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Germin and Germin-like Proteins: Evolution, Structure, and Function

Jim M. Dunwell[1], J. George Gibbings[1], Tariq Mahmood[2]*, and S. M. Saqlan Naqvi[2]*

[1]School of Biological Sciences, University of Reading, Whiteknights, Reading, U.K.

[2]Department of Biochemistry, University of Arid Agriculture Rawalpindi, Murree Road-46300, Rawalpindi, PAKISTAN

*Current Address; Department of Plant Sciences, Quaid-e-Azam University, Islamabad.

ABSTRACT: Germin and germin-like proteins (GLPs) are encoded by a family of genes found in all plants. They are part of the cupin superfamily of biochemically diverse proteins, a superfamily that has a conserved tertiary structure, though with limited similarity in primary sequence. The subgroups of GLPs have different enzyme functions that include the two hydrogen peroxide-generating enzymes, oxalate oxidase (OxO) and superoxide dismutase. This review summarizes the sequence and structural details of GLPs and also discusses their evolutionary progression, particularly their amplification in gene number during the evolution of the land plants. In terms of function, the GLPs are known to be differentially expressed during specific periods of plant growth and development, a pattern of evolutionary subfunctionalization. They are also implicated in the response of plants to biotic (viruses, bacteria, mycorrhizae, fungi, insects, nematodes, and parasitic plants) and abiotic (salt, heat/cold, drought, nutrient, and metal) stress. Most detailed data come from studies of fungal pathogenesis in cereals. This involvement with the protection of plants from environmental stress of various types has led to numerous plant breeding studies that have found links between GLPs and QTLs for disease and stress

resistance. In addition the OxO enzyme has considerable commercial significance, based principally on its use in the medical diagnosis of oxalate concentration in plasma and urine. Finally, this review provides information on the nutritional importance of these proteins in the human diet, as several members are known to be allergenic, a feature related to their thermal stability and evolutionary connection to the seed storage proteins, also members of the cupin superfamily.

Keywords: oxalate oxidase, stress response, disease resistance, multigene family, polyploidy, allergen, diagnostics

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1. INTRODUCTION

This review will summarise recent information related to the evolution, expression and function of germin and related proteins, a functionally and biochemically diverse group of plant proteins linked to programmed cell death and expressed during specific stages of plant development and in response to biotic and abiotic stress. The exploitation of this most diverse of protein families in applied plant breeding, and in biomedical applications will also be considered.

2. GERMIN AND GERMIN-LIKE PROTEINS

Germins constitute a group of homologous proteins only found in "true cereals" including barley (*Hordeum*), maize (*Zea*), oat (*Avena*), rice (*Oryza*), rye (*Secale*) and wheat (*Triticum*) (Lane, 2002). Germin was initially identified as a specific marker for the start of germination in wheat embryos, from which function it was given the name "germin" (Thompson and Lane, 1980). Many years later it was characterized as a homopentameric glycoprotein (Faye and Chrispeels, 1988: Jaikaran *et al.,* 1990) with oxalate oxidase (OxO) activity (Lane *et al.,* 1993), a process that generates hydrogen peroxide (H_2O_2) and which may help to produce refractory barriers against tissue invasion by predators (Lane, 2000; Schweizer *et al.,* 1999) (for more details see discussion below). Woo *et al.* (1998, 2000) subsequently determined the structure of germin at 1.6 Å resolution, and showed that in contrast to the suggestion from previous

biochemical studies, the mature protein comprises six β-jellyroll monomers locked into a homohexamer (a trimer of dimers) (Fig. 1). This structure accounts for its remarkable stability to various denaturing agents; all germins share unusual resistance to broad specificity proteases and to dissociation by various agents such as heat, SDS and extreme pH (Lane *et al.*, 1993; Lane, 1994; Wei *et al.*, 1998; Carter and Thornburg, 2000; Membré *et al.*, 2000).

In terms of sequence, all germins contain a characteristic peptide sequence, PHIHPRATEI, known as the germin box (Lane *et al.*, 1991). Proteins related to wheat germin and containing the characteristic germin have subsequently been found in various land plants and designated as germin-like proteins (GLPs) (Dunwell, 1998). GLPs have a maximum of 90% sequence identity to wheat germin, although the average level of identity is close to 50%. However, there is almost complete identity in the two motifs within the conserved cupin domain (see below) that contain the residues involved as ligands in the active site (Fig. 1) and a common structure is predicted (Dunwell, 1998; Dunwell *et al.*, 2000, 2001).

The structural resolution of germin confirmed the prediction concerning conserved N-glycosylation sites (NXS/T), and there is also some evidence for S-nitrosylation (Romero-Puertas *et al.*, 2008). It is assumed that the N-terminal signal peptide is associated with targeting to the cell wall/extracellular matrix (Lane *et al.*, 1992; Heintzen *et al.*, 1994; Lane 1994; Berna and Bernier 1997). However, the precise significance of these post-translational modifications and targeting sequences in modulating the activity of these proteins is unknown.

3. GERMIN AND GLPS ARE MEMBERS OF THE CUPIN SUPERFAMILY

Germin and GLPs were described as the archetypal members of the cupin superfamily (Dunwell, 1998). The cupin superfamily of proteins, named on the basis of this conserved β-

barrel fold (‘cupa’ is the Latin term for barrel), was originally discovered using the conserved motif found within germin, GLPs from higher plants, and spherulin (a stress-associated protein from the slime mould *Physarum polycephalum*) (Lane *et al*., 1991). Overall, the cupins comprise the most functionally diverse protein fold yet described with more than 60 different enzyme and non-enzyme activities (Dunwell *et al.,* 2001; 2004; unpublished). In addition to the oxidase activity represented by OxO, other enzyme activities include isomerases, decarboxylases, dioxygenases, cyclases, and epimerases. The reason for this biochemical diversity lies in the flexibility of the ‘active site’ within the centre of the eight stranded β-barrel mature cupin domain (Dunwell *et al.,* 2001; Rajavel *et al*., 2008).

4. GLPS ARE ENCODED BY A FAMILY OF RELATED GENES

Although GLPs were first identified in cereals and a small number of other higher plants, they are now known to be ubiquitous plant proteins encoded by a family of related genes. A large number of GLP genes have been discovered as expressed sequence tags (ESTs) or by genome sequencing in higher plants like *Arabidopsis* (Carter *et al.,* 1998; Membré *et al*., 1997), barley (Wu *et al.,* 2000, Druka *et al.,* 2002), and rice (Membré and Bernier, 1998). When reviewed by Bernier and Berna (2001) it was reported that “The number of different sequences related to germin is now close to a hundred”. At the time of preparing the present review GenBank contains a total of 379 proteins and 825 nucleotides annotated as germin, though the number of related but non-annotated ESTs is several thousand (Dunwell, unpublished). It is now known that the number of GLP genes in each species of higher plant exceeds 30 (Cannon et *al*., 2004) and most are present within the genome as multiple copies at a small number of loci (Kazusa *et al*., 2000; Manosalva, 2006). The most complete analysis of GLPs from lower plants is that

conducted by Nakata *et al*. (2004) who identified a family of nine genes in the moss *Physcomitrella patens*, a member of the first land plant group (Qiu and Palmer, 1999; Quatrano *et al*., 2007). Similar sequences have been isolated from other mosses including *Ceratodon purpureus* and *Barbula unguiculata* (Yamahara *et al.*, 1999; Nakata *et al*., 2002), and from a liverwort, *Marchantia polymorpha* (Nagai *et al.*, 1999). Nakata *et al.* (2004) also reported a phylogenetic analysis of the GLP family of *P. patens* and proposed two novel clades designated bryophyte subfamilies 1 and 2, which are evidently discernible from the clades of higher plants (Carter and Thornburg, 2000). Interestingly, the two cysteins molecules at conserved positions in the N-terminus of all reported germins and GLPs, are not present in bryophytes subfamily 1, or in spherulin. It would seem therefore that duplication of the progenitor GLP gene had already occurred during evolution of the first land plant, and that a further phase of duplication took place during Angiosperm evolution. Indeed the GLP gene family has been used recently in studies of ancestral polyploidy (Senchina *et al*., 2003; Pfeil *et al*., 2005), gene duplication (Canon *et al*., 2004; Adams, 2007; Roth *et al*., 2007) and subfunctionalization, the process by which a pair of duplicated genes, or paralogs, experiences a reduction of individual expression patterns or function while still reproducing the complete expression pattern and function of the ancestral gene (Federico *et al*., 2006; Zimmerman *et al*., 2006; Liu and Adams, 2007). The subject of expression patterns during development is described in more detail in section 6 below, which is then followed by sections that consider changes in the pattern of expression in response to a range of biotic and abiotic influences.

5. BIOCHEMICAL PROPERTIES

To date, three different enzymatic activities have been associated with these proteins. First, OxO activity is associated with the true germins (Berna and Bernier, 1997, Lane *et al.,* 1993); secondly, superoxide dismutase (SOD) activity is found in some germins and GLPs (Yamahara *et al.,* 1999; Woo *et al*., 2000; Carter and Thornburg, 2002; Christensen *et al.*, 2004; Zimmermann *et al.,* 2006; Gucciardo *et al.,* 2007), and finally ADP-glucose pyrophosphatase or phosphodiesterase (AGPPase) activity has been shown in a barley GLP (Rodríguez -López *et al.,* 2001). Two isoforms of AGPPase have been characterized using barley leaves, one of the isoforms designated as soluble AGGPPase1 (SAGPPase1), is soluble in low ionic strength buffer, the other SAGPPase2 is extractable by using high salt concentration solution. N-terminal and internal sequence analysis showed that both SAGPPase1 and SAGPPase2 are distinct oligomers of the previously designated *Hordeum vulgare* GLP1 (Rodríguez-López *et al.,* 2001). More recently, a maize protein *Zm*GLP1 with sequence similarity to the barley GLP with AGPPase activity has also been reported (Fan *et al.,* 2005). Additionally, a GLP which inhibits serine protease activity has been reported in the wheat apoplast and suggested to be part of a defense system against insect and bacterial proteases ((Segarra *et al.,* 2003; Cordo *et al*., 2007). Such biotic responses are discussed below.

In light of the biochemical diversity present in the cupin superfamily (Dunwell *et al*., 2004), it is very likely that some GLPs may have additional enzyme activities that have not yet been identified. In addition to their direct enzymatic role some GLPs are known to be involved in specific protein-protein interactions. For example, the DING group of phosphate binding proteins (Berna *et al*., 2008; Griffaut *et al*., 2007; Moniot *et al*., 2007; Perera *et al*., 2008; Zhang *et al*., 2007b) were first identified in plants by their binding to a specific subclass of GLP.

6. DEVELOPMENTAL REGULATION OF GLP GENE EXPRESSION

It has become obvious in recent years that germins and GLPs expression is neither restricted to cereals as originally thought nor is specific to germination. As discussed above, genes or EST sequences encoding proteins with sequence similarity to germins have been identified from all plant species from such data are available. Additionally, there are now many experimental studies from various species which show a variety of expression patterns of GLP genes. The methods available for the numerical assessment of transcripts as a means of assessing patterns of gene expression include EST quantification, microarrays and Serial Analysis of Gene Expression (SAGE) technologies (Gibbings *et al*., 2003), and the information summarised below was generated using one or more of these methods. In the context of this review, it should be noted that there are few comparative studies using different techniques on the same material (Grimplet *et al*., 2007). As well as transcriptomic data derived from the various gene expression technologies, there is now an increasing emphasis on proteomic information in which high resolution protein extraction and separation methods have been allied to mass-spectrometry to identify the presence of specific peptide fragments. Such information will be integrated in the subsections below that follow (with various additions and amendments) the pattern used in an earlier review (Dunwell *et al*., 2000) to which the reader is referred; the emphasis in the present review is on recently published information.

Several of these studies have identified GLPs amongst the population of proteins found in specific tissues including in the extracellular environment or apoplast that incorporates the cell wall, a highly dynamic compartment with a role in many important plant processes including defence, development, signaling and assimilate partitioning. For example, soluble apoplast proteins from Arabidopsis, wheat and rice were separated by two-dimensional electrophoresis

and the molecular weights and isoelectric points for the dominant proteins were established prior to excision, sequencing and identification by matrix-assisted laser-desorption time of flight mass spectrometry (MALDI - TOF MS) (Haslam *et al*., 2003). From the selected spots, 23 proteins from rice and 25 proteins from Arabidopsis were sequenced, of which nine identifications were made in rice (39%) and 14 in Arabidopsis (56%). This analysis revealed that the principal proteins identified in the aqueous matrix of the apoplast were involved in defence, i.e. GLPs or glucanases, and cell expansion, i.e. beta-D-glucan glucohydrolases.

One most important issues that must be addressed in assessing the data reported in the subsequent sections of this review is the ability to discriminate between expression from specific members of the GLP gene family. One possible method that permits such discrimination is use of the SAGE technology. In one relevant study of this type Gibbings *et al*., (2003) compared expression from mature leaves and immature seeds from the rice Nipponbare cultivar. The leaf library consisted of a total of 42,087 tags and 9,412 tags were analysed in the immature seed library. Tag identities were determined by matching them to EST members of the tentative consensus (TC) sequences of the TIGR rice gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/geneprod_search.pl). Analysis of these tags revealed ESTs coding for six unique GLPs with one represented by two fragments (from the total of 40 GLPs encoded by the rice genome). Four of these seven tags, derived from transcripts from chromosome 8 (Tani *et al*., 2008), were observed in the seed data set and three in the leaf data set (Table 1) with none of the tags shared between the two data sets. Most significantly, the higher abundance of the tags in the seed data set correlates with the expression reports for these tissues from the rice gene index. These data thus demonstrate the power of SAGE technology in discriminating between expression of different members of this large gene family.

