

Arabidopsis thaliana germin-like proteins: common and specific features point to a variety of functions

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Received: 28 June 1999 / Accepted: 6 December 1999

Abstract. Germin-like proteins (GLPs) are ubiquitous plant proteins encoded by diverse multigene families. It is not known whether they share germin's unusual biochemical properties and oxalate oxidase activity. Using specific antibodies, we have studied three GLPs (AtGER1, AtGER2 and AtGER3) in *Arabidopsis thaliana* (L.) Heynh. as well as in transgenic tobacco (*Nicotiana tabacum* L.) plants overexpressing these proteins. Like wheat (*Triticum aestivum* L.) germin, these *Arabidopsis* GLPs are associated with the extracellular matrix (ECM) and they also seem to exist as two glycosylated isoforms. However, none of them is an oxalate oxidase. Although GLPs display several conserved features, each has its specific characteristics. Both AtGER2 and AtGER3 are oligomeric proteins that share germin's resistance to pepsin and to dissociation by heat and SDS. In contrast, AtGER1 seems to exist as a monomer. The GLPs may interact with the ECM in a variety of ways, since each is efficiently extracted by different conditions. In addition, germins and GLPs all bind Cibacron Blue, a dye often but not exclusively used for the purification of enzymes having nucleotide cofactors. In the case of AtGER2, binding to the dye is so tight that it almost allows a one-step purification of this protein. The variety of sequences, expression patterns and biochemical features indicates that GLPs could be a class of receptors localized in the ECM and involved in physiological and developmental processes as well as stress response.

Key words: *Arabidopsis* – Cell wall – Extracellular matrix – Germin – Germin-like protein – Oxalate oxidase

Introduction

Germins and germin-like proteins (GLPs) constitute a large family of proteins ubiquitously distributed in the plant kingdom. They are part of a superfamily of proteins now called “cupins” that also includes seed storage globulins, sucrose-binding proteins as well as microbial and animal proteins. The overall amino acid sequence conservation of the cupins is quite low; nevertheless, all these proteins have in common a predicted β -barrel structure and histidine-containing motifs that are probably involved in binding metal ions (Braun et al. 1996; Dunwell 1998; Dunwell and Gane 1998; Shutov et al. 1998).

The phylogenetic tree for germins and GLPs has five branches (Carter et al. 1998; Neutelings et al. 1998). One of these includes only the “true” germins: this constitutes a very homogeneous group (pairwise amino acid identities always over 95%) including proteins that have only been found in cereals and that usually possess oxalate oxidase activity (Dumas et al. 1993; Lane et al. 1993). Pairwise comparisons of GLPs show amino acid identities ranging from 30 to 95% while comparisons with germins never reveal identities higher than 60% (Carter et al. 1998). Although the overall sequence identity is not very high among the germins and GLPs, they all possess an N-terminal signal sequence, and several conserved oligopeptides and key amino acid residues have been identified, including the putative metal-binding motifs (Membré et al. 1997; Carter et al. 1998; Dunwell 1998; Membré and Bernier 1998).

So far, only germins have been extensively studied at the biochemical and cellular levels. They are oligomeric proteins associated with the extra cellular matrix (ECM) and they resist protease degradation and dissociation by various denaturing agents such as heat and detergents (Lane 1991). Contrary to what their name implies, they are not germination specific. Their expression patterns and oxalate oxidase activity suggest functions both in development, more precisely in cell expansion, and in resistance to stress, especially pathogen infection (Lane

Abbreviations: ECM = extracellular matrix; GLP = germin-like protein; IWF = intercellular washing fluid; SOL = soluble fraction

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1994; Dumas et al. 1995; Zhang et al. 1995; Hurkman and Tanaka 1996; Zhou et al. 1998).

The timing of wheat germin expression and its up-regulation by auxins favor the hypothesis of a role in promoting cell expansion (Lane 1994; Berna and Bernier 1997). However, the enzymatic release of hydrogen peroxide, which could be used in cross-linking reactions, as well as the specific localization of its expression point to a role in the restriction of growth (Caliskan and Cuming 1998). This issue is still a matter of debate since it has been proposed recently that $\cdot\text{OH}$ radicals, formed by hydrogen peroxide, could well be involved in scission of polysaccharides and, consequently, in wall loosening and cell expansion (Fry 1998).

Expression of several wheat and barley germin and GLP genes has been shown to be up-regulated by fungal infection (Dumas et al. 1995; Zhang et al. 1995; Hurkman and Tanaka 1996; Zhou et al. 1998). Although the definite proof of oxalate oxidase or GLP involvement in pathogen resistance is still lacking, several observations point to such a role. First, the importance of the oxidative burst in pathogen resistance is now well documented (see Wojtaszek 1997; Alvarez et al. 1998 and references therein). Second, hydrogen peroxide accumulation has been observed in barley papillae, cell wall appositions formed to prevent powdery mildew penetration (Gregersen et al. 1997; Thordal-Christensen et al. 1997). Even though the presence of oxalate oxidase has still to be demonstrated in these papillae, GLPs have been identified (Wei et al. 1998). Third, insolubilization of some GLPs in barley leaf cell walls following heat-shock could be correlated with a type of fungal resistance not involving papilla formation (Vallélian-Bindschedler et al. 1998a,b). Fourth, a wheat oxalate oxidase gene maps within a resistance QTL (Quantitative Trait Locus; Faris et al. 1999). Recently, it has been shown that the participation of germis and GLPs in stress response is probably not restricted to fungal invasion: transcription of the wheat germin gene is highly stimulated by some heavy metals, especially cadmium, and by tobacco mosaic virus infection in transgenic tobacco plants transformed with the wheat gene (Berna and Bernier 1999).

Except for barley GLPs associated with resistance to infection, GLPs have received very little attention so far, and it is peculiar to note that almost nothing is known about dicot GLPs even though a plant with a small genome like *Arabidopsis thaliana* has at least 12 different GLP genes (Carter et al. 1998) and that rice has at least eight (Membré and Bernier 1998). Germin-like proteins or GLP mRNAs can be found in almost every organ and developmental stage. For example, the three *Arabidopsis* genes that have been studied each have a distinct expression profile (Membré et al. 1997). A few dicot GLPs have been localized at the subcellular level and have been found in the ECM (Domon et al. 1994; Heintzen et al. 1994; Wojtaszek et al. 1997; Ohmiya et al. 1998). These proteins are probably not oxalate oxidases but, without any information on their possible oligomeric structure and stability, this remains to be verified.

In order to start addressing the question of dicot GLP function(s), more comprehensive knowledge about their biochemical properties is needed. We have investigated the structure, cellular localization and purification of three *Arabidopsis* GLPs, named AtGER1, AtGER2 and AtGER3 in *Arabidopsis* as well as in transgenic tobacco plants overexpressing these proteins.

