Journal of Plant Biology, June 1999, 42(2) : 97-108 **REVIEW**

Germin-Like Proteins: Structure, Phylogeny, and Function

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Germin-like proteins (GLPs) are a large family of polypeptides present in all plants that share high identity to wheat germin. Germin is a 130 kDa homooligomeric glycoprotein composed of 26 kDa subunits, which was originally identified in monocots (McCubbin et al., 1987). It is expressed in developing seedlings, although it is not found in mature seed, nor in immature embryos. Rather, its synthesis is induced within five hours following imbibition of seeds. Its expression increases as the embryo grows (Thompson and Lane, 1980; Grzelczak and Lane, 1984) and accumulates in the apoplast (Lane et al., 1986).

Germin was originally described as a relatively rare protein that was present only in germinating wheat; however, with the advent of genome sequencing projects, it has become increasingly evident that germin and the related GLPs are indeed ubiquitous in the plant kingdom. The best characterized family of germin-like proteins is found in Arabidopsis (Table 1), where there are at least 27 individual family members (Membré et al., 1997; Carter et al., 1998; Sage-Ono et al., 1998). A similar large family is known to occur in rice, in which at least eight GLP cDNAs have been deposited in the databases and hexaploid wheat which contains at least seventeen members (Lane et al., 1991). Within the past five years, numerous reports indicate that expression of GLPs occurs in all major plant tissues as well as some unusual sources including pollen (Leitner et al., 1998) and a GLP that is secreted into floral nectar.

GLPs are also highly expressed. Of the 35,680 cDNAs that have been deposited in the *Arabidopsis thaliana* EST databases, surprisingly 39 of these encode germin-like proteins. Thus, mRNAs encoding GLPs represent about 0.1% of all of the mRNAs cloned in this project. Further, among ESTs from *Mesembryanthemum crystallinum* plants, germin-like proteins are one of the most abundant families of proteins representing 1.9% of all mRNAs examined (JC Cushman & CB Michalowski, personal communication). Thus, these highly abundant, extracellular proteins represent a unique but poorly understood feature of plant biology. This review will focus on three topics: The general structure of germin-like proteins and their genes, the phylogeny of the gene family and the potential functions of germin-like proteins.

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Structure of Germin-Like Proteins

Most GLPs have been identified from cDNA sequences. Currently, there are 58 complete cDNAs or genes from 16 plant species in the databases defining GLPs. With this abundance of protein sequences now available, we can identify related structural features that define germin-like proteins. The shared structural features among GLPs include: conserved structural elements, secretory transit peptides, protein glycosylation sites and regions of conserved sequence identity. Other physical characteristics of this family include resistance to SDS denaturation, heat stability and protease resistance (Dumas et al., 1993).

The most prominent conserved structural feature among these GLPs is a conserved amino acid sequence termed the germin box. The consensus pattern is: GxxxxHxHPxAxEh, where x is any amino acid and h is a hydrophobic amino acid. Mature germins and GLPs are all approximately 200 amino acids in length and the germin box occurs near the middle for all proteins in this family.

Models of GLPs, based upon on the structure of vicilin, indicate that this conserved sequence is located within a β-barrel structure. The histidine cluster within the germin box is structurally similar to histidine clusters found in other proteins. The copper amine oxidase from *Escherichia coli* contains one such histidine cluster. Within this enzyme, the histidine cluster is part of a metal binding site that exists at the active site (Gane et al., 1998). A similar histidine-rich sequence is conserved in auxin binding proteins (Jones et al., 1998). The histidines of this sequence participate in the binding of the carboxylate group of IAA. Whether the GLP histidine rich sequence functions to bind metal or functions to bind one of the carboxylates of oxalic acid is not clear. However the

Table 1. Analysis of the *Arabidopsis thaliana* GLP gene family.

	Identity	GenBank Identity[a]			
		Clade/Subclade[b]	cDNA	gene	#of ESTs[c]
1	At-GLP1	Subfamily 3	U75206	AF090733	13
2	At-GLP2a	Subfamily 1/GLP2	U75192	---	3
3	At-GLP2b	Subfamily 1/GLP2	X91957	---	1
4	At-K3K3.1	Subfamily 1/GLP2	---	AB010694	0
5	At-K3K3.2	Subfamily 1/GLP2	---	AB010694	0
6	At-K3K3.4	Subfamily 1/GLP2	---	AB010694	0
7	At-MXF12.11	Subfamily 1/GLP2	---	AB016892	0
8	At-GLP3a	Subfamily 3	U75188	---	2
9	At-GLP3b	Subfamily 3	U75195	U33014	4
10	At-GLP4	True Germins	U75187	[d]	1
11	At-GLP5	Subfamily 2	U75198	[d]	5
12	At-GLP6	Subfamily 1/GLP6	U75194	---	1
13	At-MXF12.9	Subfamily 1/GLP6	---	AB016892	0
14	At-MXF12.10	Subfamily 1/GLP6	---	AB016892	0
15	At-MXF12.12	Subfamily 1/GLP6	---	AB016892	0
16	At-MXF12.P4	Subfamily 1/GLP6	---	AB016892	0
17	At-GLP7	Gymnosperm GLPs	U75202	[d]	1
18	At-GLP8	Subfamily 2	U75207	[d]	1
19	At-GLP9	Subfamily 1/GLP9	U81294	Z97336	1
20	At-K15E6.9	Subfamily 1/GLP9	---	AB009048	0
21	At-K15E6.11	Subfamily 1/GLP9	---	AB009048	0
22	At-K15E6.12	Subfamily 1/GLP9	---	AB009048	0
23	At-K15E6.13	Subfamily 1/GLP9	---	AB009048	0
24	At-K15E6.14	Subfamily 1/GLP9	---	AB009048	0
25	At-MAC9.4	Outlier	---	AB010069	0
26	At-GLP10	Subfamily 2	U95036	[d]	2
27	At-GLP11	Subfamily 2	---	AF058914	0