6.1. Leaf Development

In an EST study of sugar cane leaves, two hundred fifty clones were randomly selected, subjected to single-pass sequencing, and identified by sequence similarity searches (Carson and Botha, 2000); two clones were identified clones as GLPs. Similarly, Fan *et al.* (2005) reported a green tissue specific GLP gene from *Zea mays* (*Zm*GLP1). The transcript of this gene was found to be abundant in young leaves, less frequent in mature leaves, young tassels, and cobs and not detectable in roots, immature kernels and stalks. RNA *in situ* hybridization experiments revealed that *Zm*GLP1 was only expressed in mesophyll, phloem and guard cells in the young whorl leaves.

In a study of the role of the nuclear protein DET1 in the photomorphogenesis pathway in Arabidopsis it was found that a peroxisomal protein encoded by *TED3* is essential for growth (Hu *et al*., 2002). One GLP (U75201) was among the proteins shown to be integral to this pathway. Also important in this same context are cryptochromes, nuclear proteins that mediate light control of hypocotyl elongation, leaf expansion, photoperiodic flowering, and the circadian clock. The blue light photoreceptor mutant cryptochrome1-304 (cry1-304) and Columbia wild-type 4 (col-4) of *Arabidopsis thaliana* were grown under white light and blue light, and in darkness (Yang *et al*., 2008). To study the difference in protein expression levels between cry1-304 and col-4, a proteomic approach was applied based on 2-D gel electrophoresis. A GLP (spot 42) was shown to be up-regulated in cry1-304.

Secondary growth is supported by a dividing population of meristematic cells within the vascular cambium whose daughter cells are recruited to differentiate within secondary phloem and xylem tissues. In a study of this subject Groover *et al*. (2006) cloned a *Populus* Class 1

KNOX homeobox gene, ARBORKNOX1 (ARK1), which is orthologous to Arabidopsis SHOOT MERISTEMLESS (STM). ARK1 is expressed in the shoot apical meristem and the vascular cambium, and is down-regulated in the terminally differentiated cells of leaves and secondary vascular tissues that are derived from these meristems. Transformation of *Populus* with either ARK1 or STM over-expression constructs resulted in similar morphological phenotypes characterized by inhibition of the differentiation of leaves, internode elongation, and secondary vascular cell types in stems. Analysis with a microarray comprising 55,794 nuclear and 126 mitochondrial and chloroplast gene models, and 9,995 unigenes derived primarily from *P. tremula* x *P. tremuloides* EST sequences showed that 41% of genes up-regulated in the stems of ARK1 over-expressing plants encode proteins involved in extracellular matrix synthesis or modification, including proteins involved in cell identity and signaling, cell adhesion, or cell differentiation. Three GLPs were present in this list. These gene expression differences are reflected in alterations of cell wall biochemistry and lignin composition in ARK1 over-expressing plants. The results suggested that ARK1 has a complex mode of action that may include regulating cell fates through modification of the extracellular matrix. These findings support the hypothesis that the shoot apical meristem and vascular cambium are regulated by overlapping genetic programs. Relevant data from field grown poplar have been reported recently by Sjödin *et al*. (2008).

Certain plant growth-promoting rhizobacteria (PGPR), in the absence of physical contact with a plant stimulate growth via volatile organic compound (VOC) emissions, through largely unknown mechanisms. To probe how PGPR VOCs trigger growth in plants, RNA transcript levels of Arabidopsis seedlings exposed to *Bacillus subtilis* (strain GB03) were examined using oligonucleotide microarrays (Zhang *et al*., 2007a). These authors screened over 26,000 protein-

coded transcripts and identified a group of approximately 600 differentially expressed genes related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation and other expressed proteins. These genes included certain GLPs (eg GER2, At5g39190). Transcriptional and histochemical also data indicated that VOCs from the PGPR strain GB03 trigger growth promotion in Arabidopsis by regulating auxin homeostasis.

As a complement to these transcriptomic studies, a recent proteomic study of Arabidopsis involved analysis of three set of material: leaves of fully developed rosettes containing differentiated cells; etiolated hypocotyls analyzed at the end of elongation; and 7-day-old cell suspension cultures, when cells are actively dividing and expanding (Boudart *et al*., 2005; Jamet *et al*., 2006). All three cell wall proteomes were obtained using comparable salt-extraction protocols, separation of proteins by electrophoresis, and identification by MALDI-TOF spectrometry. The 11 proteins common to all three organs included one GLP. Similarly, in a related study of a sub-proteome of Arabidopsis mature stems trapped on Concanavalin A, only one GLP was identified (Minic *et al*., 2007). Schlesier *et al*. (2004) also used a proteomic approach to compare the protein patterns of the Arabidopsis ecotypes Col-0 and Ws-2. In leaf extracts, pairs of protein spots were found to be diagnostic for each of the lines. Both pairs of spots were identified as closely related GLPs differing in only one amino acid by using peptide mass finger printing of tryptic digests and by gaining additional data from post-source decay spectra in the MALDI-TOF analysis.

In a further study of the subject, Chitetti and Peng (2007) established the proteome map of *Arabidopsis* cotyledons and investigated the dynamic change of the cotyledon proteome in the time course of cell dedifferentiation. Among the 353 distinct genes, corresponding to 500 2-DE gel protein spots identified with high confidence, 12% had over twofold differential regulation

within the first 48 h of induction of cell dedifferentiation. The distribution of these genes among different Gene Ontology categories and gene differential regulations within each of the categories was also examined. Additionally, the authors investigated the cotyledon phosphoproteome using Pro-Q Diamond Phosphoprotein in Gel Stain followed by mass spectrometry analyses. Among the 53 identified putative phosphoproteins including a GLP, nine are differentially regulated during cell dedifferentiation. These studies have revealed yet another interesting facet of GLPs, namely that their activities may involve regulation by phosphorylation

In addition to these and other studies on higher plants (Carpentier *et al*., 2005), similar methods were used in the identification of 306 proteins from the protonema of *Physcomitrella patens* (Sarnighausen *et al*., 2004). Proteins were separated by two dimensional electrophoresis, excised from the gel, analysed by means of mass spectrometry, and five GLPs were identified.

6.2. Root Development

Perhaps the most interesting recent study of this topic is that of Vellosillo *et al*. (2007) who used two Arabidopsis mutants, *noxy2* (for *nonresponding to oxylipins2*), a new mutant insensitive to 9-hydroxyoctadecatrienoic acid (9-HOT), and *coronatine insensitive1-1* (jasmonate-insensitive). These results, together with the findings that *noxy2* and mutants with defective 9-lipoxygenase (9-LOX) activity showed increased numbers of lateral roots, suggest that 9-HOT, or a closely related 9-LOX product, is an endogenous modulator of lateral root formation. Histochemical and molecular analyses revealed that 9-HOT activated events common to development and defense responses. A subset of 9-HOT-responding root genes, including GLPs (At4g14630, At5g38910) was also induced in leaves after 9-HOT treatment or pathogen inoculation. In a related investigation, a root cDNA library from pineapple was differentially

screened for mRNAs that are preferentially present in roots (Neuteboom *et al*., 2002). Over 300 randomly selected cDNAs from this library were categorized into 14 classes based on their expression characteristics. One GLP was predominant among six mRNAs verified to be preferentially present in roots by analyzing in detail their abundance during field-grown plant and fruit development. A germin EST was also recorded among the most abundant transcripts found in sorghum roots (Baerson *et al*., 2008).

At a protein level, there are several relevant studies, including one on the role of sphingolipid/sterol-rich domains so-called lipid rafts of the plasma membrane. It was shown recently that lipid raft domains, defined as Triton X-100-insoluble membranes, can be prepared from *Medicago truncatula* root PMs (Lefebvre *et al*., 2007). These domains were extensively characterized by ultrastructural studies as well as by analysis of their content in lipids and proteins, and were shown to be enriched in sphingolipids and D7-sterols, with spinasterol as the major compound. A large number of proteins (i.e. 270) were identified. Among them, receptor kinases and proteins related to signaling, cellular trafficking, and cell wall functioning, including a GLP (AC149038_25.1), were well represented whereas those involved in transport and metabolism were poorly represented. Likewise, in a study of plasma membranes from maize roots, Kukavica *et al.* (2005) reported the presence of a Mn-containing SOD that has the thermal stability characteristic of a GLP. Recently, in a study of salt-stressed pea roots Gucciardo *et al.* (2007) also reported SOD activity in a protein with an electrophoretic mobility similar to that of the *Ps*GER1 protein. Furthermore, in developing pea nodules, *Ps*GER1 transcript was detected in expanding cells just proximal to the meristematic zone and to lesser extent in the epidermis. This is the first report of a GLP with SOD activity associated with nodules. Similarly, the presence of

GLPs was shown in proteomic studies of cassava roots (Sheffield *et al*., 2006) and root-pressure xylem exudate from cut stems of *Brassica napus* (Kehr *et al*., 2005).

6.3. Floral Development

Following earlier studies on Arabidopsis (Heintzen *et al.,* 1994) and *Sinapis alba* (Heintzen *et al.,* 1994) that showed a diurnal pattern of expression of various GLPs in leaves and flowers, a more recent study of maize reported that a *Zm*GLP1 transcript varied with a circadian rhythm (Fan *et al.,* 2005). GLPs are also expressed at high levels in some specific reproductive tissue. For example, about 3% of the ESTs expressed in egg cells of maize encode germin-like (OxO) proteins (Yang *et al*., 2006). By contrast, only two of the 7,165 female gametophyte ESTs encoded GLPs. In another study of this subject, a reference map of the Arabidopsis mature pollen proteome was produced by using multiple protein extraction techniques followed by 2-DE and ESI-MS/MS. Among the 135 distinct proteins from a total of 179 protein spots, several were shown to be GLPs (Holmes-Davis *et al*., 2005; Noir *et al*., 2005).

Carter and Thornburg (2000) and Naqvi (unpublished) have demonstrated that the sugar rich nectar synthesized by the nectary glands of the ornamental tobacco contains five proteins which make act in a coordinated defense system against infection of the nectar and nectary glands. Among the nectar proteome, a GLP with Mn-SOD activity is proposed to play an important role in generation of H_2O_2 which may serve as a major defense molecule. A large scale EST study of sunflower has also reported high expression of GLP in floral tissue (Fernández *et al*., 2003), and GLP8 (At3g05930) was shown to have a reduced level of expression in male sterile Brassica flowers (Lou *et al*., 2007).

6.4. Zygotic and Somatic Embryogenesis

The most comprehensive study of zygotic embryos in this context is probably that conducted on Arabidopsis in which a total of 11,032 EST sequences obtained from isolated immature seeds were used as the initial dataset. A pilot study performed using EST virtual subtraction followed by microarray data analysis led to the identification of 49 immature seed-specific genes (Becerra *et al*., 2006). Genes that reached the maximum level of expression between late torpedo and early walking-stick embryo stages included five genes: At5g09640, encoding a serine carboxypeptidase, At5g49190, encoding a sucrose synthase, At2g34700, encoding a proline rich glycoprotein, and two genes encoding GLPs (At3g04170 and At3g04190).

There are more data concerning somatic embryos. For example, following initial studies that identified several GLPs in embryogenic cultures of Caribbean pine (*Pinus caribaea* Morelet var. *honduriensis*) (Domon *et al.*, 1995), a full-length GLP expressed in both somatic and zygotic embryos was reported by Neutelings *et al.* (1998). Similar sequences have been found to be associated with somatic embryos of Monterey pine *Pinus radiata*, a suspension culture of potato and a cell culture of lupin (Wojtaszek *et al.*, 1998). More recently, a small number of GLP genes were shown to have high mRNA transcript levels in embryogenic tissue and little or no expression in non embryogenic (roots, shoots and needles) or callus tissue (needle and fiber callus culture) of *Pinus radiata* (Bishop-Hurley *et al.*, 2003) and other conifers (Mathieu *et al*., 2006). Similar results were obtained by Lippert *et al*. (2005) with *Picea glauca*, and Çaliskan *et al*. (2004b) who described the presence of OxO expression in embryogenic wheat callus.

Additionally, proteomic methods have been employed to assess quantitatively the expression levels of proteins across four stages of somatic embryo maturation in white spruce

(*Picea glauca*) (0, 7, 21 and 35 d post abscisic acid treatment) (Lippert *et al*., 2005). Forty-eight differentially expressed proteins, including one GLP, displayed a significant change in abundance as early as day 7 of embryo development. Similar methods have also been applied, and similar results obtained, in an analysis of somatic embryogenesis in *Vitis vinifera*, by comparing embryogenic and non embryogenic calluses of the *Thompson seedless* cultivar (Marsoni *et al*., 2008). Changes in GLP expression have also been reported in early cotyledon development in Arabidopsis embryos (Chitteti and Peng, 2007), and it has been shown that GLP1 is expressed in the embryo axis during germination and is expressed neither in cotyledons nor in mature vegetative tissues of *Phaseolus vulgaris* (Aubry *et al.*, 2003). These results can be compared with similar data from wheat germination (Çaliskan and Cuming, 2000; Çaliskan *et al*., 2003, 2004a).