Materials and methods

Growth of plants. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) seeds were kindly provided by Dr. R.N. Beachy (St. Louis, Mo., USA) and seeds of *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia were obtained from Lehle seeds (Round Rock, Texas, USA). Plants were grown in the greenhouse or propagated in vitro as previously described (Berna and Bernier 1999).

Plasmid constructs and transgenic tobacco. All molecular biology manipulations were performed according to standard procedures (Sambrook et al. 1989).

The +85 (*Hind*III site) to +1127 (*Eco*RI site) fragment of the intronless wheat germin *gf*-2.8 gene (Lane et al. 1991) that includes the complete open reading frame (+105 to +776) was inserted between the *Xba*I and *Eco*RI sites of plasmid pBI121 (Clontech) by partial fill-in of the *Hind*III and *Xba*I sites to express germin under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The AtGER1 and AtGER2 cDNAs (Membré et al. 1997) were cloned between the CaMV 35S promoter and the nopaline synthase (NOS) transcriptional terminator of plasmid pBI121 (Clontech) in place of the β -glucuronidase (GUS) reporter gene.

The AtGER3 protein-coding region was amplified by polymerase chain reaction (PCR) from the original cDNA clone (GenBank Y12673) with primers covering the translation start comprising an engineered *Nco*I site and the translation stop followed by an engineered *Bam*HI site, respectively. The gel-purified 650-bp amplification product was inserted into the vector pRT104 (Töpfer et al. 1987) between the CaMV 35S RNA promoter with the duplicated enhancer fused to the tobacco mosaic virus omega element (Gallie et al. 1987) and the CaMV polyadenylation signal. The entire expression cassette was isolated by *Sph*I digest and inserted into *Sma*I-cut pBin19 (Bevan 1984) after removing the 3' overhangs with T4 DNA polymerase.

The constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by either triparental mating or freeze-thaw transformation. Tobacco plants were infected by *Agrobacterium tumefaciens* and transgenic plants were regenerated as previously described (Berna and Bernier 1997). Transgenic plants harboring the highest transgene expression levels were selected following northern blot analysis.

Cell fractionation. Tissues were ground with a pestle in cold wheat-germ (WG) buffer (20 mM Hepes, pH 7.5; 100 mM K-acetate; 3 mM Mg-acetate; 1 mM DTT) at a ratio of one ml of buffer per g of tissue. The homogenate was filtered on a nylon mesh to get a crude cell wall fraction (see below). The filtrate was centrifuged at 16 000g for 10 min: the supernatant constituted the soluble fraction (SOL) and the pellet was resuspended in a volume of WG buffer equal to the starting volume to make up fraction P16.

The crude cell wall fraction was washed three or four times with WG buffer containing 1% Triton X-100. Cell walls were then either resuspended in 10 mM Tris-HCl (pH 8) or washed in this buffer containing either 5.5 M urea, 1% SDS, 1 M NaCl, 1 M CaCl₂ or 1 M β -mercaptoethanol in order to extract GLPs. After centrifugation, the pellet was boiled in 1% SDS to recover residual proteins and both fractions were analyzed by western blot.

To obtain the fraction containing the intercellular washing fluid (IWF), leaves were vacuum-infiltrated with 10 mM Tris (pH 7.5), 1 mM CaCl₂.

Western blotting. Sequence analysis revealed that a very hydrophilic and variable region resides on the N-terminal part of all mature GLPs, just after conserved Box A (Membré et al. 1997), so this region was used to design immunogenic oligopeptides specific to each of the GLPs under study. The following peptides coupled to KLH (keyhole limpet hemacyanin) were obtained from Genosys Biotechnologies Inc (Cambridge, UK): ANLKRAETPA-GYP (amino acids 35–47 of AtGER1), KDPKRVDKDF (amino acids 49–60 of AtGER2) and DPKGPQSPSGYS (amino acids 29–40 of AtGER3). Anti-wheat-germin antibody was raised against germin overexpressed in tobacco: IWF proteins from leaves were obtained by vacuum infiltration and germin was further purified using Prep-Cell (Bio-Rad). All antibodies were raised in rabbits.

Western blots were performed as described in Berna (1995) using horseradish-alkaline phosphatase-coupled secondary antibodies.

Chromatography. Gel-filtration chromatography was performed on Sephadex G-200 in a 10-ml column with 20 mM Tris-HCl (pH 7.5) as a buffer. The volume of the sample was 1 ml and the elution rate was around 12 ml per hour.

Cibacron Blue (Sigma) affinity chromatography was performed in a 2-ml column equilibrated with 20 mM Tris-HCl (pH 7.5), 50 mM NaCl. Elution of the proteins was realized by a stepwise increase of NaCl concentration (100, 300, 500, 1000 mM) followed by a final wash with 8 M urea, 2.5 M NaCl.

Assay of oxalate oxidase. The spectrophotometric oxalate oxidase assay is based on the detection of hydrogen peroxide with horseradish peroxidase, 4-aminoantipyrine and N,N-dimethylaniline, and was performed essentially as described by Sugiura et al. (1979) except that the samples were first dialyzed in 10 mM Hepes (pH 7) in order to eliminate small molecules that interfere with enzyme activity.

Results

We have previously described three cDNAs coding for *Arabidopsis* GLPs, each one encoded by a gene having a specific expression pattern (Membré et al. 1997). Even though *AtGER1* and *AtGER3* are part of the same *Arabidopsis* sub-family (Carter et al. 1998; Neutelings et al. 1998), their products show an amino acid identity

of 64% and of only around 40% with the protein encoded by *AtGER2*. Typically for GLPs, amino acid identities with wheat germin range from 34 to 46%. In order to facilitate biochemical analyses and as a first approach to understanding GLP function, these three cDNAs were expressed in transgenic tobacco plants under the control of the CaMV 35S promoter. Even the plants showing the highest levels of protein accumulation (see below) did not display any visible phenotypic alteration at any developmental stage. These plants were selected for further biochemical characterization.

Biochemical properties of *AtGER1*, *AtGER2* and *AtGER3*. Since *AtGER1* and *AtGER3* are both expressed in *Arabidopsis* leaves (Membré et al. 1997) and since the 35S promoter gives rise to a high level of transcription in this organ type, proteins recognized by the specific antibodies were first looked for in soluble leaf protein extracts. When the samples were denatured by boiling before loading on the gel, a band of the expected MW (approximately 23 kDa) was observed with all three antibodies with *Arabidopsis* proteins and with proteins from transgenic tobacco plants expressing the corresponding transgene (Fig. 1). The amount of foreign protein detected in tobacco was much higher than endogenous protein levels in *Arabidopsis*, especially for *AtGER2* and *AtGER3*. A low level of *AtGER2* in *Arabidopsis* leaves was expected since mRNAs for this protein were not detected in leaf tissue by northern blot analysis (Membré et al. 1997). The identity of the 23-kDa proteins and the specificity of the antibodies are confirmed by the following observations: no wild-type tobacco protein of this size reacted with the antibodies (Fig. 1), the reaction of a given 23-kDa protein was abolished by the use of an excess of the corresponding peptide and, finally, antibodies did not cross-react with proteins from transgenic tobacco plants expressing a non-corresponding GLP or wheat germin (data not shown).