[a]For those members where multiple ESTs have been identified the GenBank Accession of the longest clone is presented.
[b]Refor to Figure 4.
[c]Number of ESTs or cDNAs isolated and characterized to date.
[d]The sequences shown in Figure 2 have not yet been deposited.

degree of conservation of this sequence within the GLPs indicates that it is likely to play a role in either the structure or function of this protein.

The vicilin-based models of germin also predict that GLPs contain a large amount of β-structure (Gane et al., 1998). Secondary structure predictions (Carter et al., 1998) and circular dichroism measurements (McCubbin et al., 1987) also indicate that large amounts of β-structure exist within GLPs. A secondary structural prediction for the most abundant *Arabidopsis thaliana* GLP is shown in Figure 1, Panel A. The patterns of helix-loop-sheet are typical of all GLPs that have been analyzed to date. As can be seen, the N-terminal transit peptide consists mostly of an α-helix. The N-terminal half of the mature GLP1 protein is largely unstructured and contains a conserved site of N-glycosylation. The C-terminal half is predicted to contain several short β-structures. This is the region that matches the β-barrel structure of vicilin (Gane et al., 1998) and appears to mediate oligomer formation.

The N-terminal transit sequence is another hallmark of GLPs. Almost all germins and GLPs contain an N-terminal signal peptide. Out of 27 known members of the Arabidopsis GLP family (see Table 1), only one does not appear to contain a cleavable N-terminal signal sequence (Carter et al., 1998). The signal sequences of GLPs contain all of the typical features of other signal sequences including charged residues near the N- and C-terminus of the signal peptide and a hydrophobic core sequence that forms an α-helix. As is expected for most proteins containing signal sequences, GLPs accumulate extracellularly (Dratewka-Kos et al., 1989; Lane et al., 1992; Lane et al., 1993; Neutelings et al., 1998). For those GLPs that have not been examined experimentally, predictions of subcel-

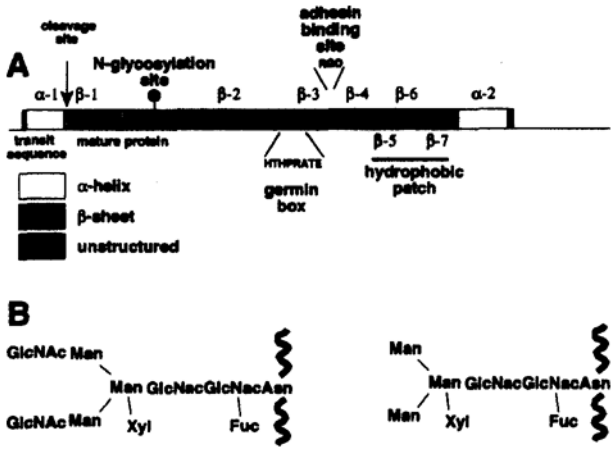

Figure 1. Panel **A.** Predicted secondary structure of Arabidopsis GLP1. α-helical regions are shown in white; β-sheet regions are shown in dark grey and unstructured regions are shown in light grey. The α-helical and β-sheet domains are consistent in all GLPs. The transit sequence cleavage site is indicated by the arrow. The conserved site of N-glycosylation is indicated by the black circle. A hydrophobic patch which may mediate oligomer formation is underlined and the location of the germin box, HTHPRATE and the putative adhesin binding sites are indicated Panel **B.** Structure of N-linked glycans present on Germin for G (at left) and G' (at right) (Jaikaran et al., 1990).

lular localization (Nakai and Kanehisa, 1992) indicate that they most likely accumulate extracellularly (Carter et al., 1998). Nevertheless, in a few Cases, germin-like proteins and oxalate oxidase activity have been found to be associated with microsomal fractions (Franceschi, 1978; Hurkman et al., 1991).

All GLPs examined to date contain at least one conserved site of N-glycosylation. This site occurs in the N-terminal half of the protein within a loop region (see Fig. 1). In a large number of studies, N-glycosylation has been shown to be crucial for effective protein secretion, solubilization, folding, activation, signaling, and protein binding (Lerouge et al., 1998). The precise function of the conserved N-linked glycan moiety in GLPs is unknown, however, both monocot and dicot GLPs contain the conserved glycosylation sites indicating that the sites have been maintained since monocot-dicot divergence over 125 million years ago. The conservation of this site over such a long time span suggests an important role for glycosylation in the function of GLPs.

Jaikaran et al examined the N-linked glycans of wheat germin (Jaikaran et al., 1990). These authors found two forms of N-linked glycans on the same protein which resulted in two distinct isoforms of germin. These isoforms were termed G and G' and had differing SDS PAGE mobilities (Jaikaran et al., 1990). Each G monomer unit contained the structure of shown in Figure 1, Panel B (left) and G' isoform lacked the antennary N-acetylglucosamine residues (Jaikaran et al., 1990) and contained the structure shown in Figure 1, Panel B (right). Both of these structures are consistent with normal plant glycoproteins. Interestingly, the G isoform was primarily found in soluble fractions, whereas G' was principally associated with cell walls.