Probably the most relevant and complete study of Arabidopsis cells *in vitro* is that conducted recently by Che *et al*. (2007). It is known that *Arabidopsis* shoots regenerate from root explants in tissue culture through a two-step process requiring preincubation on an auxin-rich callus induction medium (CIM) followed by incubation on a cytokinin-rich shoot induction medium (SIM). During CIM preincubation, root explants acquire competence to respond to shoot induction signals whereas pericycle cells in root explants undergo cell divisions and dedifferentiate and lose the expression of a pericycle cell-specific marker. These cells acquire competence to form green callus only after one day CIM preincubation and to form shoots after 2–3 days CIM preincubation. Genes requiring CIM preincubation for upregulation on SIM were identified by microarray analysis and included the GLP At3g10080. It can be assumed that the type of global modelling described by (Zeng *et al*., 2007) will be able to define the interaction between GLPs and other genes that regulate this developmental pathway.

6.5. Seed and Fruit Development.

Three subtracted cDNA libraries were prepared from the lemma/palea of barley at the elongation stage (between pollination and milky stages) through to the dough stages of kernel development (Abebe *et al*., 2004). Differential screening and northern hybridization showed that the cloned genes were highly expressed in the lemma/palea, compared with the flag leaf. High expression levels of defence-related genes, including GLPs, strongly suggested that the lemma/palea constitutively accumulate defensive molecules to inhibit invasion of florets and kernels by pathogens (see above).

In cotton (*Gossypium hirsutum* L.) a GLP (*Gh*GLP1) shows tissue-specific accumulation in fibres (Kim and Triplett, 2004; Kim *et al.*, 2004; Tu *et al*., 2007; Wu *et al*., 2007). The germins and GLPs with OxO and SOD activities both produce hydrogen peroxide (H_2O_2), which could be used in cross-linking reactions within the cell wall. The association of maximal *Gh*GLP1 expression with stages of maximal cotton fibre elongation indicates that some GLPs may be important for cell wall expansion (Kim *et al.*, 2004).

A detailed analysis of gene expression during the development of grape berries was recently conducted by Grimplet *et al*. (2007) who validated the expression profiles obtained using the Affymetrix GeneChip® *Vitis* oligonucleotide microarray by quantitative RT-PCR on 19 genes using gene specific primer pairs. Transcript abundance patterns were compared among tissues including two genes for which comparisons were confirmed between pulp and skin or pulp and seed tissues from berries harvested from well-watered and water-deficit stressed vines. Although linear regression analysis showed coefficients of variation of 0.93 or 0.94, significant differences were observed for several of these genes depending on the tissue examined. For

example, the transcript abundance in the seed for the GLP (TC45186) and 9-cis-epoxycarotenoid dioxygenase (NCED) (TC42536) were disproportionately higher when estimated by microarray.

In a similar study on fruit of *Citrus sinensis*, a putative GLP was found to be the most abundant metabolism-related transcript from the whole flavedo databank with 339 reads (0.7% transcripts) sequenced from that tissue (Berger *et al*., 2007). A similarly comprehensive transcriptome analysis using a citrus 22K oligoarray was performed to identify ethylene-responsive genes and gain an understanding of the transcriptional regulation by ethylene in mandarin fruit (*Citrus unshiu* Marc.). In the 72 h after ethylene treatment, 1493 genes, including a GLP, were identified as ethylene-responsive with more than 3-fold change in expression (Fujii *et al*., 2007). Related results have also been reported with fruit of *Citrus sinensis* (Goudeau *et al*., 2008).

6.6. Lignification and Wood Development

To gain insights into the metabolic, developmental, and regulatory events that control vascular and interfascicular fibre differentiation along the axis of bolting stems in Arabidopsis, Ehlting *et al*. (2005) employed an Arabidopsis full-genome longmer microarray. More than 5000 genes were differentially expressed, among which more than 3000 changed more than twofold, and were placed into eight expression clusters based on polynomial regression models. Analysis of differentially expressed phenylpropanoid genes identified a set known to be involved in lignin biosynthesis. These were used to anchor co-expression analyses that allowed the identification of candidate genes encoding proteins involved in monolignol transport and monolignol dehydrogenation and polymerization. Three GLP genes were identified by this process.

Wissel *et al.,* (2003) measured the mRNA levels of 11 different genes including a GLP mRNA in leaves of free-growing aspen (*Populus tremula*) throughout the growing season (bud burst to leaf abscission) and reported that GLP mRNA was only expressed in the early season rather than the whole season like other genes tested. In a larger scale study, microarrays containing approximately 16,700 unique cDNAs were used to study transcript profiles that characterize the developmental transition in apical shoots of Sitka spruce (*Picea sitchensis*) from their vegetative tips to their woody bases (Friedmann *et al*., 2007). Along with genes involved in cell-wall modification and lignin biosynthesis, a number of differentially regulated genes encoding protein kinases and transcription factors with base-preferred expression patterns were identified. These could play roles in the formation of woody tissues inside the apical shoot, as well as in regulating other developmental transitions associated with organ maturation. It is interesting that a set of genes represented on the spruce cDNA microarray with similarity to these Arabidopsis genes is also up-regulated in the xylem-enriched tissue. These five spruce genes included one annotated as encoding a GLP.

In order to elucidate the genetic regulation of secondary growth in hybrid aspen (*Populus tremula* L. x *P. alba* L.), an analysis was conducted on a series of cDNA-amplified fragment length polymorphism (AFLP)-based transcriptomes in vertical stem segments that represent a gradient of developmental stages with regard to secondary growth (Prassinos *et al*., 2005). This approach allowed the screening of >80% of the transcriptome expressed in six samples and the identification of genes differentially expressed with the progress of secondary growth, in a tissue-specific manner. By the analysis of 76,800 transcript-derived fragments, 271 were selected and sequenced based on their differential expression patterns. Many of the xylem-up-regulated genes were involved in cell wall and lignin biosynthesis, while the bark-up-regulated genes had

diverse functional roles. In the tissues forming secondary xylem a germin-like oxalate oxidase gene (*Pt1037*) was found to show high expression that coincided with that of at least one of the peroxidases.

Similarly, the presence of GLPs was shown in a SAGE study of *Eucalyptus grandis* (De Carvalho *et al*., 2008) and a proteomic study of wood production in *Pinus pinaster* (Gion *et al*., 2005). In a study of ESTs expressed during the drying process of *Cryptomeria japonica* sapwood, it was found that the second largest category of 58 sequences was annotated as being related to plant "defence" (Yoshida *et al*., 2007). This group, that represented 22 different transcripts, contained a large proportion (8 %) of ESTs assigned as GLPs.

6.7. Senescence

Changes in the activity of OxO and of the concentrations of oxalate and H_2O_2 were investigated during the ageing of leaf sheaths of ryegrass (*Lolium perenne* L.) stubble (Davoine *et al*., 2001). The accumulation of H_2O_2 during ageing coincides with the increases of both oxalate level and OxO activity. Western and Northern blot analyses using protein and RNA extracts of the different categories of leaf sheaths suggested that OxO gene expression, as well as Ca-oxalate synthesis, are crucial events of ageing for leaf sheaths. Immunocytochemistry experiments revealed that OxO, which is an extracellular enzyme, is nearly always present in the parenchymatous cells surrounding the vascular bundles and in the cells of the lower epidermis. Overall, results suggest that in ryegrass that synthesizes both Ca oxalate and OxO, the production of H_2O_2 and Ca^{2+} during ageing of stubble might be involved in the constitutive defences against pathogens, thus allowing the phloem mobilization of nutrient reserves from the leaf sheaths

towards elongating leaf bases of ryegrass. GLPs were also among the set of genes found to be expressed in a recent study of post-harvest deterioration in cassava (Reilly *et al*., 2007).

One of the more comprehensive recent studies of leaf senescence in plants was that conducted on Arabidopsis (Masclaux-Daubresse et al., 2007). The extent to which leaf senescence is induced by nitrogen deficiency or by sugar accumulation varies between natural accessions of this species. Analysis of senescence in plants of the Bay-0 x Shahdara recombinant inbred line (RIL) population revealed a large variation in developmental senescence of the whole leaf rosette, which was in agreement with the extent to which glucose (Glc) induced senescence in the different lines. To determine the regulatory basis of genetic differences in the Glc response, the authors investigated changes in gene expression using Complete Arabidopsis Transcriptome MicroArray (CATMA) analysis. Genes whose regulation did not depend on the genetic background, as well as genes whose regulation was specific to individual RILs, were identified. In addition to sugar accumulation, nitrogen starvation can induce leaf senescence and regulation of senescence mainly depends on the relative availability of nitrogen and carbon. To test whether nitrogen deficiency can elicit similar effects as Glc feeding, the authors determined gene expression in plants grown with low (2 mM nitrate) or high (10 mM nitrate) nitrogen supply. Induction of the senescence marker SAG12 confirmed that senescence was induced by low nitrogen supply. The germin gene GER1, the Asn synthetase gene ASN2, and the Gln synthetase gene GS2, which were downregulated by Glc in the CATMA experiment, were repressed as plants senesced at low nitrogen supply.

Another form of senescence is that associated with the formation of heart wood. This process, a determining factor in wood quality, is a form of senescence that is accompanied by a variety of metabolic alterations in ray parenchyma cells at the sapwood–heartwood transition

zone. Although senescence has been studied at the molecular level with respect to primary growth, the cell maturation and death events occurring during heartwood formation have been difficult to study because of their location and timing. In a transcriptomic study of this process, cDNA microarrays carrying 2567 unigenes derived from the bark/cambium region, sapwood and transition zone of a mature black locust tree were used to characterize seasonal changes in the expression patterns of 1873 genes from the transition zone of mature black locust trees (Yang *et al*., 2004). When samples collected in summer and fall were compared, 569 genes showed differential expression patterns: 293 genes were up-regulated (> twofold) in summer and 276 genes were up-regulated in the autumn. More than 50% of the secondary and hormone metabolism-related genes on the microarrays were up-regulated in summer. Twenty-nine out of 55 genes involved in signal transduction were differentially regulated, suggesting that the ray parenchyma cells located in the innermost part of the trunk wood react to seasonal changes. The authors also established the expression patterns of 349 novel genes, of which 154 were up-regulated in summer and 195 were up-regulated in the autumn. Two GLPs (GenBank BI677524, BI642242) were among the list of genes whose expression was up-regulated more than twofold in transition zones harvested in summer.

7. BIOTIC INTERACTIONS

Plants suffering from stress, mechanical injury or pathogen attack often react with enhanced production of active oxygen species (AOS), a phenomenon often referred to as the "oxidative burst". An important response of plants to salt stress is the accelerated production of AOS such as superoxide, hydrogen peroxide, and the hydroxyl radical, which constitutes oxidative stress (Bellaire *et al.*, 2000; Hernández *et al.*, 2001; Pastori and Foyer, 2002). SOD,

which catalyses the dismutation of superoxide to H_2O_2 and O_2, is of major importance in protecting living cells from superoxide anion toxicity produced under oxidatively stressed circumstances. Various types of SODs are thought to have important roles in controlling oxidative stress in many organelles such as mitochondria, chloroplast and peroxisomes (Alscher *et al.,* 2002, del Río *et al.,* 2002). However, the occurrence and role of SOD in the cell wall are not well known (Alscher *et al.,* 2002). Superoxide (i.e. the SOD substrate) can be generated from NADPH oxidase activity in the cell wall (Christensen *et al.,* 2000). Although the superoxide and H_2O_2 themselves have relatively low toxicity, their metal ion-dependent conversion to the highly toxic hydroxyl radical via the Haber-Weiss reaction is thought to be responsible for the majority of biological damage associated with reactive oxygen species (Wojtaszek, 1997; Zhao *et al*., 2007a). Since SOD rapidly dismutes superoxide to H_2O_2, the enzyme is thought to inhibit hydroxyl radical production and protect living cells from its toxicity. In plant cell walls, SOD could have a role in cell wall stiffening, through the possible action of dismutating H_2O_2 toward lignification (Christensen *et al.,* 2000). In fact, extracellular SOD was reported to exist in lignin-accumulated tissues (Karpinska *et al.,* 2001). Yamazaki *et al.* (2004) identified nine novel thioredoxin target candidate proteins, including a GLP, in the cytosol together with several target proteins for chloroplast thioredoxin of the anti-oxidative stress system in dark grown *Arabidopsis thaliana*.

Similarly, another GLP (*Hv*GLP1) without OxO activity that becomes insolubilized in H_2O_2 treated leaves (Vallélian-Bindschedler *et al.,* 1998), indicates the role of GLPs in plant defence. Both germin and *Hv*GLP1 are tightly associated with the cell wall (Berna and Bernier, 1997; Lane *et al.,* 1992; Vallélian-Bindschedler *et al.,* 1998) and may thus play a structural role in stressed leaves for cell wall reinforcement. Wheat germin was identified as functional OxO

(Berna and Bernier, 1997; Lane *et al.*, 1993). OxO converts oxalate $(COOH)_2$ into CO_2 and H_2O_2 and releases free Ca^{++} from Ca-oxalate. Therefore, germin (OxO) might be involved in defense reactions, the production of active oxygen species for cross-linking reactions (Bradley *et al.*, 1992; Otte and Barz, 1996) and stress-related cell signaling by enhancing extracellular Ca^{++} levels (Bayles and Aist, 1987). Specific responses to a range of individual pathogens are summarised below.