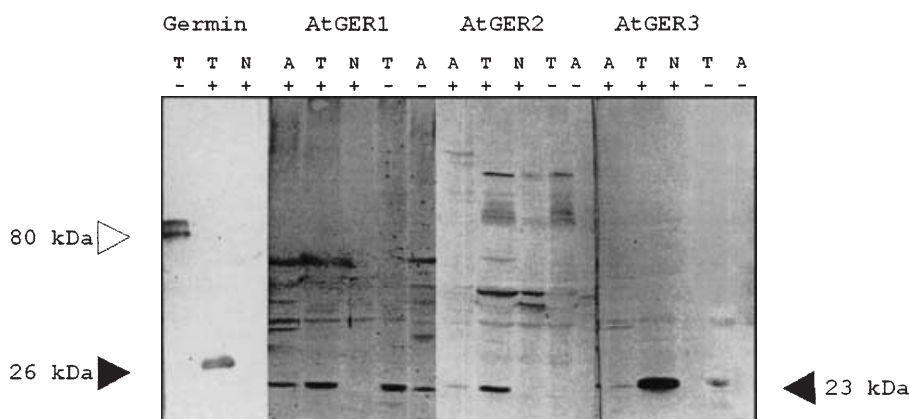


Fig. 1. Detection of *AtGER1*, *AtGER2* and *AtGER3* in *Arabidopsis* and in transgenic tobacco plants with specific antibodies. Soluble proteins (approximately 200 µg) from *Arabidopsis* (lane A), transgenic tobacco (lane T) or wild-type tobacco (lane N) leaves were separated on a composite polyacrylamide gel (8% acrylamide in the top part of the running gel and 11% acrylamide in the bottom part), transferred

onto a nitrocellulose membrane and reacted with anti-*AtGER1*, anti-*AtGER2* or anti-*AtGER3* antibody. In parallel, anti-wheat germin antibody was used to detect heterologous germin in transgenic tobacco plants expressing wheat germin *gf-2.8* gene. + sample denatured by boiling; – sample not denatured by boiling

In wheat, germin is present in the form of very stable oligomers that run as a doublet on SDS-PAGE when the protein sample is not heat-denatured before running the gel. The two germin isoforms are named germin G and germin G' and only differ at their glycan moiety (Lane et al. 1986). In transgenic tobacco plants expressing the wheat germin *gf-2.8* gene, the typical oligomeric doublet of 80 kDa was indeed present (Fig. 1; see also Berna and Bernier 1999) and the 26-kDa monomer was detected when the sample was boiled (Fig. 1). If AtGER1 oligomers exist *in planta* they do not share exceptional stability of wheat germin since monomeric proteins of 23 kDa were also detected in unboiled proteins from either *Arabidopsis* or tobacco plants expressing this protein (Fig. 1). On the other hand, neither AtGER2 nor AtGER3 protein could be detected without prior denaturation (Fig. 1). The most likely explanation is that these two GLPs exist as oligomers in which the epitopes recognized by our antibodies are hidden.

If epitopes recognized by the antibodies are indeed not accessible in the oligomers it should be possible to expose them after migration of unboiled samples. Boiling the gel for 5 min in electrophoresis buffer before transfer did allow antigenic reaction of AtGER2 and AtGER3 oligomers with their specific antibodies (Fig. 2). The AtGER3 migrated as a doublet whereas AtGER2 formed a fuzzy band that also suggested the presence of two isoforms.

The molecular weight (MW) of AtGER2, AtGER3 and wheat germin oligomers estimated by SDS-PAGE varied according to the percentage of acrylamide in the gel. Discrepancies regarding germin MW estimation have been reported previously (Zhang et al. 1995). In our hands, wheat germin and AtGER2 ran as 80-kDa proteins in 8% acrylamide gels whereas AtGER3 had an estimated MW of 130 kDa. In 12% acrylamide gels, AtGER3 had an estimated MW of 140 kDa and both wheat germin and AtGER2 behaved as 110-kDa oligomers. Because of these discrepancies, it can only be concluded that AtGER2 probably resembles wheat germin, which has recently been shown to be a hexamer (Woo et al. 1998), whereas AtGER3 is comprised of a higher number of subunits.

In order to determine whether GLP oligomers share the remarkable stability of wheat germin, soluble protein extracts from tobacco plants overexpressing AtGER2, AtGER3 or wheat germin were subjected to various denaturing treatments (see Table 1) and analyzed by western blot. Resistance was assessed by comparing

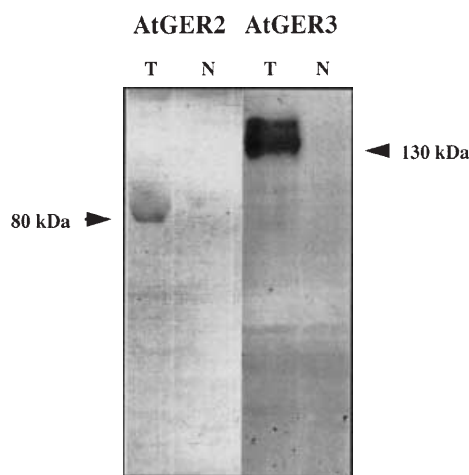


Fig. 2. Detection of AtGER2 and AtGER3 oligomers with specific antibodies after heat-denaturation of the proteins in the gel. Leaf soluble proteins from tobacco plants overexpressing either AtGER2 or AtGER3 were extracted and, in the case of AtGER2, further purified by heating at 60 °C followed by pepsin digestion. Proteins were then loaded onto an 8% polyacrylamide gel without heat denaturation. After migration, part of the gel was boiled for 5 min in electrophoresis buffer prior to transfer onto nitrocellulose and reaction with specific antibodies. *Lane T*, transgenic tobacco proteins; *lane N*, wild-type tobacco proteins (heated at 60 °C and digested by pepsin in the case of anti-AtGER2 antibody reaction)

band intensities with untreated samples. Table 1 shows that AtGER2 oligomers resist heat, chemical denaturing agents and pepsin degradation as well as wheat germin. The AtGER3 oligomers also show good resistance to denaturation and degradation but are nevertheless dissociated by β -mercaptoethanol and urea.