The quaternary structure of GLPs has also been addressed by several groups. Based upon SDS PAGE and ultracentrifugation, wheat germin was originally proposed to be a homopentamer of approximately 130 kDa (McCubbin et al., 1987). However, Sugiura et al. have reported that the native barley enzyme exists as a dimer of identical subunits (Sugiura et al., 1979). Recently x-ray crystallographic and modeling methods have suggested that in solution germin probably exists as a trimer of dimers (Gane et al., 1998; Woo et al., 1998). It should be noted that differential glycosylation and adventitious binding of cell wall carbohydrate may effect the observed molecular weights (Woo et al., 1998).

The sites of interaction between monomer subunits have also been investigated. A highly hydrophobic patch shown in Figure 1 that is located within the C-terminal β-structure region has been proposed to mediate monomer interactions (Dratewka-Kos et al., 1989; Carter et al., 1998). However, the vicilin-based models of GLPs have suggested that an α-helical region within the C-terminal half of the protein may be responsible for multimer formation (Gane et al., 1998).

Structure of GLP Genes

To date all of the germin or GLP genes exist in one of two structural organizations. A few of the genes are transcribed from a single exon; however, the majority appear to be encoded by two exons interrupted by a single intron in the transcriptional unit. Among the 21 Arabidopsis GLPs whose complete genomic sequences are available (presented in Table 1 and Fig. 2) only 5 genes GLP1, GLP3b, GLP7, K15E6.13 and MAC9.4 lack an intron . Even the exact site of exon splicing is conserved in the closely related GLP5 and GLP10 genes of Arabidopsis and also in a tobacco GLP gene, Nectarin I. The remaining 16 genes show the two exon-one intron genomic structure. Interestingly, the reported cereal germins, are encoded on a single exon (Lane et al., 1991).

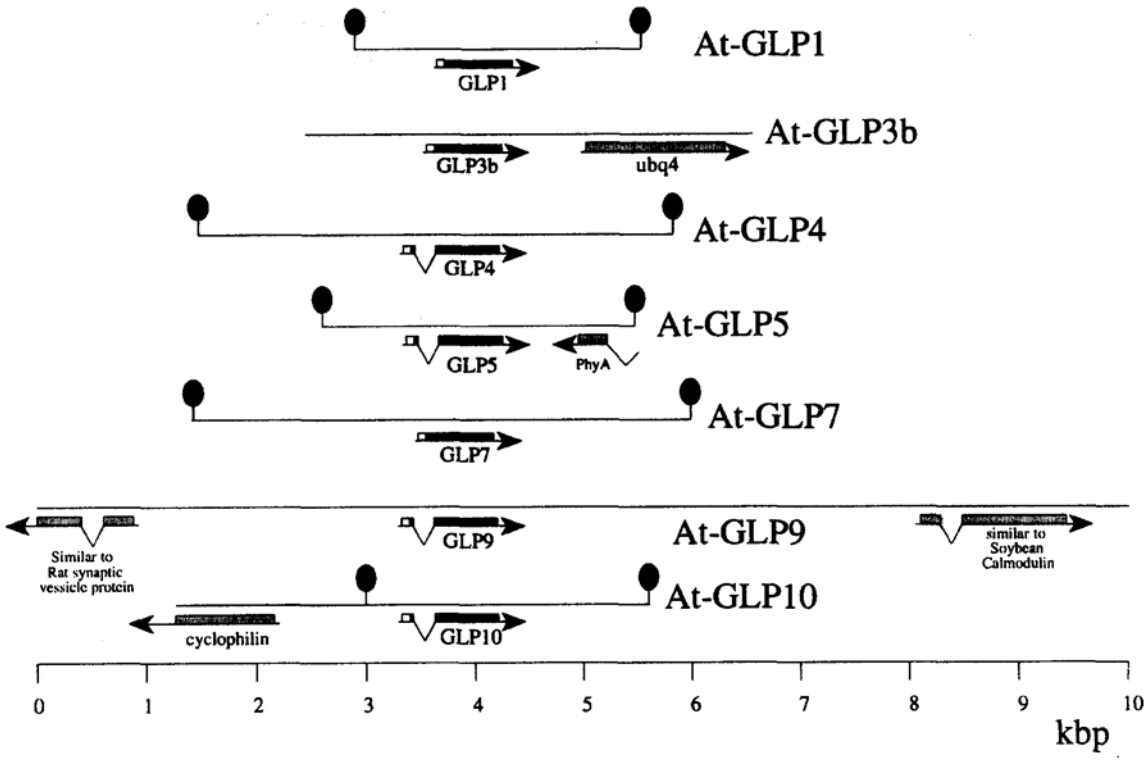

Figure 2. A comparison of the structures of some Arabidopsis GLP genes. The location of the GLP coding regions and identified non-related genes are indicated and labeled. The ruler across the bottom of the figure represents the size of the DNA fragments in kilobases.

Sequence analysis of the promoters of the wheat germin genes, gf-2.8 and gf-3.8, has revealed several regions within the promoters that resemble auxin-responsive elements (Lane et al., 1991; Berna and Bernier, 1997). When analyzed in plants, Hurkman and Tanaka demonstrated that germin could be induced by treatment with IAA (Hurkman and Tanaka, 1996a). Later, in studies with transgenic plants containing germin-reporter gene constructs the germin promoters were independently shown to be induced by IAA (Berna and Bernier, 1997). Several other GLP genes, including Arabidopsis GLP2b, GLP5, GLP7, GLP8, and GLP9 also contain putative auxin-responsive promoter elements (Carter et al., 1998).