7.1. Viruses

The first study implicating GLPs with response to viruses was that of Park *et al*. (2004) with the hot pepper plant (*Capsicum annuum* L. cv. Bugang), which exhibits an HR in response to infection by Tobacco mosaic virus pathotype P0 (TMV-P_0). A full-length cDNA clone was isolated by differential screening of a cDNA library that was constructed with mRNA extracted from leaves during the resistance response to TMV-P_0. The predicted amino acid sequence of the cDNA clone exhibited a high sequence similarity to a GLP, and was classified as belonging to a new family of PR proteins, 'PR-16' (see also van Loon *et al*., 2006). More recently, Cristofani-Yaly *et al*. (2007) constructed expressed sequence tag (EST) libraries with tissues collected from *Poncirus trifoliata* plants, inoculated or not with Citrus tristeza virus, and found a GLP among the genes upregulated at 90 d after inoculation (see also Gandía *et al*., 2007). Similar results have been reported for *Capsicum chinense* plants infected with pepper mild mottle virus (PMMoV) (Elvira *et al*., 2008) and pepper Huasteco yellow vein virus (PHYVV) (Gasca-González *et al*., 2008).

Infection of *Cucumber mosaic virus* (CMV) and D satellite RNA (satRNA) in tomato plants induces rapid plant death, which has caused catastrophic crop losses. A long serial

analysis of gene expression (LongSAGE) investigation of control and virus-infected plants was conducted to identify the genes that may be involved in the development of this lethal tomato disease (Irian *et al*., 2007). The transcriptomes of mock-inoculated plants and plants infected with CMV, CMV/D satRNA, or CMV/Dm satRNA (a nonnecrogenic mutant of D satRNA with three mutated nucleotides) were compared, and the analysis revealed both general and specific changes in the tomato transcriptome including GLP genes after infection with these viruses.

7.2. Bacteria

In an extensive bioinformatic survey, Vargas *et al*. (2003) searched the sugarcane database for all expressed sequence tags (ESTs) preferentially or exclusively expressed in cDNA libraries constructed from sugarcane plants inoculated with the diazotrophic and plant hormone-producing endophytic bacteria *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans*. It was found that various GLP homologues were exclusively or preferentially represented in the libraries from infected material. More recently, GLP expression was shown in *Citrus sinensis* (De Souza *et al*., 2007) and grape tissues (Lin *et al*., 2007) in response to infection with *Xylella fastidiosa*.

Bacterial effector proteins secreted through the type III secretion system (TTSS) play a crucial role in causing plant and human diseases. Although the ability of type III effectors to trigger defense responses in resistant plants is well understood, the disease-promoting functions of type III effectors in susceptible plants are largely enigmatic. Previous microscopic studies suggest that in susceptible plants the TTSS of plant-pathogenic bacteria transports suppressors of a cell wall-based plant defense activated by the TTSS-defective *hrp* mutant bacteria. However, the identity of such suppressors has remained elusive. It is now known that the *Pseudomonas*

syringae TTSS down-regulate the expression of a set of Arabidopsis genes encoding putatively secreted cell wall and defense proteins in a salicylic acid-independent manner (Hauck *et al*., 2003). The strong bias of TTSS-repressed genes toward those encoding secreted proteins can best be explained by suppression of extracellular plant defense. Indeed, it was found that the majority of TTSS-repressed genes are apparently associated with plant cell wall functions including hydroxyproline-rich proteins or extensins, which are known components of papillae; and at least four genes which share sequence similarities with genes encoding known extracellular defense-associated proteins:- a GLP, a nonspecific lipid transfer protein, and two acid phosphatases. In a related study of the same system, Mohr *et al*. (2007) have show that ABA induces susceptibility of Arabidopsis towards an avirulent strain of *P. syringae* pathovar (pv.) *tomato* via suppression of the accumulation of components crucial for a resistance response. Lignin and salicylic acid concentrations in leaves were increased during a resistant interaction but reduced when plants were treated with ABA. This reduction in lignin and salicylic acid production was independent of the development of the hypersensitive response (HR), indicating that in this host-pathogen system HR is not required for resistance. Genome-wide gene expression analysis using microarrays showed that treatment with ABA suppressed the expression of many defence-related genes, including those important for phenylpropanoid biosynthesis and those encoding resistance-related proteins such as oxalate oxidase. Together, these results show that resistance induction in Arabidopsis to an avirulent strain of *P. syringae* pv. *tomato* is regulated by ABA. Relevant recent information by Kűrkcűoglu *et al.* (2007) indicate that ESTs rapidly up-regulated after the application of a non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to *Malus domestica* include those encoding GLPs.

Fire blight is caused by the Gram-negative bacterium, *Erwinia amylovora*. Hosts, which include apple, pear and other members of the Rosaceae, are most susceptible during blooming. Genes found in a sampling of 66 clones from an enriched *E. amylovora*-inoculated apple leaf cDNA pool included one GLP (Bonasera and Beer, 2002). Another study of bacterial pathogenesis utilized an *indica* rice cultivar IET8585 (Ajaya) resistant to diverse races of the *Xanthomonas oryzae pv oryzae* pathogen attack, and often cultivated as bacterial leaf blight (blb) resistant check in India. An analysis of the transcriptional profile of the blb infected resistant cultivar using a rice 22K oligo array revealed differential expression of numerous genes at both early (6 h) and late (120 h) stages of infection in the resistant IET8585 cultivar over the susceptible IR24 (Kottapalli *et al*., 2007). Some of the differential gene expressions were validated by both RT-PCR and western analysis. Genes encoding two harpin-induced 1 family proteins, a germin (AK059817), lipid transfer protein/par allergen, E-class P450, FAA hydrolase, t-snare, and NAM proteins were up-regulated, while the down-regulated genes included the GLP, AK060864.

Evidence of a role for GLPs in the relationship between plants and microbes has also come from studies of nodulation in legumes, as well as from investigations of specific pathogen responses in cereals and other species. Effects seems to be exerted by production of extracellular reactive oxygen species which may either oxidize the infection apparatus, strengthen the cell wall or may perform both roles. However the association with nodule development is very interesting since both host and microbe seem to have evolved in such a way that neither microbe is killed nor it is prevented from the host. However, it is encapsulated in tough walled nodules. Following earlier studies, summarised in Dunwell *et al*. (2000), Gucciardo *et al.* (2007) recently

reported *Ps*GER1 transcript in developing pea nodules, detected in expanding cells just proximal to the meristematic zone and to lesser extent in the epidermis.

In the establishment of symbiosis between the legume *Medicago truncatula* and the nitrogen-fixing bacterium *Sinorhizobium meliloti*, the lipopolysaccharide (LPS) of the microsymbiont plays an important role as a signal molecule. It has been shown in cell cultures that the LPS is able to suppress an elicitor induced oxidative burst. To investigate the effect of *S. meliloti* LPS on defense associated gene expression, a microarray experiment was performed (Tellstrom *et al*., 2007). The results indicated that *M. truncatula* cell cultures do not respond strongly to the LPS of the symbiotic partner. In contrast, in suspension cells treated with invertase, 336 genes were identified to be at least 2-fold induced with a statistical significance of $P \leq 0.05$, whereas 43 genes were identified to be repressed at least 2-fold. Furthermore, two GLPs were among the 20 genes most highly expressed in the elicited cell cultures compared with water controls.

Similar methodology was applied in a study of the endophyte *Azoarcus* sp. strain BH72 that expresses nitrogenase (*nif*) genes inside rice roots. A proteomic approach was used to dissect responses of rice roots toward bacterial colonization and also to jasmonic acid (JA) treatment (Miché *et al*., 2006). Proteins strongly induced in roots in both varieties by JA were identified as Bowman-Birk trypsin inhibitors, a GLP (Accession 5852087), a putative endo-1,3-beta-D-glucosidase, a glutathione-*S*-transferase, a 1-propane-1-carboxylate oxidase synthase, peroxidase precursor, PR10-a, and a RAN protein previously not found to be JA induced. Data suggested that plant defense responses involving JA may contribute to restricting endophytic colonization in grasses. Remarkably, in a compatible interaction with endophytes, JA-inducible stress or defense responses were apparently not important.

7.3. Mycorrhizae

The large majority of land plants are capable of undergoing a tight symbiosis with arbuscular mycorrhizal (AM) fungi. During this symbiosis, highly specialized new structures called arbuscules are formed within the host cells, indicating that during interaction with AM Glomalean fungi, plants express AM-specific genetic programmes. Despite increasing efforts, the number of genes known to be induced in the AM symbiosis is still low. Doll *et al.* (2003) characterized unique features of a cDNA sequence from *M. truncatula* (*MtGLP1*) and identified a putative orthologue in *Lotus japonicus* that suggested an important role of the gene product during AM symbiosis. This finding was confirmed by several similar studies (Wulf *et al*., 2003; Hohnjec *et al*., 2005; Kistner *et al*., 2005).

In a similar attempt to identify novel AM-induced genes which have not been listed before, 5,646 ESTs were generated from two *M. truncatula* cDNA libraries: a random cDNA library (MtAmp) and a suppression subtractive hybridization (SSH) library (MtGim), the latter being designed to enhance the cloning of mycorrhiza-upregulated genes (Frenzel *et al*., 2005). *In silico* expression analysis was applied to identify those tentative consensus sequences (TCs) of The Institute for Genomic Research *M. truncatula* gene index (MtGI) that are composed exclusively of ESTs derived from the MtGim or MtAmp library, but not from any other cDNA library of the MtGI. This search revealed 115 MtAmp- or MTGim-specific TCs. For the majority of these TCs with sequence similarities to plant genes, the AM-specific expression was verified by quantitative reverse-transcription polymerase chain reaction. In this study, two GLPs were demonstrated to be upregulated in mycorrhizal roots. Together with the MtGLP described above, a total of three different AM-induced GLPs seem to be involved in AM symbiosis.

In a similarly comprehensive study, whole transcriptome analysis using a custom-designed Affymetrix Gene-Chip and confirmation with real-time RT-PCR revealed 224 genes affected during arbuscular mycorrhizal symbiosis in rice (Güimil *et al*., 2005). It is noteworthy that three of the rice mycorrhiza responsive genes encode GLPs (*OsAM4*, *OsAM9*, *OsAM85*); all were strongly induced upon mycorrhizal colonization and cluster to a 6-kb region on chromosome 9. The first report of similar gene induction by a fluorescent pseudomonad and a mycorrhizal fungus in roots supports the hypothesis that some plant cell programmes may be shared during root colonization by these beneficial microorganisms (Sanchez *et al*., 2004). Less similarity existed in expression of the gene set with nodulation by *S. meliloti*. To determine common gene expression in root interactions with rhizobacteria, alterations in expression patterns in *P. fluorescens*-colonized roots were compared with *S. meliloti* nodulated roots by semiquantitative RT-PCR. Five patterns of differential gene regulation were observed for the 12 plant genes upregulated in mycorrhiza. Expression of 12 plant genes activated in mycorrhizal roots of *M. truncatula* (Brechenmacher *et al*., 2004) was first compared by reverse Northern hybridization of corresponding ESTs using cDNA probes from *M. truncatula* roots inoculated or not inoculated with *G. mosseae*, *P. fluorescens* or *S. meliloti*. Activation of all 12 genes was confirmed in mycorrhizal roots. Genes corresponding to six of the ESTs, including a GLP, showed at least 2.5-fold greater expression in *P. fluorescens*-inoculated roots than in uninoculated roots. Expression was increased ($P = 0.05$) in both *P. fluorescens* and *S. meliloti* inoculated roots for genes encoding the putative wound-induced protein, nodulin 26-like aquaporin and glutathione-*S* transferase, while genes encoding a GLP, MtGmLs11, MtGmLs291 and glutamine synthetase, which were activated in *P. fluorescens*-inoculated roots, were downregulated ($P = 0.05$) in nodulated roots.