Interaction of AtGER1 with tobacco proteins from the soluble fraction. Since only AtGER1 monomers could be detected after SDS-PAGE of unboiled samples, we reasoned that it might exist as an unstable oligomer, unable to resist SDS and reducing conditions even at room temperature. Soluble extracts from tobacco plants overexpressing AtGER1 were fractionated by gel filtration on Sephadex. The AtGER1 always eluted in the void volume, even with Sephadex G-200, meaning that the MW would be over 600 kDa (data not shown). To our surprise, AtGER2, AtGER3 and wheat germin behaved similarly although we had determined their MW to be well below 200 kDa by SDS-PAGE (see above). For these three proteins, omitting the void

Table 1. Resistance of wheat germin and *Arabidopsis* GLP oligomers to various denaturing agents and to pepsin degradation. Leaf soluble protein extracts from tobacco plants overexpressing wheat germin or *Arabidopsis* AtGER2 or *Arabidopsis* AtGER3 (see *Materials and methods*) were treated as indicated and analyzed by western blotting with specific antibodies. The intensities of the bands were compared with those obtained from untreated samples

Treatment	Resistance of oligomer		
	Wheat germin	AtGER2	AtGER3
100 °C, 1 min	+	+	+
60 °C, 2 h	+	+	+
60 °C, 2 h, in 0.1% SDS	+	+	+
60 °C, 2 h, in 1 M β -mercaptoethanol	+	+	–
60 °C, 2 h, in 5.5 M urea	+	+	–
37 °C, 3 h, 50 μ g/ml pepsin at pH 2	+	+	+

volume marker Blue Dextran in the sample restored the expected elution profile and we were able to determine that proteins bound in fact to the Cibacron Blue dye of Blue Dextran (see section *purification of wheat germin and Arabidopsis AtGER1, AtGER2 and AtGER3*). For AtGER1 however, the high-MW complexes were not caused only by Blue Dextran since it still eluted in the void volume of Sephadex G-200 even in the absence of this marker.

To determine whether AtGER1 exists in the form of such a complex in the ECM or whether the association forms with components of the soluble fraction after grinding, AtGER1 obtained from a cell wall fraction washed with CaCl_2 (see next section) was also analyzed by gel filtration. In this case, AtGER1 was retained on the column and eluted in the expected fractions for a monomeric protein whether or not it was first dialyzed to remove calcium ions (Fig. 3A). However, the addition of a wild-type tobacco soluble fraction to this AtGER1 preparation resulted in the formation of a Sephadex G-200-excluded high-MW complex (Fig. 3B). This complex was unstable in the presence of calcium ions since it was not obtained using non-dialyzed cell wall AtGER1 fraction. The complex was also dissociated by 5.5 M urea, but not by 1 M urea (data not shown).

Subcellular localization of *Arabidopsis* GLPs. As a first approach to determine the subcellular localization of the three GLPs under study, cell fractionations were carried out as described in *Materials and methods*. The AtGER1 protein was predominantly found in the cell wall fraction of transgenic tobacco leaves as well as in *Arabidopsis* leaves and siliques. Part of the protein was also found in the SOL fraction: this represents either intracellular protein or polypeptides that were only loosely bound to the cell surface so that they were washed out from the cell wall fraction even in low-ionic strength buffer (Fig. 4). The situation was the same for AtGER3 except that it was almost undetectable in *Arabidopsis* siliques (Fig. 4). This was expected since, based on previous northern blots, there is little or no expression of *AtGER3* during embryogenesis (Membré et al. 1997).

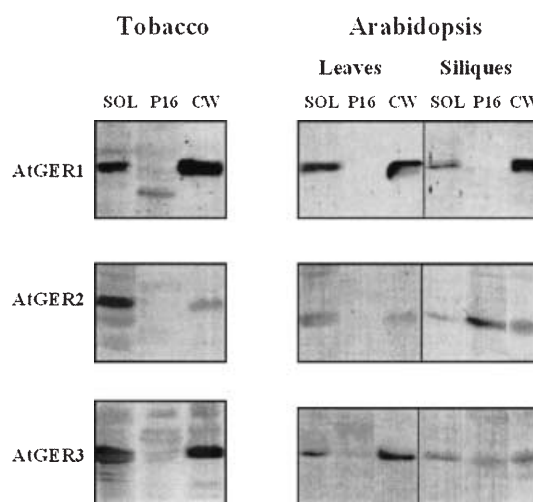


Fig. 4. Subcellular localization of AtGER1, AtGER2 and AtGER3. *Arabidopsis* leaves and siliques, and transgenic tobacco leaves, were ground and fractionated as described in *Materials and methods*. The proteins were then separated on an 11% polyacrylamide gel, blotted onto nitrocellulose and reacted with antibodies specific for each GLP. An amount of sample corresponding to 20 mg (fresh weight) of the initial organ was loaded in each lane to allow a direct semi-quantitative comparison of the different fractions. Lane SOL, soluble fraction; lane P16, 16 000g pellet; lane CW, cell wall

A different picture emerged for AtGER2. Northern blots had shown an almost exclusive expression of *AtGER2* in developing siliques (Membré et al. 1997) and, expectedly, antigenic reaction with *Arabidopsis* leaf proteins was very weak (Fig. 4). In *Arabidopsis* siliques, AtGER2 seemed to be associated with membranes since it predominated in the P16 fraction although it was also detected in the SOL and cell wall fractions (Fig. 4). In contrast, AtGER2 was dominant in the tobacco leaf SOL fraction and not detectable in the P16 fraction (Fig. 4). This discrepancy could be attributed to the fact that, in tobacco, AtGER2 is being synthesized in an organ (leaf) where it does not usually accumulate.

Except for AtGER2 in *Arabidopsis* siliques, GLPs were present in cell wall and SOL fractions. Since the SOL fraction could still contain some membrane mate-