In addition to IAA, germin levels are also inducible by treatment with NaCl, salicylate, methyl salicylate, methyl jasmonate, abscisic acid, and pathogen attack but not wounding (Dumas et al., 1995; Hurkman

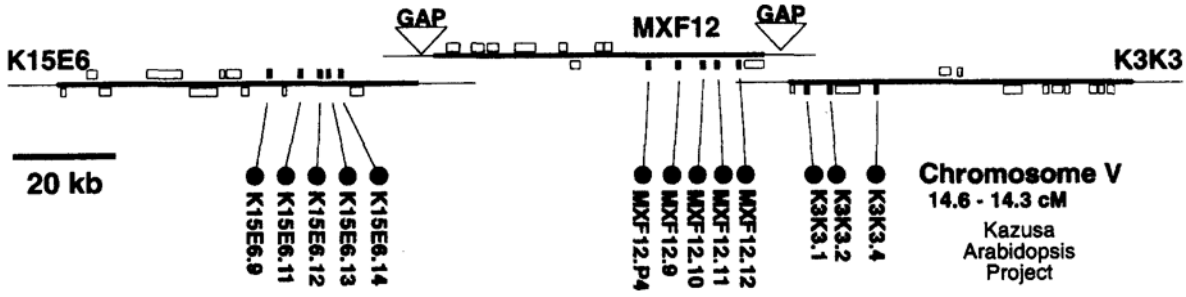

Figure 3. The Arabidopsis GLP2/6/9 locus located on chromosome V. The BAC clones K15E6, MXF12, and K3K3 were sequenced by the Kazusa Arabidopsis Genome Project (Sato et al., 1998). The open boxes indicate non-GLP genes. The black boxes represent GLP genes and each is identified below the map. The bar at the bottom-left of the figure represents the 20 kilobases of DNA.

and Tanaka, 1996a).

Structure of the GLP2/6/9 Locus

We routinely search the Arabidopsis genome databases to identify additional Arabidopsis GLPs. One of these searches identified a locus that had been sequenced by the Kazusa Arabidopsis Project (Sato et al., 1998). This locus, present on three BAC clones is located between 14.3 and 14.6 cM on Arabidopsis Chromosome V and contains 13 GLP genes in three clusters (See Fig. 3). Interestingly, all of the genes in each of the clusters are transcribed from the same strand indicating that they may have arisen by direct duplication. Further we have performed a cladistic analysis of the Arabidopsis GLP genes and each of these clusters of genes appears to reside in a distinct clade of the phylogenetic tree. These findings suggest that this locus may have recently undergone the duplication events that have given rise to this large number of GLP genes.

Phylogeny

Figure 4 shows a cladistic, phylogenetic analysis of 56 complete GLP sequences that have to date been deposited in the GenBank/EMBL/DDBJ. Approximately 30 additional GLP ESTs from various species are also present in the databases. However, these clones have not yet been completely sequenced so they are not included in this analysis. Based upon this analysis, we conclude that the GLP gene family is rapidly expanding and conclusions may change with time. Still, by comparing the amino acid sequences of the GLP proteins we can evaluate the degree of relatedness among these proteins.

As can be seen from Figure 4, the GLPs fall into five major clades. GLPs appear to be found in all plant species going as far back as gymnosperms. With the exception of the Arabidopsis GLP7, the gymnosperm GLPs form a separate clade of the GLP family tree. It will be especially interesting to determine whether additional gymnosperm GLPs are also members of this clade or whether gymnosperms contain as diverse a family of GLPs as do the angiosperms. Preliminary evidence indicates that the gymnosperm GLPs may not be as disperse as the angiosperm GLPs, because southern blot hybridizations suggest that PcGER1 is probably unique in the pine genome (Neutelings et al., 1998).

Likewise, the true germins form a major clade of the phylogenetic tree. As shown in Figure 4, most of the wheat and barley GLP sequences that have been identified to date belong to the true germin clade of the phylogenetic tree. The conserved wheat germin sequence is found throughout the Triticeae family, although sequence diversity was observed among these sequences (Monte et al., 1998). In addition, one rice clone (Os-GLP7) and one Arabidopsis clone (At-GLP4) also lie within this clade.