7.4. Fungal Pathogens

Germin and GLPs are well documented for their involvement in defense against a great diversity of fungal pathogens including powdery mildew, *Blumeria* (syn. *Erisyphe*) (see Dunwell *et al*., 2000 for summary) (Wei *et al.*, 1998; Zimmermann *et al.*, 2006). Furthermore, a related sequence has been isolated from barley, which shows papilla-mediated resistance to this disease (Wei *et al.*, 1998). This particular transcript peaks at about 18 to 24 h after infection, specifically in the epidermal cells. Detailed analysis shows that this temporal and spatial pattern of expression closely follows the formation of papillae, which are produced as cell wall appositions below the primary wall and the appressorial germ tubes at the site of fungal penetration attempt and are thought to be composed of cross-linked proteins (Thordal-Christensen *et al.*, 1997). Their presence is associated with the presence of hydrolytic enzymes (Takahashi *et al.*, 1985) and is dependent on H_2O_2 production. Christensen *et al.* (2004) identified *Hv*GLP4 as a functional SOD, and observed that *Hv*GLP4 and *Ta*GLP4 contribute to disease resistance in wheat and barley. Resistance and susceptibility in barley to the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) is determined at the single-cell level. Even in genetically compatible interactions, infected plant epidermal cells defend themselves against attempted fungal penetration by localized responses leading to papilla deposition and reinforcement of their cell wall. This conveys a race-nonspecific form of resistance. However, this defense is not complete, and a proportion of penetration attempts succeed in infection. The resultant mixture of infected and uninfected leaf cells makes it impossible to relate powdery mildew-induced gene expression in whole leaves or even dissected epidermal tissues to resistance or susceptibility. A method for generating transcript profiles from individual barley epidermal cells has now been established

and proved to be useful for the analysis of resistant and successfully infected cells separately (Gjetting *et al*., 2004, 2007). Contents of single epidermal cells (resistant, infected, and unattacked controls) were collected, and after cDNA synthesis and PCR amplification, the resulting sample was hybridized to dot-blots spotted with genes, including some previously reported to be induced upon pathogen attack. Transcripts of several genes, (e.g., PR1a, encoding a pathogenesis related protein, and GLP4) accumulated specifically in resistant cells, while GRP94, encoding a molecular chaperone, accumulated in infected cells. Thus, the single-cell method allows discrimination of transcript profiles from resistant and infected cells. The method will be useful for microarray expression profiling for simultaneous analysis of many genes. In barley and wheat, the spatial and temporal expression pattern of GLP4, together with sequence similarity to the H_2O_2-generating oxalate oxidase (GLP1), led to speculation that this gene may have a role in producing the penetration associated H_2O_2 (Schweizer *et al.,* 1999; Wei *et al.,* 1998). Additionally Zimmermann *et al.,* (2006) have reported the role of *Hv*GER5 to encode an extra-cellular SOD with antifungal activity against powdery mildew. The interaction between Arabidopsis and *Golovinomyces cichoracearum* (formerly *Erysiphe cichoracearum*) is an ideal system for exploring the compatibility between plants and obligate biotrophs. GLP (AtGER3 - At5g20630) was shown to one of the genes with reduced expressed during the early phase of inoculation with this organism (Fabro *et al*., 2008). Related information is available from studies involving the infection of grape berries with the important powdery mildew pathogen (*Uncinula necator*) (Ficke *et al*., 2002, 2004). Grape berries are highly susceptible to powdery mildew one week after bloom but acquire ontogenic resistance two to three weeks later. It was recently demonstrated that germinating conidia of *U. necator* cease development before penetration of the cuticle on older resistant berries. As part of this study it was concluded that a GLP (*Vv*GLP3)

was expressed within 16 h of inoculation in resistant, but not in susceptible berries (Godfrey *et al*., 2007).

A related study in wheat was that examining the effect of salicylic acid (SA) on OxO and peroxidase activities and hydrogen peroxide (H_2O_2) production in leaf cells of the susceptible cultivar Zhnitsa infected by *Septoria nodorum*, a pathogen of wheat leaf blotch (Troshina *et al*., 2007). The results show that fungal hyphae spread into interstices between mesophyll cells and that infected tissues contain H_2O_2. Treatment with SA results in enhanced H_2O_2 production in mesophyll cells, which is due to activation of oxalate oxidase and peroxidase in the cell wall. The differentially virulent race T1 of common bunt (*Tilletia tritici*) was used to inoculate the wheat lines Neepawa (compatible) and its sib BW553 (incompatible) that are nearly isogenic for the *Bt-10* resistance gene (Lu *et al*., 2005). Inoculated crown tissues were used to construct a suppression subtractive hybridization (SSH) cDNA library. Of the 1920 clones arrayed from the SSH cDNA library, approximately 10% were differentially regulated. A total of 168 differentially up-regulated and 25 down-regulated genes were identified and sequenced; one GLP was amongst this latter group.

The barley leaf stripe disease is caused by the seed-borne fungus *Pyrenophora graminea*. Haegi *et al*. (2008) investigated the reaction of barley embryos to this pathogen at a molecular and microscopic level. In the resistant genotype NIL3876-*Rdg2a*, fungal growth ceased at the scutellar node of the embryo, while in the susceptible near-isogenic line (NIL) Mirco-*rdg2a* fungal growth continued past the scutellar node and into the embryo. Also potentially related to cell-wall reinforcement is the production of reactive oxygen species. In this study, genes encoding H_2O_2- generating enzymes (germin F and OxO) were found to be induced in both resistant and susceptible barley genotypes but at a higher level in the resistant NIL.

To assess relative gene expression after pathogen attack, expressed sequence tag redundancy was compared between EST collections from susceptible *Malva pusilla* and *Medicago truncatula* inoculated with *Colletotrichum gloeosporioides* f. sp. *malvae* and *C. trifolii*, respectively, and resistant and susceptible *Sorghum bicolor* inoculated with *C. graminicola* (=*C. sublineolum*) (Goodwin *et al*., 2004). EST redundancies from the fungal-inoculated *S. bicolor* and *M. truncatula* were also compared to healthy plants of the same species. Although most of the ESTs examined in this work had counterparts in the other *Colletotrichum*–plant interactions, there were also many that had very few or no matches between the EST collections. For example, ESTs for GTP-binding protein, auxin-binding protein 19a and a GLP occurred with relatively high redundancy in the Ct-*Medicago* interaction but were either not observed or observed much less frequently in the other EST collections examined in this study.

The necrotrophic fungal pathogen *Fusarium pseudograminearum* (*F. pseudograminearum*) causes crown rot disease (CR) in wheat. In a recent study using real-time quantitative PCR, the expression of 26 selected wheat genes was examined 1, 2 and 4 d after inoculation of wheat seedlings of the CR susceptible cultivar Kennedy and the partially field-resistant cultivar Sunco (Desmond *et al*., 2006). Reproducible induction of eight defence genes including the GLP *TaGLP2a* was observed. Methyl jasmonate (MJ) treatment also induced these pathogen responsive defence genes in both cultivars while benzo(1,2,3)thiadiazole-7-carbothionic acid *S*-methyl ester (BTH) treatment weakly induced them in Kennedy only. Similarly, treatment with MJ before inoculation significantly delayed the development of necrotic symptoms for 2 weeks in both wheat cultivars, while BTH pre-treatments delayed symptom development in Kennedy only. The chemically induced protection, therefore,

correlated with induction of the *F. pseudograminearum*-responsive genes. These results support the emerging role of jasmonate signalling in defence against necrotrophic fungal pathogens in monocots. A related study used the Chinese cultivar Ning 7840, one of a few wheat cultivars with resistance to the pathogen. GeneCalling™, an open-architecture mRNA-profiling technology, was used to identify differentially expressed genes induced or suppressed in spikes of Ning 7840 after infection by *F. graminearum*. One hundred and twenty-five cDNA fragments representing transcripts, including those from GLP genes, differentially expressed in wheat spikes were identified (Kong *et al*., 2007). Using the same system, it was shown that the Fusarium-associated mycotoxin deoxynivalenol (DON) also induced GLP expression (Boddu *et al*., 2006; Desmond et *al*., 2008).

In a study of the rice blast pathogen (*Magnaporthe grisea*), a rice cDNA library was screened using OSK3 protein kinase as bait in a yeast two-hybrid system (Takano, 2004). The gene encoding germin OxO was one of the positive clones interacting with OSK3 protein kinase. The interactions were verified by detecting expression of the reporter gene *lacZ*. The results suggest that OxO is a downstream element in the disease resistance signal cascade mediated by OSK3 protein kinase in rice. An interesting study that demonstrates the complexity of transcript variation, including GLP genes, in response to rice blast infection is that published by Gowda *et al*. (2007). They exploited the power of SAGE technology (see above) and identified a high frequency of A-to-G and U-to-C nucleotide conversions in transcripts, as well as many antisense transcripts, in the *M. grisea*-infected plants.

There are several additional instances of such a link between GLP expression and plant defence that suggest GLPs to be markers in the plant defence mechanism. A GLP was found to be expressed in microstems of chestnut (*Castanea sativa*) infected with chestnut blight fungus

(*Cryphonectria parasitica*) (Schafleitner and Wilhelm, 2002), and two GLPs were amongst 1057 differentially expressed genes in *Arabidopsis* leaves infected with *Peronospora parasitica* (Mahalingam *et al.*, 2003).

During the parasitic interaction between the model legume *M. truncatula* and the oomycete *Aphanomyces euteiches*, a plant protease inhibitor (PI)-encoding gene, *MtTi2*, was found to be induced. Sequence and database analyses showed that *MtTi2* belongs to a group of at least four PIs, which are all specifically expressed upon pathogen infection or elicitor treatment. In order to investigate whether suppression of *MtTi2* results in transcriptional changes, transcriptome profiles of *MtTi2i* roots and control roots were analyzed using an 8000 gene *M. truncatula* microarray (Nyamsuren *et al*., 2007). A cluster analysis of genes regulated in roots upon infection in two *MtTi2i*-lines or two vector lines revealed groups of genes showing different regulation in *MtTi2i*- and wild type roots. There were two GLPs among the list of genes down-regulated by *A. euteiches* infection in vector control and *MtTi2i*-silences roots.

Two Brassica lines derived from an interspecific cross between *Brassica napus* and *B. carinata* were evaluated for tolerance to the fungal pathogen *Alternaria brassicae* (Sharma *et al*., 2007). Pathogen-induced chlorosis and necrosis spread significantly in one line whereas it remained localized in the other. Proteome-level changes in response to the fungal pathogen were investigated using two-dimensional electrophoresis. The antioxidant enzymes identified in this study included a GLP in the Alternaria-tolerant line observed to be elevated at 12 h after infection. Interestingly, this study also identified a GLP whose intensity was reduced in the Alternaria-susceptible line 24 h following pathogen challenge. This suggests that the enhanced levels of specific GLPs may have a significant role in mediating the observed responses to the pathogen in these two lines.

A recent study of Norway spruce seedlings inoculated with *Rhizoctonia* showed that after pathogen inoculation, there were significant and early increases in many root transcripts encoding defense-related proteins including GLPs (GLP1 and GLP2) (Jøhnk *et al.*, 2005; Fossdal *et al*., 2007). The expression of these transcripts gradually increased in roots as a local response after inoculation, and reached a peak 96-192 h after treatment. In roots subjected to drought alone, increased levels of the same transcripts occurred later than in roots inoculated with Rhizoctonia, with peak values at 192 h after cessation of irrigation. In the treatment group with combined pathogen inoculation and drought stress, the same transcripts were elevated as in the other two stress groups, but the elevations were detected significantly earlier (48 h) and were maintained through the remaining experimental period. Related results on infection of spruce with the pathogenic fungus *Heterobasidion annosum* have been reported (Karlsson *et al*., 2007).

In an interesting recent study on *Moniliophthora perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*, Ceita *et al*. (2007) examined active oxalate degradation and its possible source of origin in infected tissues. Degradation of calcium oxalate crystals is recognized to be due to germin OxO activity (G-OxO), and a corresponding *T. cacao* gene transcript was identified in a cDNA library produced from mRNA from infected tissue. The expression of this gene was analyzed by semi-quantitative RT-PCR using mRNA collected from meristems of infected stems, and showed significant expression in the diseased tissues. In a related study of this system, a total of 6884 ESTs were generated from libraries obtained from resistant and susceptible material (Gesteira *et al*., 2007). One GLP was identified as showing significant expression levels.

Elicitors are molecules known to trigger plant defence responses against pathogens. A large-scale microarray analysis in Arabidopsis in response to treatment with the elicitor chitin

showed that genes encoding several stress proteins, a GLP and a leucine-rich repeat protein, are down-regulated from 10 min to 6 h after chitin treatment and are induced only at the 24 h time point (Ramonell *et al*., 2002). This is consistent with the model that these genes are late-acting, occurring at the end of signal transduction pathways. Another study of this type involved the first proteomic analysis of rice defense response induced by probenazole (PBZ), an agricultural chemical that has been widely used to protect rice plants from rice blast and the bacterial blight pathogen. Two-dimensional gel electrophoresis (2-DE) on extracts from seedlings identified a total of 40 protein spots including nine that were up-regulated by PBZ and 31 abundant protein spots that include a GLP (Lin *et al*., 2008). Additional data on this topic have been reported by Renard-Merlier *et al*. (2007). In a search for new sources of elicitors from marine algae, an extract was prepared from green algae, *Ulva* spp., and its elicitor activity was established on the model legume, *M. truncatula* (Cluzet *et al*., 2004). When infiltrated into plant tissues or sprayed onto the leaves, this extract induced the expression of the defence related marker gene *PR10* without provoking necrosis. Using a cDNA array enriched in genes potentially involved in plant defence, the expression of 152 genes was monitored after one or two consecutive treatments. A broad range of defence-related transcripts was found to be up-regulated, notably genes involved in the biosynthesis of phytoalexins, pathogenesis related proteins and cell wall proteins. One GLP was present in this list.

Expression of defense-associated genes was analyzed in leaf tissues of near-isogenic resistant and susceptible barley cultivars upon infection by *Rhynchosporium secalis* (Steiner-Lange *et al*., 2003). The genes encoding pathogenesis-related (PR) proteins PR-1, PR-5, and PR-9 are specifically expressed in the mesophyll of resistant plants, whereas a GLP (OxOGLP) is synthesized in the epidermis irrespective of the resistance genotype. At least two additional

elicitors, therefore, must be postulated, one for the non-specific induction of OxOGLP and one for the resistance-specific induction of LoxA, pI2-4, and SD10.