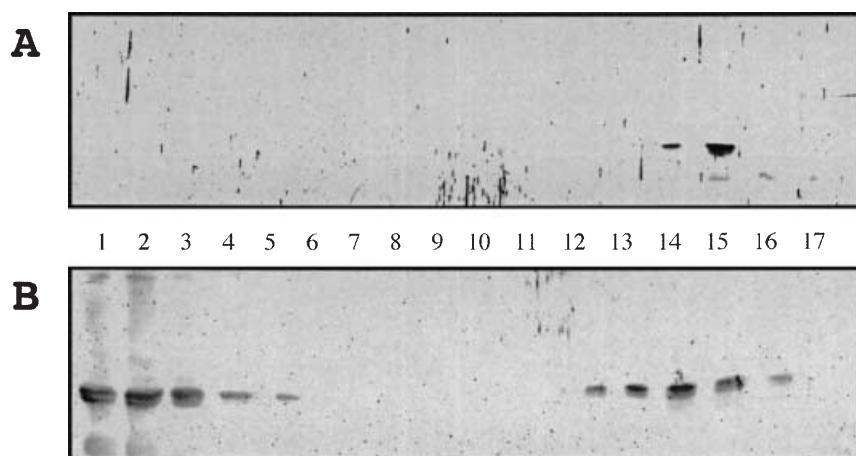


Fig. 3A,B. Analysis of AtGER1 by gel-filtration chromatography. AtGER1 was obtained by a CaCl_2 wash of the cell wall fraction (see *Materials and methods*) and dialyzed to remove calcium ions. It was then applied to a Sephadex G-200 column without further treatment (A) or after preincubation with wild-type tobacco soluble fraction (B). The eluted fractions were separated on an 11% polyacrylamide gel, blotted onto nitrocellulose and reacted with anti-AtGER1 antibody. Bromophenol blue eluted starting at fraction 16. The arrowheads indicate the position of AtGER1

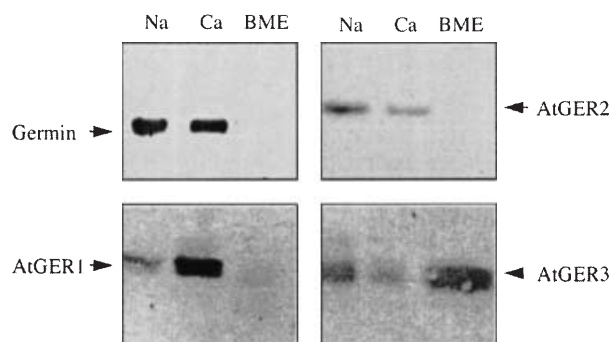


Fig. 5. Extraction of wheat germin and *Arabidopsis* AtGER1, AtGER2 and AtGER3 from the cell wall fraction. The cell wall fraction of transgenic tobacco leaves overexpressing *Arabidopsis* GLPs was washed with 1 M NaCl (lane Na), or 1 M CaCl₂ (lane Ca) or 1 M β -mercaptoethanol (lane BME) as described in *Materials and methods*. After centrifugation, proteins from the supernatant were separated on an 11% polyacrylamide gel, blotted onto nitrocellulose and reacted with antibodies specific for each GLP. An amount of sample corresponding to 20 mg (fresh weight) of the initial organ was loaded in each lane to allow a direct semi-quantitative comparison of the different fractions

rial, an additional centrifugation, at 100 000g, was performed on leaf SOL fractions from transgenic tobacco plants. Germin-like proteins were always found exclusively in the supernatant, indicating that they are not associated with membranes (data not shown).

In order to determine how GLPs are held in the cell wall and whether all GLPs were involved in the same kind of interactions, cell wall fractions were washed with various salts and denaturing agents. Triton X-100 at 1% was used to wash crude cell wall fractions (see *Materials and methods*) and we verified that the use of this detergent did not release GLPs or wheat germin. Urea was also ineffective in removing GLPs from the wall fraction whereas SDS extracted only part of these. Boiling the cell wall fractions in SDS released most of the three GLPs (data not shown).

Germin and GLPs could also be extracted with NaCl and CaCl₂. Calcium chloride was much more effective for extracting AtGER1 whereas NaCl was more effective for extracting AtGER2 and AtGER3 (Fig. 5). The AtGER3 was the only protein that could be extracted with β -mercaptoethanol (Fig. 5); this could be related to the fact that AtGER3 oligomers, unlike the others, are dissociated by this denaturing agent (see previous section).

Wheat germin has previously been shown to be only loosely associated with the ECM since a simple wash sufficed to obtain it from cultured embryos (Lane et al. 1986). Germin and maybe GLPs could thus be expected to be found in the IWF following vacuum infiltration with a low-ionic-strength buffer. Wheat germin and *Arabidopsis* AtGER3 each represented by far the most abundant IWF protein in transgenic tobacco plants (Fig. 6, IWF and IWF-den) when leaves were vacuum-infiltrated with 1 mM CaCl₂. Furthermore, a simple treatment with pepsin digested most of the other proteins (Fig. 6, IWF-pep). In contrast, appreciable amounts of AtGER1 or AtGER2 were not obtained by

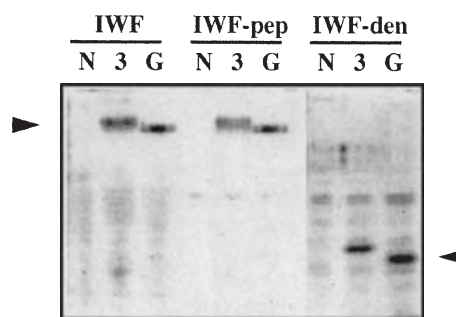


Fig. 6. Analysis of IWF proteins of transgenic tobacco plants. Leaves from wild-type tobacco (lane N) or transgenic tobacco plants expressing wheat germin (lane G) or *Arabidopsis* AtGER3 (lane 3) were vacuum-infiltrated as described in *Materials and methods*. Proteins from the IWF were then separated by SDS-PAGE without further treatment (IWF) or after pepsin digestion (IWF-pep). Heat-denatured proteins were also analyzed by SDS-PAGE (IWF-den). Proteins were stained with Coomassie Brilliant Blue. An amount of sample corresponding to 20 mg (fresh weight) of leaf tissue was loaded in each lane. Arrowheads indicate the position of the heterologous proteins

vacuum infiltration, even by increasing the CaCl₂ concentration up to 200 mM (data not shown).

Purification of wheat germin and Arabidopsis AtGER1, AtGER2 and AtGER3. In order to further characterize the biochemical properties and biological functions of germin and GLPs, purified proteins will be needed. Germins and GLPs are usually not abundant and an overexpression system would greatly facilitate purification. Attempts to express germin or GLPs in *E. coli* have either been unsuccessful or have failed to yield oligomeric proteins (Heintzen et al. 1994). Since all these proteins are probably glycosylated, a procaryotic expression system is not convenient. Yeast does not appear to be well suited either since, even though it can synthesize wheat germin, only monomers are produced (our unpublished observations).

We have shown that high amounts of germin and GLPs can be produced in transgenic tobacco lines and that the properties of these proteins are conserved in this host. Thus, by taking advantage of this plant heterologous system and of the characteristics of the GLPs it should be possible to devise simple purification schemes based on two or three easy steps for most of these proteins.

For wheat germin and *Arabidopsis* AtGER3, a purification strategy can take advantage of the fact that the IWF is already highly enriched in these proteins and that they resist pepsin degradation under controlled conditions (see Fig. 6). Since normal tobacco IWF proteins do not display the same pepsin resistance, this two-step procedure yields a polypeptide that is almost pure (Fig. 6, IWF-pep). Using this procedure, about 30 μ g of AtGER3 was recovered per gram of leaf tissue whereas the yield for wheat germin was around 10 μ g per gram.