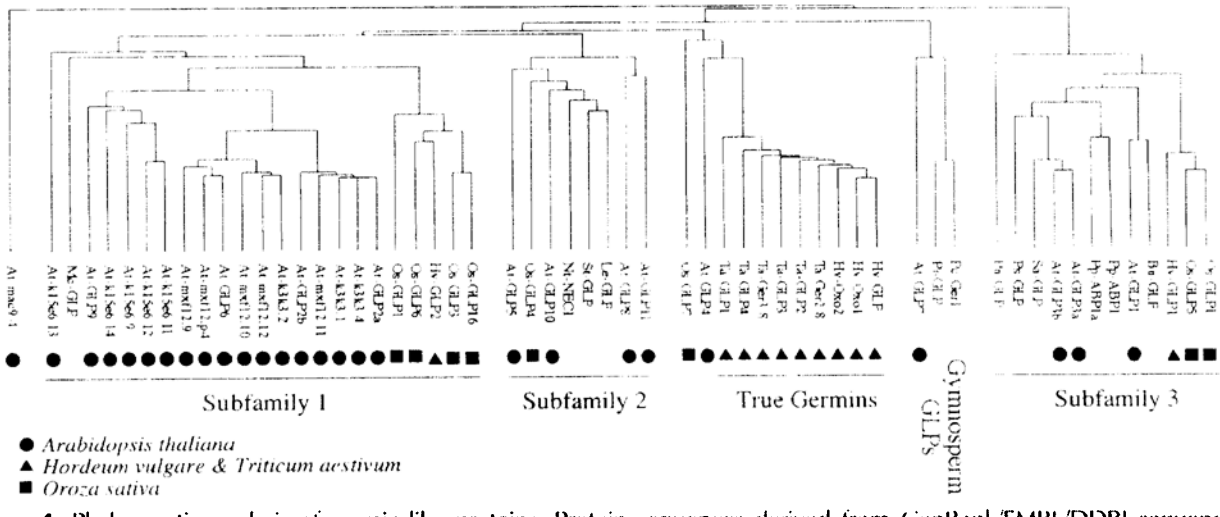

Figure 4. Phylogenetic analysis of germin-like proteins. Protein sequences derived from GenBank/EMBL/DDBJ sequences were compared using the Wisconsin GCG program "Pile-Up". One outlier (At-mac9.4) and 5 clades were identified. The various subfamilies are indicated. Those sequences derived from *Arabidopsis thaliana* GLPs are indicated by the black circles below the sequence name. Those sequences derived from barley and wheat are indicated by the black triangles. Those sequences derived from rice are indicated by the black squares.

The remaining GLP clones fall into three distinct clades that compose three major subfamilies. Subfamily 1 contains the largest number of sequences. There are 23 members of subfamily 1 present in the databases. Seventeen of these GLP subfamily 1 sequences are Arabidopsis clones. Interestingly, as shown in Figure 3, thirteen of them reside in a locus that is present on three BAC clones (K15E6, MXF12, and K3K3) on Arabidopsis chromosome V (Sato et al., 1998). The other genes appear to be scattered throughout the genome. Within this subfamily, individual subclades are formed that contain GLP2s (5 members), GLP6s (6 members), and GLP9s (5 members). The rice and barley members of subfamily 1 are less closely related to the Arabidopsis GLPs than the Arabidopsis GLPs are to each other indicating that the divergence of the Arabidopsis GLPs may be relatively recent.

GLP subfamily 2 contains the fewest members of the remaining GLP subfamilies. There are five Arabidopsis members of this subfamily, GLP4, GLP5, GLP8, GLP10, and GLP11. In addition, one rice clone and three GLPs from solanaceous plants, the tomato GLP, the potato GLP, and an unusual GLP that is expressed in the nectar of tobacco plants are also members of this subfamily.

The GLP subfamily 3 is the most cosmopolitan of the GLP subfamilies. Of the twelve subfamily 3 members, eight species are represented. There have not been enough species analyzed to determine whether the GLP subfamily 3 is more dispersed in plants than the other subfamilies; however, we discount this. Rather, there is evidence that GLP subfamily 3 members are easier to isolate because their level of expression is higher. In Arabidopsis, subfamily 3 GLPs represent 58% of all cloned GLP ESTs. Based upon this frequency, we have speculated that the subfamily 3 GLPs are transcribed at 2.5 to 14 times the rate of other Arabidopsis GLPs (Carter et al., 1998). If this is true for most plants, then the higher levels of expression would result in more frequent isolation of GLP subfamily 3 members.

Both sequence identity and protein structure have led to the classification of germins and GLPs into a superfamily of proteins termed 'cupins' (Dunwell, 1998). The main attribute of cupins is the formation of a small β-barrel. Other representative cupin proteins include phosphomannose isomerases, polyketide synthases, sucrose binding proteins, auxin binding proteins, and seed vicilins. Members of this superfamily are represented primarily by plant and microbial proteins and include uni- and bi-domain (bicupin) polypeptides, with the latter containing a duplication of the first domain. Germins have been classified as 'long cupins' of the single domain type (Dunwell, 1998). There is little sequence identity between most cupins and germin-like proteins.

Expression

By far the best characterized GLPs are the original wheat and barley germin. Germin was originally identified as an early marker of embryo germination (Lane, 1991). Approximately 5-10 h postimbibition, germin mRNA levels increase greatly. This increase in germin mRNA levels occurs simultaneously with secondary embryo hydration (Lane, 1991). In wheat, the initial expression is found within the embryonic axis, and by two days post-imbibition, the expression is found throughout the roots. By 9 days post imbibition, expression is found in the vascular bundles of the leaves (Caliskan and Cuming, 1998).

Studies of transgenic tobacco containing either the intact wheat germin gene or promoter-GUS fusions have also been performed. Both GUS and oxalate oxidase activity was primarily found in developing seeds and in seedlings (Berna and Bernier, 1997). Germin gene transcription was also induced by auxin and this stimulation was not limited to developing seeds and seedlings, as auxin treatment also stimulated expression in young leaves (Berna and Bernier, 1997). Germin expression was also driven by fungal pathogens, polyamines, and certain abiotic stresses such as Al^{+3}, Cd^{+2}, Cu^{+2}, and Co^{+2} (Hamel et al., 1998; Berna and Bernier, 1999). This finding is interesting as oxalate has been reported to detoxify high levels of aluminum in buckwheat (Ma et al., 1998; Zheng et al., 1998).