It is known that cell wall protein fractions (CWPs) of the biocontrol agent *Pythium oligandrum* have elicitor properties in sugar beet and wheat. Recently, the effect of treatment with the D-type of CWP, a fraction that contains two major forms (POD-1 and POD-2), on the induction of defence-related genes in sugar beet was examined (Takenaka *et al*., 2006). Using PCR-based cDNA library subtraction, the authors identified five genes that were highly expressed in response to CWP treatment. The five genes are probably of OxO-like germin (OxOLG), glutathione S-transferase (GST), 5-enol-pyruvylshikimate-phosphate synthase (EPSPS), phenylalanine ammonia-lyase (PAL) and aspartate aminotransferase (AAT). In addition, they purified and characterized POD-1 and POD-2 and found that POD-1 induced all five genes, whereas POD-2 induced only three of the genes excluding OxOLG and GST.

A particularly interesting recent report concerns the possible role of microRNAs (miRNAs) in pathogenesis. These molecules are endogenous small RNAs that can have large-scale regulatory effects on development and on stress responses in plants. The subject of the study was the endemic rust fungus *Cronartium quercuum* f. sp. *fusiforme* that causes fusiform rust disease in pines, resulting in the development of spindle-shaped galls (cankers) on branches or stems; this disease is the most destructive disease of pines in the southern USA. To test whether miRNAs play roles in fusiform rust gall development, 26 miRNAs were cloned and identified from stem xylem of loblolly pine (*Pinus taeda*). These 26 belonged to four conserved and seven loblolly pine-specific miRNA families. Forty-three targets, including a GLP (similar to At3g05930), were experimentally validated *in vivo* for nine of these 11 families and it is suggested that indeed these miRNAs may be implicated in disease progression (Lu *et al*., 2007).

Resistance in the host is often manifested by a hypersensitive response, which results in localized cell death at the site of infection, structural alterations, and production of a wide range of plant defense molecules such as antimicrobial proteins (Broekaert *et al.,* 1997; Yun *et al.,* 1997; Grant and Mansfield, 1999). It has been suggested that the H_2O_2 produced by the OxO members of germin and GLP family may act as a messenger for the initiation of other defense related genes in the same cell or in neighboring cells and activate an effective defensive response. It is also relevant to note the tenacious association between wheat germin and the arabinose-rich hemicelluloses (arabinoxylans or arabinogalactans) of cereal walls (Jaikaran *et al.,* 1990). There is increasing evidence to support common links between the transduction pathways for the detection/assessment and response to biotic and abiotic stresses (see next section 8 below) and that active oxygen species are involved in the plant-environment interaction (Thordal-Christensen *et al.,* 1997; Wojtaszek, 1997). For example, it has been demonstrated by Dixon *et al.* (1991) that certain pathogenesis-related proteins accumulate within crystal idioblasts (cells that contain crystals of calcium oxalate and occur throughout the leaves of many plants), and supply of oxalate in these cells would provide a source of H_2O_2 depending on the levels of OxO.

In general plant pathogenesis related (PR) proteins, PR-15 and -16 are typical of monocots and comprise families of germinlike OxOs and OxO-like proteins with SOD activity, respectively (Park *et al*., 2004; van Loon *et al*., 2006). These proteins generate hydrogen peroxide that can be toxic to different types of attackers or could directly or indirectly stimulate plant-defense responses (Quan *et al*., 2007). PR-15 and PR-16 are known to be widely expressed in a range of Citrus tissues (Campos *et al*., 2007). In an interesting, though relatively small scale evolutionary study, the genes encoding 13 classes of these PR proteins were examined for positive selection using maximum-likelihood (ML) models of codon substitution (Scherer *et al*.,

2005). The study involved 194 sequences from 54 species belonging to 37 genera. Although the sizes of the sequences examined varied from 237 bp for PR12 to 1,110 bp for PR7, most classes (9 out of 13) contained sequences made up of more than 400 nucleotides. Signs of positive selection were obtained for sites in PR proteins 4, 6, 8, 9 and 15 (OxO) using an ML-based Bayesian method and likelihood ratio tests. These results confirm the importance of positive selection in proteins related to defense mechanisms already observed in a wide array of organisms.

7.5. Insects

Germins and GLPs are well known to function in pathogen resistance (previous section), but their involvement in defense against insect herbivores is more poorly understood. In the native tobacco *Nicotiana attenuata*, attack from the specialist herbivore *Manduca sexta* or elicitation by adding larval oral secretions (OS) to wounds up-regulates transcripts of a GLP (Hermsmeier *et al*., 2001). To understand the function of this gene, which occurs as a single copy, the full-length *Na*GLP was cloned and its expression silenced in *N. attenuata* by expressing a 250-bp fragment in an antisense orientation by transformation and by virus-induced gene silencing (VIGS) (Lou and Baldwin, 2006). Homozygous lines harboring a single insert and VIGS plants had significantly reduced constitutive (measured in roots) and elicited *Na*GLP transcript levels (in leaves). Silencing *Na*GLP improved *M. sexta* larval performance and *Tupiocoris notatus* preference, two native herbivores of *N. attenuata*. Silencing *Na*GLP also attenuated the OS-induced H_2O_2, diterpene glycosides, and trypsin proteinase inhibitor responses, which may explain the observed susceptibility of antisense or VIGS plants to herbivore attack. Silencing also increased nicotine contents, but did not influence the OS-elicited

jasmonate and salicylate bursts, or the release of the volatile organic compounds (limonene, cis-alpha-bergamotene, and germacrene-A) that function as an indirect defense. This suggests that *Na*GLP is involved in H_2O_2 production and might also be related to ethylene production and/or perception, which in turn influences the defense responses of *N. attenuata* via H_2O_2 and ethylene-signaling pathways. In a similar study of gene expression in the same species of tobacco, increased expression of a GLP was observed in plants infested with *Myzus nicotianae* aphids (Voelckel *et al*., 2004).

In a related analysis of genes induced by insect feeding, 108 cDNA clones representing genes related to plant responses to chewing insect-feeding, pathogen infection, wounding and other stresses were collected, and Northern blot and cDNA array analysis were employed to investigate gene expression regulated by the piercing-sucking insect, brown planthopper (BPH) *Nilaparvata lugens* (Homoptera: Dephacidae) on both the resistant and susceptible rice genotypes (Zhang *et al*., 2004). After BPH feeding in rice for 72 h, the expression of most tested genes was affected; 14 genes in resistant rice variety B5 and 44 genes in susceptible MH63 were significantly up- or down-regulated. Most of the well-regulated genes were grouped in the categories of signaling pathways, oxidative stress/apoptosis, wound-response, drought-inducible and pathogenesis-related proteins. Those related to the flavonoid pathway, aromatic metabolism and the octadecanoid pathway were mostly kept unchanged or down-regulated. The results indicated that BPH feeding induced plant responses which would take part in a jasmonic acid-independent pathway and crosstalk with those related to abiotic stress, pathogen invasion and phytohormone signaling pathways. Expression of the one GLP (Accession number D40254) was not affected by BPH feeding.

As part of an approach to elucidate molecular interactions between plants and the Hessian fly, a cDNA library from near-isogenic lines infested by larvae of biotype L of this insect was constructed for expressed sequence tag (EST) analysis (Jang *et al*., 2003). Based on their putative identification, 730 ESTs that showed significant similarity with known coding sequences were divided into 13 functional categories. Defense- and stress-related genes represented about 16.1% of the total, including protease inhibition, oxidative burst, lignin synthesis, and phenylpropanoid metabolism. This list included three GLP sequences. It was suggested that these EST clones may provide valuable information related to the molecular interactions between plant and larva of the Hessian fly larval infestation.

A whole-genome microarray was used to study the expression profile of Arabidopsis leaves after oviposition by two pierid butterflies (Little *et al*., 2007). For *Pieris brassicae*, the deposition of egg batches changed the expression of hundreds of genes over a period of 3 d after oviposition. The transcript signature was similar to that observed during a hypersensitive response or in lesion-mimic mutants, including the induction of defense and stress-related genes and the repression of genes involved in growth and photosynthesis. Deposition of single eggs by *Pieris rapae* caused a similar although much weaker transcriptional response. The overlap between oviposition- and herbivory-repressed genes (after feeding by *P. rapae* larvae) was even smaller. One of the 10 genes repressed by both treatments was a GLP (At5g20630). Only four genes were down-regulated 72 h after oviposition by *P. rapae*. However, three of these genes, including one GLP (At5g20630), were also down-regulated after oviposition by *P. brassicae***.**

An extension of this approach has recently been demonstrated in a microarray analysis of field-grown soybean (*Glycine max*), in which it was shown that the number of transcripts in the leaves affected by herbivory by Japanese beetles (*Popillia japonica*) was greater when plants

were grown under elevated CO_2, elevated O_3 and the combination of elevated CO_2 plus elevated O_3 than when grown in ambient atmosphere (Casteel *et al*., 2008). Interestingly, expression of a GLP sequence (Affymetrix IDGma.15727.1.S1_at) was reduced in response to insect damage.

The response of roots to insect pests is an area of plant defense research for which less information is available. In one relevant study, Puthoff and Smigocki (2007) identified more than 150 sugar beet root ESTs enriched for genes responding to sugar beet root maggot feeding from both moderately resistant F1016, and susceptible F1010, genotypes using suppressive subtractive hybridization. The largest number of identified F1016 genes grouped into the defense/stress response (28%) and secondary metabolism (10%) categories with a polyphenol oxidase gene, from F1016, identified most often from the subtractive libraries. A GLP gene was also amongst those most frequently observed.

One study that approached plant-insect interactions from a novel perspective involved an examination of the stability of plant proteins in the insect gut. It was found that wound-inducible and jasmonate-inducible proteins comprised the largest group of tomato proteins in *M. sexta* frass (Chen *et al*., 2007). Among the proteins previously implicated in defense against lepidopteran insects was a GLP similar to a GLP isozyme from *N. attenuata* (Lou and Baldwin, 2006).

7.6. Nematodes

The soybean cyst nematode (SCN) *Heterodera glycines* is the most devastating pest of soybean in the U.S.A. The resistance response elicited by SCN in soybean is complex, and genes involved in the response to a large extent are unknown and not well characterized. In a study of cDNA libraries made from mRNA extracted from roots of the resistant soybean *Glycine max* L.

Merr. ‘Peking’ at 12 h, 2 to 4 d, and 6 to 8 d post inoculation with SCN, population NL1-RHp, a total of 3454 cDNA clones were examined (Alkharouf *et al*., 2004). A GLP was among the list of genes representing at least 0.3% of the ESTs in the 6- to 8-d cDNA library. This list included many other stress-induced proteins.

Related, but more precise, studies of the same material have recently been published (Klink *et al*., 2007a, 2007b). In this case, syncytial cells in soybean roots infected by incompatible and compatible populations of SCN were collected using laser capture microdissection and gene transcript abundance was assayed using Affymetrix® soybean GeneChips®, each containing 37,744 probe sets. By 8 d, several genes of unknown function and genes encoding a GLP, peroxidase, LOX, GAPDH, 3-deoxy-D-arabino-heptolosonate 7-phosphate synthase, ATP synthase and a thioesterase were abundantly expressed. These observations suggest that gene expression is different in syncytial cells as compared to whole roots infected with nematodes. Very similar results with this system have been reported by Ithal *et al*. (2007a, 2007b) and it is also reported in Zhang *et al*. (2008) that germin is involved in the response to nematodes in sugar beet.

7.7 Parasitic plants

A recent addition to the variety of biotic interactions relevant to this review is a study of *Striga hermonthica*, a root hemiparasite of cereals that causes devastating loss of yield. Recently, a rice cultivar, Nipponbare, was discovered, which exhibits post-attachment resistance to this parasite and quantitative trait loci (QTL) associated with the resistance were identified. Changes in gene expression in susceptible (IAC 165) and resistant (Nipponbare) rice cultivars were profiled using rice whole-genome microarrays, and in addition to a functional categorization of

changes in gene expression, genes that were significantly up-regulated within a resistance QTL were identified (Swarbrick *et al*., 2008). The resistance reaction was characterized by up-regulation of defence genes, including pathogenesis-related proteins (including Os04g5272 GLP subfamily 1 member 11 precursor), pleiotropic drug resistance ABC transporters, genes involved in phenylpropanoid metabolism and WRKY transcription factors. It was suggested that these changes in gene expression resemble those associated with resistance to microbial pathogens (see above).

8. ABIOTIC INTERACTIONS

The variety of studies that report the expression of GLPs in response to biotic stress is almost equal to that from biotic investigations, and some of these are summarised below.