Alternatively, wheat germin and *Arabidopsis* AtGER3 can be purified from transgenic tobacco SOL

fraction since they are both readily observed in it (Fig. 7A, SOL). Digestion with pepsin (Fig. 7A, SOL-pep) or incubation at 60 °C (Fig. 7A, SOL-60) is sufficient to remove the majority of the tobacco proteins and a combination of these two simple treatments yields protein fractions highly enriched in the foreign protein (Fig. 7A, SOL-pep60). The same observations were made for germin and AtGER3 monomers when the protein samples were heat-denatured before SDS-PAGE (Fig. 7B).

Arabidopsis AtGER2 was most efficiently purified from a soluble fraction heated at 60 °C. The proteins left in solution were affinity-purified on a Cibacron Blue-Sepharose column (see *Materials and methods*). The AtGER2 protein possesses an extremely high affinity for this dye: a combination of 8 M urea and 2.5 M NaCl was required to elute the protein whereas other GLPs were washed away by 500 mM NaCl. Since very few proteins have such a strong affinity for Cibacron Blue, an almost pure preparation was obtained from this simple purification scheme, with only one or two major

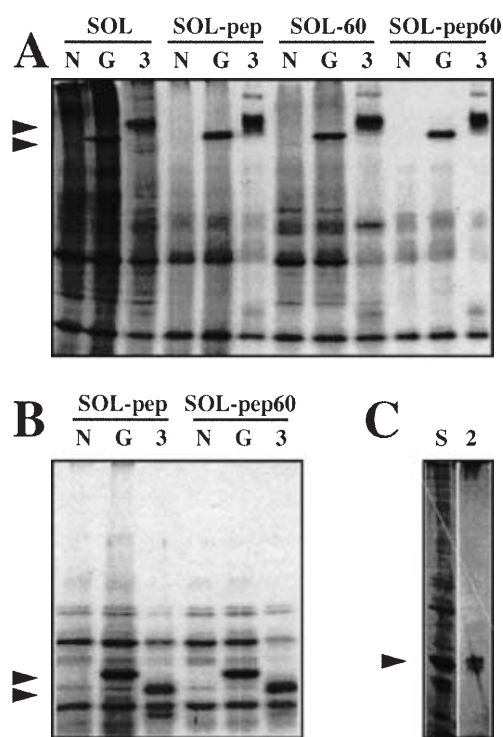


Fig. 7A–C. Partial purification of germin and GLPs from transgenic tobacco plants. Soluble proteins (SOL) were obtained from wild-type tobacco (lane N) or transgenic tobacco plants expressing wheat germin (lane G) or *Arabidopsis* AtGER3 (lane 3) as described in *Materials and methods*. They were then separated by SDS-PAGE on an 11% polyacrylamide gel after pepsin digestion (SOL-pep) or heating at 60 °C (SOL-60) or both (SOL-pep60) and stained with Coomassie Brilliant Blue. An amount of sample corresponding to 20 mg (fresh weight) of leaf tissue was loaded in each lane. **A** Samples not heat-denatured before migration. **B** Samples denatured by boiling before migration. **C** Silver-stained soluble proteins from transgenic tobacco plants expressing AtGER2 after heating at 60 °C (lane S) and after further purification by affinity chromatography on Cibacron Blue (lane 2). The arrowheads indicate the position of the heterologous proteins

Table 2. Determination of oxalate oxidase activity for wheat germin and *Arabidopsis* GLPs overexpressed in transgenic tobaccos. Various amounts of soluble extracts from transgenic tobacco leaves were assayed for oxalate oxidase activity by a spectrophotometric method as described in *Materials and methods*. All assays were performed at pH 4. nd, not detectable

Protein	Relative amount (μ l of extract)	Time	Δ DO ₅₅₅
Wheat germin	0.1	30 min	0.8
AtGER1	0.1; 1; 10 or 50	up to 16 h	nd
AtGER2	0.1; 1; 10 or 50	up to 16 h	nd
AtGER3	0.1; 1; 10 or 50	up to 16 h	nd

bands being visible on a silver-stained polyacrylamide gel (Fig. 7C). When two strong bands were obtained, as shown on Fig. 7C, only the top one reacted with the anti-AtGER2 antibody (not shown). The yield was around 0.1 μ g of AtGER2 per gram of leaf tissue.

Do Arabidopsis GLPs have oxalate oxidase activity? Wheat germin is a remarkably stable oxalate oxidase that can be assayed on nitrocellulose after SDS-PAGE electrophoresis and electroblotting (Dumas et al. 1993; Lane et al. 1993). Using this assay, no oxalate oxidase activity was obtained with wild-type *Arabidopsis* and tobacco plants as well as with transgenic tobacco plants overexpressing AtGER1, AtGER2 or AtGER3, even when using high amounts of protein and long reaction times. In contrast, the same assay allowed easy detection of oxalate oxidase activity in wheat and transgenic tobacco plants transformed with the wheat *gf-2.8* gene (Berna and Bernier, 1997).

Since not all GLPs share wheat germin's structure and stability, the use of an assay relying on SDS stability is questionable. Accordingly, we optimized a spectrophotometric oxalate oxidase assay (see *Materials and methods*) and repeated the assays for transgenic tobacco plants. Even though the amounts of heterologous protein differ for each one, we have shown that AtGER1, AtGER2 and AtGER3 accumulated in these lines. The results summarized in Table 2 clearly illustrate that the three *Arabidopsis* GLPs under study do not exhibit oxalate oxidase activity. Identical results to those shown in Table 2 were obtained for AtGER1, AtGER2 and AtGER3 at pH 6 and pH 8.

Discussion

Germins are remarkably stable oligomeric glycoproteins of the apoplast that have oxalate oxidase activity (Lane 1994). Although some GLPs have been localized to the extracellular space (Domon et al. 1994; Heintzen et al. 1994; Wojtaszek et al. 1997; Ohmiya et al. 1998; Vallélian-Bindschedler et al. 1998b), only three of those have also been studied at the structural level and shown to be oligomers: a barley GLP (Vallélian-Bindschedler et al. 1998b) and two very closely related peach auxin-binding proteins (ABP19 and ABP20; Ohmiya et al. 1998). The question of resistance was only addressed for the barley

GLP which, like wheat germin, was shown to be a heat-stable, SDS-resistant oligomer (Vallélian-Bindschedler et al. 1998b; Wei et al. 1998). A pea rhicadhesin receptor, the only GLP that has been purified so far and one which has yet to be cloned, was shown to exist as a monomer (Swart et al. 1994).