Pseudogermin is a closely related isoform of germin that is found in developing wheat seeds that shows unusual stability (Lane et al., 1991). N-terminal sequence shows a high degree of identity between germin and pseudogermin, however, SDS PAGE analysis reveals that the two proteins run as distinct isoforms with differing mobilities and stabilities. Upon germination pseudogermin is incorporated into cell walls, and is highly protease resistant and thermostable (relative to germin), retaining its activity at 100°C with t1/2 = six minutes (Lane et al., 1992). The reason for pseudogermins unique thermostability is not yet clear.

Other GLPs

The most abundant Arabidopsis germin-like protein

is GLP1. It is most highly expressed in leaves and is present at low levels in siliques at the start of embryogeny (Membré et al., 1997). Arabidopsis GLP3b is also highly expressed and is found within stems and hypocotyls. In addition, its concentration varies in a circadian cycle (Membré et al., 1997). This is similar to the *Sinnapis alba*, *Hordeum vulgare*, and *Pharbitis nil* GLPs which are also expressed in response to light (Heintzen et al., 1994; Ono et al., 1996; Vallelain-Bindschedler et al., 1998). While the mRNA levels vary, the GLP steady-state protein concentration remains constant over light/dark cycles (Heintzen et al., 1994). Other subfamily 3 GLPs are expressed in peach shoot apices (Ohmiya et al., 1998).

In the ice plant, *M. crystallinum*, a germin-like protein, (McGLP) is expressed in roots and decreases in response to salt stress (Michalowski and Bohnert, 1992). Extracellular GLPs that share both immunological identity and sequence identity with wheat germin have also been identified in embryogenic cell cultures of Caribbean Pine, *Pinus caribaea* (Domon et al., 1995). These proteins are expressed in non-embryogenic cell cultures. Recently, a radish GLP was identified as an EST. It was more highly expressed in leaves and roots than in flower buds (Moon et al., 1998). GLPs have been identified in the extracellular matrix of lupine (Wojtaszek et al., 1998). The lupine GLPs are also induced in response to fungal elicitors or to Cu^{+2} ions (Wojtaszek et al., 1997).

Recently, a novel tobacco GLP was characterized that is secreted into the nectar of tobacco flowers. This protein, termed Nectarin I, was not identified in western blot analysis of leaves, stems, roots, or other floral organs with the exception of floral nectaries. These studies also demonstrated that GLPs are secreted into the nectar of a variety of species. Whether the closely related potato and tomato GLPs represent Nectarin I isologs must await further analysis. While Nectarin I was not detected in other floral organs, other GLPs have been identified in pollen. N-terminal amino acid sequence analysis of the main pollen allergen in pepper, a 28 kDa protein revealed identity with wheat germin (Leitner et al., 1998).

Functions

Because of the extremely high occurrence of these proteins in plants, coupled with the large size of the gene families, even in streamlined species such as Arabidopsis, the roles that these proteins play in plant physiology/biochemistry becomes increasingly important. Several widely diverse functions have been proposed for germin-like proteins. These include a role in cell wall restructuring, osmotic regulation, and in a number of stress responses including fungal attack and metal stresses.

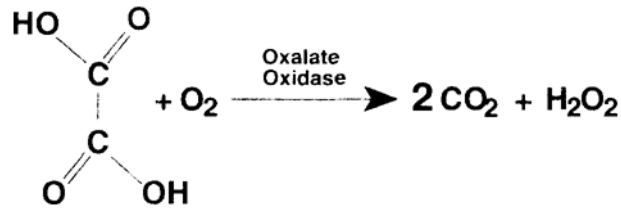

Figure 5. Enzymatic activity of oxalate oxidase. Some germin-like proteins show oxalate oxidase activity.

Recently, two groups independently reported that germin is an oxalate oxidase (Dumas et al., 1993; Lane et al., 1993). This enzyme catalyzes the oxidative breakdown of oxalate to carbon dioxide and hydrogen peroxide (Fig. 5). To date, the oxidation of oxalate to CO_2 and H_2O_2 has been the only catalytic activity reported for this protein family. Interestingly, the majority of GLPs appear to be devoid of oxalate oxidase activity. This may indicate that non-oxalate oxidase GLPs utilize alternative substrates to generate hydrogen peroxide or play another role in oxalate metabolism.

Although intensely studied, the cofactors required for catalysis have not yet been confirmed. A requirement of metal ions for activity has been demonstrated as metal chelating agents inactivate this enzyme (Satyapal and Pundir, 1993; Kotsira and Clonis, 1998). Certain metals such as Cu^{+2}, Mn^{+2}, Ca^{+2}, Pb^{+2}, and Fe^{+2} have been implicated as required cofactors (Pundir, 1991; Lane, 1993; Satyapal and Pundir, 1993; Kotsira and Clonis, 1998; Wojtaszek et al., 1998). Also, early studies reported that oxalate oxidase is a flavoprotein and several reports indicate that flavins activate the enzyme from sorghum (Pundir, 1991; Satyapal and Pundir, 1993). However, it has recently been reported that barley oxalate oxidase does not contain flavin cofactors (Kotsira and Clonis, 1998) and that Mn^{+2} and Fe^{+2} are the most likely cations required for activity (Requena and Bornemann, 1998).