8.1. Salt

The first evidence for induction of GLP expression by abiotic stress was provided by studies of salt stress in barley roots (Hurkman *et al*., 1991; Hurkman *et al.*, 1994; Hurkman and Tanaka 1996). Previously, Ramagopal (1987) in barley and Naqvi *et al.*, (1993) in rice observed induction of apparently similar GLPs during salt stress by two-dimensional electrophoresis. However, they did not characterize these proteins by sequencing. Similar results were reported in *Mesembryanthemum crystallinum* during the induction of Crassulacean acid metabolism during water and salt stress (Bohnert *et al.* 1988; Michalowski and Bohnert, 1992). In a more recent study of this species, a total of 9733 expressed sequence tags (ESTs) from cDNAs derived from leaf tissues of well-watered and salinity-stressed (0.5 M NaCl for 30 and 48 h) were characterized (Kore-eda *et al*., 2004). Clustering and assembly of these ESTs resulted in the

identification of a total of 3676 tentative unique gene sequences (1249 tentative consensus sequences and 2427 singleton ESTs) expressed in leaves under unstressed and salinity stressed conditions. Two 'tentative clusters' encoding proteins homologous to GLPs were highly abundant in the 0 h cDNA library (0.47% and 0.4%), whereas only five such ESTs in total were present in the 30 h cDNA library and no such ESTs were found in the 48 h cDNA libraries. The model plant Arabidopsis has also been used in such studies. For example, in a proteomic analysis, it was shown that two GLPs, GLP9 (At4g14630) and OxO-like protein (At5g38940), increased in abundance in Arabidopsis roots subjected to NaCl treatment (Jiang *et al*., 2007). Similar results have also been reported from an investigation of the gene expression profile of third leaves of rice (cv. Nipponbare) seedlings subjected to salt stress (130 mM NaCl) (Kim *et al*., 2007).

The most comprehensive of these studies is that utilizing a promoter-glucuronidase (GUS) fusion (Berna and Bernier, 1997; 1999) and showing induction of the wheat germin promoter in transgenic tobacco treated with salt, heavy metals, aluminium and plant growth regulators, specifically auxin and gibberellin. The significance of GLP expression in stress conditions has also been discussed in a study of seedlings of two sugar beet varieties, in which three full-length GLP gene classes were recovered from stress-germinated seedlings of a superior emerging variety (De Los Reyes and McGrath, 2003). Expression of these genes, together with OxO enzyme activity and the H_2O_2 content of stressed seedlings, were induced by stress during germination conditions in this good emerging hybrid and were not induced in a variety that emerged poorly. It was postulated that a block in oxalate metabolism contributed to lower germination under stress in the low emerging variety. Selection for such stress induced GLP expression, or for down stream targets, presents the first direct target to enable breeding for

improved field emergence of sugar beet (De Los Reyes and McGrath, 2003; McGrath *et al*., 2007).

Tamarix androssowii is one of the most remarkable salt-tolerance woody plant species in China. To investigate the gene expression profile of this species in response to $NaHCO_3$, a large scale EST analysis was conducted (Wang *et al*., 2006). This involved the construction a cDNA library from material treated with 0.4 mol/l $NaHCO_3$ and the sequencing of more than 3000 randomly selected clones. A total of 2455 high-quality ESTs were generated, 1268 (51.65%) of which showed significant similarity to the sequences in the NCBI Nr database. Nearly 400 ESTs were found to be involved in salt-tolerance; among them, a metallothionein-like protein and a GLP were the most abundant transcripts, and accounted for 2.44 and 1.91% of total ESTs, respectively. The same type of approach has been taken in a study of a mangrove plant, *Acanthus ebracteatus* Vahl. By using a bacterial functional assay, this study identified and isolated 107 salinity tolerant candidate genes, including a GLP, (Nguyen *et al*., 2007). Similarly, an EST study of *Aeluropus littoralis* (Gouan) Parl., a C4 perennial halophyte monocotyledonous plant, identified GLP transcripts (Zouari *et al*., 2007). A larger scale transcriptome analysis in grape has been reported by Cramer *et al*. (2007) who also found GLP sequences among those genes affected by salt (and cold).

Rice yield is most sensitive to salinity stress imposed during the panicle initiation (PI) stage. A recent study focused on physiological and transcriptional responses of four rice genotypes exposed to salinity stress during PI (Walia *et al*., 2007). The genotypes selected included two *indica* (IR63731 and IR29) and two *japonica* (Agami and M103) rice subspecies with contrasting salt tolerance. In an effort to search for transcripts which may be associated with salt tolerance of Agami, the authors identified 12 probe sets which were induced in Agami but

not in M103. Two of these probe sets (Os.11975.2.A1_at and OsAffx.25546.1.S1_s_at) were induced in Agami but down-regulated in M103. Whereas probe set Os.11975.2.A1_at had no sequence match to known genes, probe set OsAffx.25546.1.S1_s_at represented a GLP gene (*GPL4*), Os03g44880.

Although barley and rice belong to the same family Poaceae, they differ in their ability to tolerate salt stress. In an attempt to understand the molecular bases of such differences, Ueda *et al*. (2006) compared changes in transcriptome between barley and rice in response to salt stress using barley cDNA microarrays. Among significantly responsive genes under salt stress only ten, including OxO (AU252397), were commonly observed in both species. Similarly, Affymetrix GeneChip1 Wheat Arrays were used to identify differentially expressed genes in roots and leaves of five salt-tolerant wheat lines under salt stress at electrical conductivity of 30 dS/m (Mott and Wang, 2007). Probe sets showing the same pattern of changes in expression level due to salt treatments in all five tested lines included the GLP sequences Ta.24453 and TaAffx.53596.

In a related study Dani *et al*. (2005) used *Nicotiana tabacum* plants as a model to investigate changes in the soluble apoplast composition induced in response to salt stress. Apoplastic fluid was extracted using a vacuum infiltration procedure from leaves of control plants and plants exposed to salt stress. Two-dimension electrophoretic analyses and mass spectrometry revealed the identity of 20 polypeptides whose abundance changed in response to salt stress. While the levels of some proteins were reduced by salt-treatment, an enhanced accumulation of protein species known to be induced by biotic and abiotic stresses was observed. In particular, two chitinases and a GLP increased significantly. Similarly, expression of a germin protein was shown to be altered in tomato seedlings in response to salt stress (Amini *et al*., 2007).

In addition to the physiological studies of OxO and GLP expression *in vivo*, one study has reported the effect of NaCl on the activity of the enzyme *in vitro* (Singh *et al*., 2006). The effect of NaCl stress on molecular and biochemical properties of OxO was studied in the seedling leaves of a grain sorghum hybrid. There was no effect on molecular weight and number of subunits of the enzyme but it did show some important changes in its kinetic parameters such as Km for oxalate and V-max. Optimum pH (5.8), activation energy (5.08 kcal $mole^{-1}$), time of incubation (6 min) and Km for oxalate ($1.21x10^{-4}$M) were increased, while V-max (0.18 mmole min^{-1}) decreased and no change in optimum temperature was observed. This showed that substrate affinity and maximum activity of the enzyme were adversely affected. The specific activity of OxO was also increased in seedlings grown in a NaCl containing medium compared to normal, which reveals the increased *de novo* synthesis of the enzyme to sustain oxalate degradation.

8.2. Water Stress

In a study of gene expression in response to water stress in Arabidopsis, two GLPs were among the group of genes down-regulated in all three experimental conditions (Bray, 2004). More detailed results were achieved from *Lupinus albus* plants, which can withstand severe drought stress and show signs of recovery 24 h after rewatering (RW). Two-dimensional gel electrophoresis was used to evaluate the effect of water deficit (WD) on the protein composition of the two components of the lupin stem (stele and cortex) (Pinheiro *et al*., 2005). This was performed at three distinct stress levels: an early stage, a severe WD, and early recovery. Protein characterisation was performed through mass spectrometric partial sequencing. One GLP (gi1171937) was found to be present in stressed plants 13 d after watering. Investigations on

other crops including grapevine, (Hausmann *et al*., 2003), rice (Wang *et al*., 2007b), *Phaseolus vulgaris* (Kavar *et al*., 2008) and cassava (Lokko *et al*., 2007) gave similar results.

To gain a comprehensive understanding of how cell wall protein (CWP) composition changes in association with the differential growth responses to water deficit in different regions of the elongation zone of maize roots, a proteomics approach was used to examine water soluble and loosely ionically bound cell wall CWPs (Zhu *et al*., 2007). The results revealed major and predominantly region-specific changes in protein profiles between well-watered and water-stressed roots. In total, 152 water deficit-responsive proteins were identified and categorized into five groups based on their potential function in the cell wall: reactive oxygen species (ROS) metabolism, defense and detoxification, hydrolases, carbohydrate metabolism, and other/unknown. A large number of the proteins for which water stress-induced changes in abundance are known to be localized in cell walls; these include two putative OxOs and two probable GLP4s. In a similar proteomic study, the xylem sap of maize plants was screened using mass spectrometry to quantify the changes in new and previously identified sap constituents under extended drought (Alvarez *et al*. 2008). The authors detected and quantified the changes in the concentration of 31 compounds, including two GLPs present in sap under progressively increasing drought stress.

Similarly, proteomic studies of *Physcomitrella* brachycyte formation identified specific changes in the extracellular protein spectrum, including GLPs, in response to the drought-associated compound ABA (Cuming *et al*., 2007).

8.3. Cold and Heat

The first relevant study of this subject was one designed to isolate and characterize ABA-responsive cDNAs associated with ABA induced freezing tolerance in bromegrass cell cultures. A putative homolog (pBGA56) of the desiccation-related wheat germin gene (Lane *et al*., 1991) was shown to be expressed in this material (Lee and Chen, 1993). More recently, Lee and Lee (2003) characterized the global gene expression patterns of Arabidopsis pollen using SAGE methods. The expression patterns of the cold-responsive transcripts identified by SAGE were confirmed by microarray analysis. Amongst the genes highly expressed in pollen, one was closely related to nectarin (At.30848) and among those repressed by cold was another GLP (At.23312).

In a related study of the effect of cold on anther development in rice, a cDNA microarray containing 8,987 rice ESTs was utilized (Yamaguchi *et al*., 2004). 160 of these ESTs were up- or down- regulated by chilling (12°C for 120 h). One GLP (AU174256) was among the ESTs significantly up-regulated by the treatment applied. In contrast, Cheng *et al*. (2007) in a transcriptome analysis in chilling tolerant japonica rice identified a subset of 121 '*early response*' genes that were upregulated in seedlings during the initial 24 h at 10°C. These genes did not include the rice GLP Os08g08970. However, cold stress has been shown to induce increased expression of a germin (related to At1g18980) in roots of Rangpur lime (*Citrus limonia*) (Boscariol-Camargo *et al*., 2007).

In one of the few studies devoted to effects of heat, a microarray analysis was conducted using the 22K Barley1 GeneChip to monitor transcriptional alterations during high-temperature injury in barley (Oshino *et al*., 2007). Expression profiles were captured at four time points during the early development of panicles, and during vegetative growth of seedlings as a control, with or without high-temperature treatment. Abiotic or biotic stress related genes, including

GLPs, were equally or more dominantly up-regulated in the seedlings exposed to high temperatures compared with the panicles.

8.4. Metals

In order to characterise the possible mechanisms involved in aluminium (Al) toxicity functional characteristics were analysed in young barley (*Hordeum vulgare* L.) seedlings cultivated between moistened filter paper. The time course analysis of *OxO* gene expression and OxO activity showed that 10 mM Al increased OxO activity as soon as 3 h after exposure of roots to Al reaching its maximum at about 18 h after Al application. These results indicate that expression of OxO is activated very early after exposure of barley to Al, suggesting its role in oxidative stress and subsequent cell death caused by Al toxicity in plants (Tamás *et al*., 2004). The function of root border cells (RBC) during aluminum (Al) stress and the involvement of OxO, peroxidase and H_2O_2 generation in Al toxicity were studied in barley roots. The results suggested that RBC effectively protect the barley root tip from Al relative to the situation in roots cultivated in hydroponics where RBC are not sustained in the area surrounding the root tip. The removal of RBC from Al-treated roots increased root growth inhibition, Al and Evans blue uptake, inhibition of RBC production, the level of dead RBC, peroxidase and OxO activity and the production of H_2O_2. Results suggested that even though RBC actively produce active oxygen species during Al stress, their role in the protection of root tips against Al toxicity is to chelate Al in their dead cell body (Tamás *et al*., 2005).

In another investigation of this subject, wheat (*T. aestivum* cv. Kalyansona) seedlings were exposed to 0, 10, 50, and 100 µM Al in a 250 µM $CaCl_2$ solution for 24 h at pH 4.5 (Hossain *et al*., 2005). Root elongation decreased gradually, while the Al content markedly

increased with increasing supply of Al in the solution. The generation of H_2O_2 during Al stress was investigated biochemically and histochemically, together with other events related to the decomposition of H_2O_2. After 24 h treatment, root elongation inhibition was detected at all the concentrations of Al. The level of lipid peroxidation at 10 µM Al after 24 h treatment was similar to that of the control, while a significant increase in lipid peroxidation was detected at 50 and 100 µM Al. H_2O_2 generation was higher at 50 and 100 µM Al than that at 10 µM Al. These results suggest that Al-inducible lipid peroxidation may require excessive yield of H_2O_2. A time course experiment with 10 µM Al indicated that there is a strongly positive correlation between root elongation inhibition and cellular H_2O_2 yield. There were no significant changes in the activities of catalase (CAT) and ascorbate peroxidase (APX) in roots at 10 µM Al after 24 h treatment. However, a significant decrease in the activities of CAT and APX and a large increase in the activities of oxalate oxidase (OxO), guaiacol peroxidase (GPX), and coniferyl alcohol peroxidase (CA-POX) as well as lignin deposition were observed at 100 µM Al. These results suggest the possible involvement of OxO in the production of a large amount of H_2O_2 under severe Al stress, whereas GPX and CA-POX may be involved in the degradation of H_2O_2 and the subsequent deposition of lignin. The potential value of OxO has also been promoted in studies of aluminium tolerance in alfalfa (Narasimhamoorthy *et al*., 2007).

