- Schweizer P, Christoffel A, Dudler R.** 1999. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. *The Plant Journal* **20**, 540–552.
- Segarra CI, Casalongue CA, Pinedo ML, Ronchi VP, Conde RD.** 2003. A germin-like protein of wheat leaf apoplast inhibits serine proteases. *Journal of Experimental Botany* **54**, 1335–1341.
- Swart S, Logman TJJ, Smit G, Lugtenberg BJJ, Kijne JW.** 1994. Purification and partial characterization of a glycoprotein from pea (*Pisum sativum*) with receptor activity for rhicadhesin, an attachment protein of Rhizobiaceae. *Plant Molecular Biology* **24**, 171–183.
- Tabuchi T, Kumon T, Azuma T, Nanmori T, Yasuda T.** 2003. The expression of a germin-like protein with superoxide dismutase activity in the halophyte *Atriplex lentiformis* is differentially regulated by wounding and abscisic acid. *Physiologia Plantarum* **118**, 523–531.
- Trinchant JC, Rigaud J.** 1996. Bacteroid oxalate oxidase and soluble oxalate in nodules of faba beans (*Vicia faba* L.) submitted to water-restricted conditions: possible involvement in nitrogen fixation. *Journal of Experimental Botany* **47**, 1865–1870.
- Vallelian-Bindschedler L, Mosinger E, Metraux J-P, Schweizer P.** 1998. Structure, expression and localization of a germin-like protein in barley (*Hordeum vulgare* L.) that is insolubilized in stressed leaves. *Plant Molecular Biology* **37**, 297–308.
- Voinnet O, Lederer C, Baulcombe DC.** 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167.
- Voinnet O, Rivas S, Mestre P, Baulcombe D.** 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949–956.
- Whittaker MM, Whittaker JW.** 2002. Characterization of recombinant barley oxalate oxidase expressed by *Pichia pastoris*. *Journal of Biological Inorganic Chemistry* **7**, 136–145.
- Wisniewski JP, Brewin NJ.** 2000. Construction of transgenic pea lines with modified expression of diamine oxidase and modified nodulation responses with exogenous putrescine. *Molecular Plant–Microbe Interactions* **13**, 922–928.
- Woo EJ, Dunwell JM, Goodenough PW, Marvier AC, Pickersgill RW.** 2000. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nature Structural Biology* **7**, 1036–1040.
- Yamahara T, Shiono T, Suzuki T, Tanaka K, Takio S, Sato K, Yamazaki S, Satoh T.** 1999. Isolation of a germin-like protein with manganese superoxide dismutase activity from cells of a moss, *Barbula unguiculata*. *Journal of Biological Chemistry* **274**, 33274–33278.
- Zhang ZG, Yang J, Collinge DB, Thordalchristensen H.** 1996. Ethanol increases sensitivity of oxalate oxidase assays and facilitates direct activity staining in SDS gels. *Plant Molecular Biology Reporter* **14**, 266–272.
- Zimmermann G, Bäumlein H, Mock HP, Himmelbach A, Schweizer P.** 2006. The multigene family encoding germin-like proteins of barley: regulation and function in basal host resistance. *Plant Physiology* **142**, 181–192.