Defense Related Functions

There is a growing body of evidence that germins are involved in plant defense responses. Even before its hydrogen peroxide producing activity was discovered, germin was implicated for its presence in domesticated strains of pathogen resistant cereals

(Lane et al., 1986). Hydrogen peroxide can act as an anti-microbial agent itself (Lachman, 1986) and acts as a secondary messenger in plant defense responses. H_2O_2 has been reported to be involved in phytoalexin biosynthesis, hypersensitive responses and PR responses (for recent reviews: Bolwell and Wojtaszek, 1997; Ebel and Mithöfer, 1998). Furthermore, H_2O_2 can be utilized in lignification processes to oxidatively crosslink various cell wall components in a manor analogous to that of cell wall restructuring in developing seedlings, thus creating a barrier to prevent further pathogen infection.

Oxalate is produced as a toxin by a number of plant pathogenic fungi (e.g. *Sclerotinia sclerotiorum*) (Magro et al., 1988; Dickman and Chet, 1998) and is well know for its metal chelating abilities, especially in the case of Ca^{+2}. Calcium has been reported to be involved in plant defense signaling (Poovaiah and Reddy, 1987; Lane, 1993; Flego et al., 1997; Long and Jenkins, 1998). Although the role of oxalate in plant diseases remains unclear, the oxalate secreted by various pathogens may act by impeding calcium mediated defense responses. Plant oxalate oxidases may therefore liberate bound Ca^{+2} from calcium oxalate crystals and confer resistance.

It should be noted that some plants naturally contain high amounts of oxalate, and calcium oxalate crystals are often found as an intracellular store of calcium (McNair, 1932; Franceschi, 1978; Kausch, 1983). Furthermore, other defense related genes (chalcone synthase, PAL) are responsive to light conditions which have been shown to be mediated through a calcium mediated signaling pathway (Long and Jenkins, 1998). The expression of several GLPs have also been shown to vary with light conditions. Thus the timely expression of cytoplasmic oxalate oxidases may act to release calcium stores for signal transduction purposes.

Identity between germins and certain vicilins may further indicate a role for germins in plant defenses. Vicilins are common seed storage proteins. In one report, legume vicilins (7S storage globulins) inhibited yeast growth and glucose stimulated acidification of the medium by yeast cells (Gomes et al., 1998). Another report found that the same vicilins bound various forms of chitin and this association may mediate resistance to *Callosobruchus maculatus* in cowpea seeds (Sales et al., 1996). Although the homology between germins and vicilins is tenuous and biochemical activities are likely different, their similarities in structure and expression might indicate a role in plant defense for both.

Recently a number of groups have reported that germin and GLP gene expression and oxalate oxidase activity increase upon pathogen attack (Dumas et al., 1995; Zhang et al., 1995; Hurkman and Tanaka, 1996b; Gregersen et al., 1997; Vallelain-Bindschedler et al., 1998; Wei et al., 1998; Zhou et al., 1998; Berna and Bernier, 1999). Twenty-four hours after infection of barley with the powdery mildew fungus, oxalate oxidase activity increases and by 48 h post inoculation there is approximately 10-fold higher oxalate oxidase activity in treated vs untreated plants (Zhang et al., 1995). Furthermore, this activity precedes PR1 accumulation by 1-3 days. This suggests a role for germin and H_2O_2 signaling in mediating defense responses. Interestingly, expression levels increase in both compatible and incompatible reactions (Hurkman and Tanaka, 1996b ; Zhou et al., 1998). This may indicate that the increase in germin gene expression is not an indicator of cultivar resistance, but rather is caused by the infection process (Hurkman and Tanaka, 1996b). However, several studies have reported that transgenic plants in which oxalate oxidase is constitutively produced have increased resistance to pathogens (Thompson et al., 1995; Dunwell, 1998). Nevertheless, the increased resistance may be due to systemic acquired resistance induced by constitutive H_2O_2 production rather than oxalate degradation.

Bacterial Interactions

One of the earliest steps in plant bacterial interactions is the attachment of the bacterium to plant cells. A cell surface protein from *Rhizobium leguminosarum* , rhicadhesin, appears to be the bacterial protein that is involved in attachment of the bacteria to the root hair surface (Smit et al., 1989; Smit et al., 1991, 1992).

A putative plant rhicadhesin receptor was isolated and submitted to N-terminal sequencing. The resulting sequence revealed that this rhicadhesin receptor was a germin-like protein (Swart et al., 1994). Thus, GLPs appear to be the plant partners in the initial interactions between rhizobia and pea root hairs. Whether other GLPs mediate bacterial attachment remains unexplored. GLPs with the highest amino acid identity to the pea root rhicadhesin receptor are the Arabidopsis genes, GLP5 and GLP10.

Rhicadhesin appears to be similar to other adhesive proteins such as vitronectin that can bind a tripeptide sequence Arg-Gly-Asp (RGD). Swart et al., also demonstrated that rhicadhesin can also bind an RGD con-

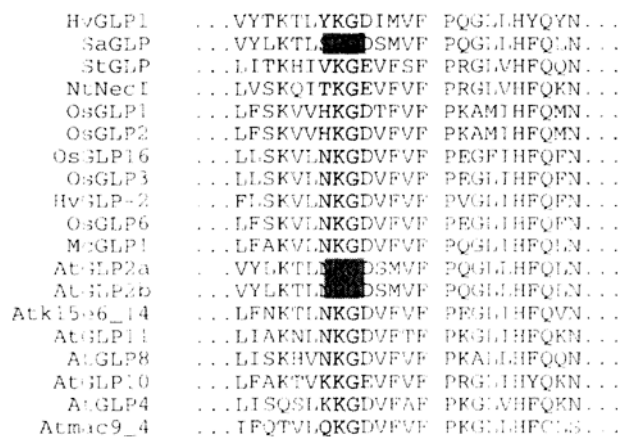

Figure 6. Putative adhesin binding sites within germin-like proteins. Those sequences containing the RGD tripeptide are boxed in dark grey. Those sequences containing variant tripeptides, KGD or KGE are boxed in light grey.

taining hexapeptide (Swart et al., 1994). They further speculated that the rhicadhesin receptor may contain an RGD tripeptide sequence. When we compare the GLP sequences from the databases, we do indeed find three GLPs (AtGLP3a, AtGLP3b, and SaGLP) that contain a single internal RGD tripeptide (Fig. 6). Several other GLPs contain conserved substitutions in the same region. While rhicadhesin appears to be specific for the RGD sequence, adhesin molecules from other bacterial species could interact with plant cell walls by binding the RGD variant GLPs.