Taken together, the current information on GLPs allows us to start making generalizations about this family of proteins. It is now quite obvious that all GLPs are somehow associated with the ECM. Except for AtGER1 and the pea rhicadhesin receptor (Swart et al. 1994), the other GLPs that have been studied were found to exist as oligomers and these oligomers showed high resistance to dissociation and to protease degradation. The case of AtGER1 is interesting since it could never be seen as an oligomer in the cell wall fraction but nevertheless had the capacity to form a very stable high-MW complex with components from the soluble fraction.

Wheat germin is N-glycosylated and it was shown that the product of the single wheat germin *gf-2.8* gene can give rise to the two isoforms that show only a slight difference in the glycan moiety (Lane et al. 1986; Berna and Bernier 1997). These two isoforms (G and G') run as a doublet on SDS gels. The same phenomenon was observed with a barley GLP although, in this case, it remains to be formally proven that both bands represented products from the same gene (Vallélian-Bindschedler et al. 1998a,b). Similarly, all GLPs possess at least one putative N-glycosylation site but the presence of a glycan moiety has been formally demonstrated only for pine GLP (Domon et al. 1994) and for the pea rhicadhesin receptor (Swart et al. 1994). By expressing single cDNAs in a transgenic host, we have been able to show that *AtGER1*, *AtGER2* and *AtGER3* coding elements each produce two proteins with slightly different mobilities although electrophoresis conditions have to be carefully adjusted in order to evidence this. Thus, it is likely that all GLPs are glycosylated and that, like wheat germin, the glycan moiety can exist under two slightly different forms. The exact nature of the glycans remains to be determined as well as the reason for having two isoforms.

All these similarities should not make us overlook the differences between GLPs. As mentioned, AtGER1 probably exists as a monomer as does the pea rhicadhesin receptor (Swart et al. 1994). In addition, the precise interactions of germins and GLPs with the ECM differ between AtGER2, AtGER3 and AtGER1. Ionic bonds probably predominate for all. However, AtGER1 was preferentially extracted from a cell wall fraction by divalent cations (Ca^{2+}) whereas, for AtGER2 and AtGER3, monovalent cations (Na^+) were more effective. Wheat germin and AtGER3 links to the wall are especially weak since, unlike AtGER1 and AtGER2, they could be recovered in the IWF after vacuum infiltration with a very low-ionic-strength buffer.

Wheat germin has always been characterized by the unusual resistance of its oligomers to dissociation and to degradation (Lane 1991), and the same conclusion was reached for one barley GLP (Wei et al. 1998). Resistance

was always assessed by the use of SDS, heat and pepsin. By these criteria alone, AtGER2 and AtGER3 oligomers displayed the same resistance as germins. However, the use of other denaturing agents (urea, β -mercaptoethanol) allowed us to demonstrate differential sensitivities to dissociation, AtGER3 being less stable than AtGER2 and wheat germin.

In barley, two GLPs have been implicated in a pathogen-defense mechanism involving cell wall modifications following a heat-shock. In non-treated plants, these prominent proteins were easily obtained in the IWF whereas they became insoluble following heat-shock (Vallélian-Bindschedler et al. 1998a,b). The same treatment was applied to our transgenic tobacco plants expressing IWF-extractable proteins, wheat germin and *Arabidopsis* AtGER3, but the heterologous proteins did not become insoluble (data not shown). It thus seems that insolubilization is a property shared by only a subset of GLPs, probably those that are mainly involved in pathogen resistance. Alternatively, this cell wall protection mechanism might not exist in dicotyledons.

Finally, we have searched for an oxalate oxidase activity associated with the three *Arabidopsis* proteins under study. Indeed, when the first GLPs were identified a few years ago, they were first hypothesized to constitute dicotyledon oxalate oxidases. However, it seems very unlikely that all GLPs exhibit this activity since we now know that plants with small genomes such as *Arabidopsis* and rice possess more than ten very different GLPs (Carter et al. 1998; Membré and Bernier 1998). In this study, we have been unable to detect oxalate oxidase activity in plants overexpressing AtGER1, AtGER2 or AtGER3, even in assay conditions not requiring the proteins to feature an exceptional stability like wheat germin. This obviously does not preclude the possibility that some of the other GLPs are oxalate oxidases but such a GLP has yet to be evidenced.

So, what is the precise function of GLPs? Because they are related to and share most of the properties of oxalate oxidase and because of their affinity for Cibacron Blue, which usually binds enzymes having nucleotide cofactors, they could be thought as a family of extracellular matrix enzymes having a wide range of activities or substrates. However, not all Cibacron Blue-binding proteins are enzymes so this question is still a matter of debate. The possibility that GLPs are cell wall structural proteins does not seem likely since the latter, in contrast to GLPs, are usually made of repetitive sequences. However, this hypothesis cannot be ruled out, especially in the case of those GLPs whose insolubilization seems to participate in pathogen resistance (Vallélian-Bindschedler et al. 1998a,b).

The most interesting possibility is that GLPs constitute a class of receptors localized in the ECM. Two observations argue in favor of this hypothesis. First, N-terminal sequencing identified a GLP as a receptor for the *Rhizobium* attachment protein rhicadhesin (Swart et al. 1994). Second, a GLP from peach was recently identified in a search for auxin-binding proteins (Ohmura et al. 1998). Interestingly, homology can be found between the well-conserved motif B of germins and

GLPs, also called the “germin signature” in PROSITE, and the putative auxin-binding site, named Box A, found in maize ABP1 as well as in several ABP1 homologs (Ohmiya et al. 1998). Both these elements contain two histidines (HXH), a glutamic acid residue followed by five consecutive hydrophobic amino acids, and are bordered by glycines. It is tempting to speculate that conserved residues constitute the core of a ligand-binding domain defined by the glycines and that variable residues determine the specificity of the interaction.

A role as receptors would also explain the diversity of GLP sequences and expression patterns: each cell type is exposed to different neighbors and/or external factors and has to be able to respond accordingly so a whole battery of diverse receptors is required to fully sense its environment. Some of these receptors would be involved in physiological and developmental processes whereas other would play a role in stress responses such as pathogen infection. Even though the details of GLP functions in the plant are still missing, accumulating evidence such as the biochemical data presented here point toward a dynamic and important role for this protein family in the extracellular matrix.

We thank Drs. Francis Durst and Jean-Pierre Salaun (IBMP, Strasbourg, France) for valuable help in the preparation of the figures. We also acknowledge the extensive correction of the style realized by one of the reviewers.

References

- Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92: 773–784
- Berna A (1995) Involvement of residues within putative α helix motifs in the behavior of the alfalfa and tobacco mosaic virus movement proteins. *Phytopathology* 85: 1441–1448
- Berna A, Bernier F (1997) Regulated expression of a wheat germin gene in tobacco: oxalate oxidase activity and apoplastic localization of the heterologous protein. *Plant Mol Biol* 33: 417–429
- Berna A, Bernier F (1999) Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H_2O_2 -producing enzyme. *Plant Mol Biol* 39: 539–549
- Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12: 8711–8721
- Braun H, Czihal A, Shutov AD, Bäumlein H (1996) A vicilin-like seed protein of cycads: similarity to sucrose binding proteins. *Plant Mol Biol* 31: 35–44
- Caliskan M, Cuming AC (1998) Spatial specificity of H_2O_2 -generating oxalate oxidase gene expression during wheat embryo germination. *Plant J* 15: 165–171
- 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 Mol Biol* 38: 929–943
- Domon JM, Dumas B, Lainé E, Meyer Y, David A, David H (1994) Three glycosylated polypeptides secreted by several embryogenic cell cultures of pine show highly specific serological affinity to antibodies directed against the wheat germin apoprotein monomer. *Plant Physiol* 108: 141–148
- Dumas B, Sailland A, Cheviet J-P, Freyssinet G, Pallett K (1993) Identification of barley oxalate oxidase as a germin-like protein. *C R Acad Sci, Life Sci* 316: 793–798
- Dumas B, Freyssinet G, Pallett K (1995) Tissue-specific expression of germin-like OxO during development and fungal infection of barley seedlings. *Plant Physiol* 107: 1091–1096
- Dunwell JM (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotech Genet Eng Rev* 15: 1–32
- Dunwell JM, Gane PJ (1998) Microbial relatives of seed storage proteins: conservation of motifs in a functionally diverse superfamily of enzymes. *J Mol Evol* 46: 147–154
- Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. *Theor Appl Genet* 98: 219–225
- Fry SC (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochem J* 332: 507–515
- Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA (1987) The 5'-leader sequence of tobacco mosaic virus enhances the expression of foreign gene transcripts in vitro and in vivo. *Nucleic Acids Res* 15: 3257–3273
- Gregersen PL, Thordal-Christensen H, Förster H, Collinge D (1997) Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria graminis* f. sp. *hordei* (syn. *Erysiphe graminis* f. sp. *hordei*). *Physiol Mol Plant Pathol* 51: 85–97
- Heintzen C, Fischer R, Melzer S, Kappeler S, Apel K, Staiger D (1994) Circadian oscillations of a transcript encoding a germin-like protein that is associated with cell walls in young leaves of the long-day plant *Sinapis alba* L. *Plant Physiol* 106: 905–915
- Hurkman WJ, Tanaka CK (1996) Germin gene expression is induced in wheat leaves by powdery mildew infection. *Plant Physiol* 111: 735–739
- Lane BG (1991) Cellular desiccation and hydration: developmentally regulated proteins, and the maturation and germination of seed embryos. *FASEB J* 5: 2893–2901
- Lane BG (1994) Oxalate, germin, and the extracellular matrix of higher plants. *FASEB J* 8: 294–301
- Lane BG, Grzelczak Z, Kennedy T, Kajioka R, Orr J, D'Agostino S, Jaikaran A (1986) Germin: compartmentation of the two forms of the protein by washing growing wheat embryos. *Biochem Cell Biol* 64: 1025–1037
- Lane BG, Dunwell JM, Ray JA, Schmitt MR, Cuming AC (1993) Germin, a protein marker of early plant development, is an oxalate oxidase. *J Biol Chem* 268: 12239–12242
- Membré N, Bernier F (1998) The rice genome expresses at least six different genes for oxalate oxidase/germin-like proteins (Genbank AF032971, AF032972, AF032973, AF032974, AF032975, AF032976) (PGR98-021). *Plant Physiol* 116: 868
- Membré N, Berna A, Neutelings G, David A, David H, Staiger D, Sáez Vásquez J, 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 Mol Biol* 35: 459–469
- Neutelings G, Domon JM, Membré N, Bernier F, Meyer Y, David A, David H (1998) Characterization of a germin-like protein gene expressed in somatic and zygotic embryos of pine (*Pinus caribaea* Morelet). *Plant Mol Biol* 38: 1179–1190
- Ohmiya A, Tanaka Y, Kadowaki K, Hayashi T (1998) Cloning of genes encoding auxin-binding proteins (ABP19/20) from peach: significant peptide sequence similarity with germin-like proteins. *Plant Cell Physiol* 39: 492–499
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Shutov AD, Braun H, Chesnokov YV, Bäumlein H (1998) A gene encoding a vicilin-like protein is specifically expressed in fern spores. Evolutionary pathway of seed storage globulins. *Eur J Biochem* 252: 79–89
- Sugiura M, Yamamura H, Hirano K, Sasaki M, Morikawa M, Tsuboi M (1979) Purification and properties of oxalate oxidase from barley seedlings. *Chem Pharm Bull* 27: 2003–2007
- 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 of Rhizobiaceae. *Plant Mol Biol* 24: 171–183
- Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J* 11: 1187–1194
- Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss H-H (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res* 15: 5890
- Vallélian-Bindschedler L, Mössinger E, Métraux JP, Schweizer P (1998a) Structure, expression and localization of a germin-like protein in barley (*Hordeum vulgare* L.) that is insolubilized in stressed leaves. *Plant Mol Biol* 37: 297–308
- Vallélian-Bindschedler L, Schweizer P, Mössinger E, Métraux JP (1998b) Heat-induced resistance in barley to powdery mildew (*Blumeria graminis* f. sp. *hordei*) is associated with a burst of active oxygen species. *Physiol Mol Plant Pathol* 52: 185–199
- Wei Y, Zhang Z, Andersen CH, Schmelzer E, Gregersen PL, Collinge DB, Smedegaard-Petersen V, Thordal-Christensen H (1998) An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. *Plant Mol Biol* 36: 101–112
- Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. *Biochem J* 322: 681–692
- Wojtaszek P, Stobiecki M, Bolwell GP (1997) Changes in the composition of exocellular proteins of suspension-cultured *Lupinus albus* cells in response to fungal elicitors or $CuCl_2$. *J Exp Bot* 48: 2015–2021
- Woo E-J, Dunwell JM, Goodenough PW, Pickersgill RW (1998) Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography. *FEBS Letters* 437: 87–90
- Zhang Z, Collinge DB, Thordal-Christensen H (1995) Germin-like OxO, a H_2O_2 -producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *Plant J* 8: 139–145
- Zhou F, Zhang Z, Gregersen PL, Mikkelsen JD, de Neergaard E, Collinge D, Thordal-Christensen H (1998) Molecular characterization of the oxalate oxidase involved in the response of barley to the powdery mildew fungus. *Plant Physiol* 117: 33–41