Cell Wall Restructuring of Seedlings and Embryos

Cell wall restructuring requires hydrogen peroxide and peroxidase dependent crosslinking of cell wall components (Lane et al., 1993; Olson and Varner, 1993; Zhang et al., 1995). Germin's peroxide producing capabilities as well as expression patterns (being expressed primarily in germinating seedlings) have led to the hypothesis that this enzyme acts to produce H_2O_2 at the cell wall which can be utilized by peroxidases in the cross-linking of cell wall components thereby restricting cell growth (Lane et al., 1993).

When isolated, wheat germin usually co-purifies with adventitiously bound glycans (Jaikaran et al., 1990; Lane et al., 1992). These authors reported that the bound glycans are highly substituted glucuronogalactoarabinoxylans and are similar to monocot cell wall hemicelluloses (Jaikaran et al., 1990; Lane et al., 1992). This finding supports the hypothesis that germin plays a role in cell wall restructuring, as these polysaccharides serve as precursors to cell wall hemicelluloses (Lane et al., 1992).

Desiccation and Hydration

The germin protein is an early marker of embryo germination. Approximately 5-10 h post-imbibition, germin mRNA levels and subsequently protein levels increase greatly. The increase in transcript accumulation occurs simultaneously with secondary embryo hydration (Lane, 1991). Furthermore, germin shares homology with spherulins 1a and 1b of the slime mold *Physarum polycephalum* (Lane et al., 1991). Spherulins are thought to be important proteins in the sporulation/encystment processes of *Physarum* (Bernier et al., 1986). Spherulins and germins also share other features: both are associated with cell walls and they accumulate under conditions of osmotic and environmental stress. Spherulins are expressed at very high levels as are the germins and this expression occurs at times when water management within cells is important. Thus, these correlations have led to the hypothesis that GLPs may function in the processes of desiccation and hydration.

Salt and Heavy Metal Responses

A number of reports have indicated that germin gene expression is effected by salt stress (Hurkman et al., 1991; Michalowski and Bohnert, 1992; Hurkman et al., 1994; Hurkman and Tanaka, 1996a). For example, two 26 kDa proteins (Gs1 and Gs2) were identified as germin-like proteins that increase in salt-stressed barley roots (Hurkman et al., 1991). The majority of protein was expressed in the soluble fraction of root extracts, however, some protein was also found in microsomal and cell wall fractions. The Gs2 protein accumulated upon treatment with abscisic acid whereas Gs1 did not (Hurkman et al., 1991) and thus may reveal functional differences. Other findings indicate that germin mRNA levels were reported to maintain maximal levels for an extra day (2 vs 3 d) for barley seedlings sown in the presence of 200 mM NaCl and that roots of 6 d old seedlings exposed to salt shock increased to a maximal level within 8 h (Hurkman and Tanaka, 1996a). These findings, as well as GLP responses to heavy metals are not necessarily surprising as oxalate is known to play a role in ionic homeostasis in plants (Franceschi, 1978; Franceschi and Horner, 1980).

Oxalate Metabolism in Plants

In plants, oxalic acid is produced via at least five different pathways (Franceschi and Horner, 1980). A major source of oxalate generation in plants is through glycolate and glyoxylate, which arise through photorespiration, the glyoxylate shunt, or purine degradation. Appreciable amounts of oxalate may also originate through enzymatic cleavage of both oxaloacetate and ascorbate (Kausch, 1983). Of these pathways, photorespiration and ascorbate degradation are thought to be the major suppliers of glycolate and glyoxylate (Franceschi, 1978; Franceschi and Horner, 1980). Indeed, oxalate generation been has associated with photosynthesis and carbohydrate metabolism (Franceschi, 1978; Franceschi and Horner, 1980). Furthermore, oxalate formation in *Oxalis corniculata* was found to increase during the hours of peak photosynthesis and decrease during the evening and night (Franceschi, 1978). Because some GLPs have oxalate oxidase activity, the circadian oscillations of oxalate may have significance for the circadian fluctuations of GLPs in plants.

The formation of calcium oxalate crystals is widespread in plants. In 1932, McNair listed 215 different plant families that were known to contain calcium oxalate crystals (McNair, 1932). Further, most tissue types investigated have been reported to contain calcium oxalate crystals, including woody tissues, anthers, ovaries, fruits, floral buds, roots, leaf tissues, bark layers, callus of wounds, pedicels, and the elongation region below the apical meristem of shoots (Franceschi, 1978; Franceschi and Horner, 1980).

The ubiquitous accumulation of oxalate crystals in plants mirrors the apparent ubiquitous nature of GLPs, some of which have oxalate oxidase activity. Whether there is a relationship between these two observations remains one of the interesting unsolved mysteries of germin-like proteins.

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