Similar results from studies of barley roots (Šimonovičová *et al*., 2005), maize roots (Maron *et al*., 2008), and rice leaves (Hsu and Kao, 2007) in response to cadmium (Cd) exposure have also been reported. In the former study, activity of OxO in several fractions of barley root tips was observed after 48 and 72 h of Cd-treatment. No OxO activation was detected in the extracellular fraction, whereas a minor increase in OxO activity was detected in soluble as well as in all five cell wall fractions after 48 h, and it was more pronounced and concentration-

dependent after 72 h of Cd treatment. A significant correlation between the enhanced OxO activity and root growth inhibition was evident after 72 h, which was accompanied also by an increase in Evans blue uptake indicating the loss of plasmalemma integrity and cell death. It was suggested that the OxO-catalysed H_2O_2 production might contribute to cell wall strengthening resulting in root growth inhibition but also to the induction of oxidative stress leading to cell death. A related study examined the transcript profiles of roots of *Arabidopsis* and *Thlaspi caerulescens* plants exposed to Cd and zinc (Zn), with the main aim being to determine the differences in gene expression between the Cd-tolerant Zn-hyperaccumulator *T. caerulescens* and the Cd-sensitive non-accumulator *Arabidopsis* (Van de Mortel *et al*., 2008). In total, 48 genes in five major clusters responded to both Zn and Cd in *Arabidopsis* when comparing Zn deficiency, Zn sufficiency, Zn excess and Cd exposure. One cluster contained five genes that were more highly expressed under Zn deficiency, Zn excess and Cd exposure: these genes encode two GLPs, the transcription factor *WRKY59* and a protein kinase. A study of iron homeostasis in Arabidopsis found that among the 50 genes increasing their expression in roots at least 2X under Fe deficient conditions was the GLP At1g09560 (Maurer, 2006).

Contrasting results were obtained with the Arabidopsis mutant *ars4*, which contains a single T-DNA insertion, which co-segregates with arsenic tolerance and is inserted in the Phytochrome A (*PHYA*) gene, strongly reducing the expression of PHYA (Sung *et al*., 2007). The GLP5 (At1g09560) gene, located slightly more than 5 kb from the tag, was also studied. The expression levels of the GLP5 and At1g09575 mRNA in *ars4ars5* were examined using RT-PCR. GLP5 mRNA showed a small increase in mRNA levels in the mutant (1.32- and 1.17-fold in two experiments), but this apparent change is unlikely to account for the strong arsenic

tolerance of the double *ars4ars5* mutant, and the *ars4* and *ars5* single mutants showed a recessive arsenic tolerance, suggesting that they are not gain-of function mutants.

8.5. Nutrients

Recently, the power of large scale transcript profiling techniques has been widely exploited to study the effects of various abiotic stress and variations in nutritional conditions. In addition to sugar accumulation, nitrogen starvation can induce leaf senescence (see above) and regulation of senescence mainly depends on the relative availability of nitrogen and carbon. To test whether nitrogen deficiency can elicit similar effects as glucose feeding, gene expression was determined in plants grown with low (2 mM nitrate) or high (10 mM nitrate) nitrogen supply (Masclaux-Daubresse *et al*., 2007). Induction of a senescence marker SAG12 confirmed that senescence was induced by low nitrogen supply. The germin gene GER1, one of the three genes which were down-regulated by glucose in the Complete Arabidopsis Transcriptome MicroArray experiment, was also repressed as plants senesced at low nitrogen supply.

Another large study on Arabidopsis utilized microarrays comprising 21,500 genes (Müller *et al*., 2007) and examined gene expression in leaf segments derived from phosphorus (P)-starved and P-replenished plants incubated with or without sucrose in order to obtain tissues with contrasting combinations of P and carbohydrate levels. Among the 40 most up-regulated genes in response to P starvation were two GLPs (At5g39130, At5g39160).

Full genome microarrays were used to assess transcriptional responses of Arabidopsis seedlings to changing external supply of the essential macronutrient potassium (K^+) (Armengaud *et al*., 2004). Rank product statistics and iterative group analysis were employed to identify differentially regulated genes and statistically significant coregulated sets of functionally related

genes. One analysis demonstrated a strong up-regulation of some genes in roots and/or in shoots after K^+ resupply with weak or non detectable change during long-term starvation. Transcripts presenting this profile included two GLPs (At1g72610, At5g20630).

Methylamine, an ammonia analogue, has been used to investigate ammonia uptake (Shiraishi *et al*., 2002). This compound competes with ammonia to be taken up by plants and also inhibits the nitrate assimilation pathway. In a study of the effect of MA on rice plants it was shown that MA-treated plants had increased aerial growth and reduced root growth. Expression of one GLP was reduced in MA-treated shoots.

8.6. Other Physical Stresses

There are a range of other stresses that have been investigated in studies relevant to this review. In one of these studies, transcript profiling identified upregulation of an OxO type germin in response to hypoxia in wheat roots (Lee *et al*., 2007). A more unusual type of stress, namely the effect of altered gravitational forces, was the subject of a study by Centis-Aubay *et al*. (2003). These authors compared the expression patterns of selected genes from *Arabidopsis thaliana* (L.) Heynh. grown either at 1 g or on a clinostat (horizontally or vertically inverted, 1 rpm), and either used directly or after hypergravity stimulation. They showed that one class of genes comprised those whose expression was either apparently unchanged (actin) or significantly modified in response to hypergravity (GLP, pectin methylesterase, extensin, and auxin-repressed dormancy associated protein). They were sensitive or insensitive to clinostat treatment but, in contrast to other classes of genes, they became unresponsive to a subsequent centrifugation at 5x g.

In another study of the effects of exposure to stress, oligonucleotide microarray technology was used to identify genes that respond after exposure to UV-C radiation and to other

agents causing genotoxic stress (Molinier *et al*., 2005). The effect of these conditions on recombinational DNA repair was monitored in parallel. Global changes in gene expression were investigated in *Arabidopsis* wild-type plants challenged with UV-C, bleomycin, another abiotic agent and xylanase (a biotic factor); all lead to elevated homologous recombination frequencies. The comparison of the expression profile of each treatment allowed the identification of genes specifically involved in the dynamic response to UV. The expression profile of plants treated with xylanase was expected to reveal changes in genes involved in induction of pathogen defense response, and indeed, genes involved in signaling and regulatory pathways, such as receptor like kinases, non-receptor protein kinases, protein phosphatases, calcium binding proteins and transcription factors were found to be transiently up-regulated in the first 2 h following the treatment. In parallel, genes encoding proteins involved in the generation of reactive oxygen species, such as OxO (H_2O_2 production), respiratory burst oxidase protein C (O_2^- production), peroxidases (H_2O_2, O_2^-) or enzymes involved in ROS scavenging, such as superoxide dismutase (SOD) were rapidly up-regulated.

8.7. Integration of Stress Responses

When stress causes protein folding in the endoplasmic reticulum (ER) to be slowed, the temporary presence of an abundance of unfolded proteins in the ER triggers the unfolded protein response (UPR). This response results in the first instance in the enhanced expression of those genes known to encode proteins that create the optimal polypeptide-folding environment, such as protein disulfide isomerase (PDI), calreticulin, calnexin, and binding protein (BiP). A detailed study of the breadth of the UPR in Arabidopsis has been conducted using gene expression analysis with Affymetrix GeneChips (Martínez and Chrispeels, 2003). With tunicamycin and

DTT as ER stress–inducing agents, the authors identified sets of UPR genes that were induced or repressed by both stresses. One GLP (At1g72610) was among the 31 genes repressed by both tunicamycin and DTT, and is therefore likely to be involved in the UPR response.

One study of GLP expression has combined an investigation of abiotic and physical variables (Tabuchi *et al*., 2003). The cDNA clone encoding a putative GLP (designated as AlGLP, *A. lentiformis* GLP) was isolated from the halophyte *Atriplex lentiformis* (Torr.) Wats. It was found that AlGLP has SOD activity by in-gel assays after immunoprecipitation, and that it is a glycosylated oligomer in native form. The AlGLP was strongly expressed in calli and weakly in roots, but not in stems or leaves. Interestingly, the transcript level in roots was decreased by salt or abscisic acid (ABA) treatment. Evidence was also presented that the accumulation level of AlGLP mRNA in leaves is increased by methyl jasmonate treatment or wounding, and that the induction is suppressed by ABA treatment. These results suggest that AlGLP expression is increased to fulfil H_2O_2 supply to modify the structure of cell wall in response to wounding.

Abscisic acid (ABA) is a hormone that modulates a variety of agronomically important growth and developmental processes and various stresses responses, but its signal transduction pathways remain poorly understood. ROP10, a member of ROP small GTPases in Arabidopsis, is a plasma membrane-associated protein specifically involved in negative regulation of ABA responses. To dissect the ROP10-mediated ABA signaling, transcriptome analysis was conducted with the Arabidopsis full-genome chip (Xin *et al*., 2005). The analysis revealed a total of 262 and 125 genes that were, respectively, up- and downregulated (≥2-fold cutoff) by 1 mM ABA in wild type (Wassilewskija [Ws]); 42 up-regulated and 38 down-regulated genes have not been identified in other studies. Among the ABA-repressed group were two GLPs (AT5G38930, AT5G38940). A similar approach involves jasmonates, a family of plant hormones that regulate

gene expression to modulate diverse developmental and defensive processes. To screen a set of jasmonate-responsive Arabidopsis genes expressed in the rosette leaves of 5-week-old Arabidopsis plants, a microarray analysis was performed using an Affymetrix GeneChip® containing about 8,300 gene probes including GLPs (Jung *et al*., 2007).

An alternative approach to the investigation of global responses to abiotic stress has been described recently by Dinneny *et al*. (2008) who generated genome-wide expression maps of *Arabidopsis* roots exposed to either a high-salt medium (osmotic stress) or an iron-deficient medium (nutrient stress) at three organizational levels - intact roots, roots divided into four longitudinal zones as proxies for developmental time, and root cells segregated along the radial axis. Much larger numbers of regulated genes were found in the second and third of these sets relative to the first set, indicating a serious dilution of information when only intact roots are analyzed. A major finding from this comprehensive study was that cell identity determines the gene pool that is regulated during stress, as reflected by the high degree of cell specificity in functional gene categories. The significance in the context of this review is that the expression of 20 GLPs was examined and differences were found in their relative responses to the stress imposed.

All responses to the environment, whether it be biotic or abiotic stress, must be mediated through cell signalling pathways. Arabinogalactan proteins (AGPs) are hydroxyproline-rich glycoproteins present at the plasma membrane and in extracellular spaces. A synthetic chemical, β-glucosyl Yariv reagent (β-GlcY), binds specifically to AGPs. A recent study examined the universality and specificity of the inhibitory effect of β-GlcY on gibberellin signaling using microarray analysis and found that β-GlcY was largely effective in repressing gibberellin-induced gene expression (Mashiguchi *et al*., 2008). In addition, >100 genes, including an OxO,

were up-regulated by β-GlcY in a gibberellin-independent manner, and many of these were categorized as defense-related genes. These results indicated that gibberellin signaling is under the regulation of defense-related signaling in aleurone cells. It is also probable that AGPs are involved in the perception of stimuli causing defense responses. The specific patterns of response to nitric oxide, another important molecule in cell signaling, have been investigated recently in *Medicago truncatula* (Ferrarini *et al*., 2008). GLPs were included in this analysis.

9. WOUND RESPONSES

The plant wound response includes establishment of a diffusion barrier, repair of damage, stimulation of new growth and differentiation around the wound region, and induction of defense mechanisms against potential biological invaders. In a detailed study of the role of OxO in wounding in *Lolium perenne* two bursts of H_2O_2 production were detected after cutting the leaf blades (Le Deunff *et al*., 2004). The second burst, which was initiated several hours later, coincided with the induction of germin-like OxO (G-OxO) activity. This wound-dependent increase of activity was the result of differential induction of four *g-OxO* genes. Moreover, expression of these genes was enhanced by an exogenous supply of H_2O_2 or methyl jasmonate (MeJa). The pattern of their expression *in planta* was identical to that occurring in senescing leaf sheaths (Davoine *et al*., 2001). These results emphasize the importance of G-OxOs in H_2O_2 production in oxalate-producing plant species such as ryegrass, and suggest that these enzymes might be crucial during critical events in the life of plants such as cutting and senescence by initiating H_2O_2-mediated defences against pathogens and foraging animals.

The promoters of genes that are induced by wounding could be valuable in regulating the expression of beneficial genes. The promoter of a GLP gene has recently been shown, for the

first time, to be induced by wounding; the *Os*RGLP2 promoter was observed to have a strong and rapid wound inducible activity in transgenic tobacco (Naqvi, unpublished). To the best of our knowledge this is the first ever report of wound inducibility of a GLP promoter and thus indicates that GLPs may have some role to play at the site of injury.

10. GENETICS AND PLANT BREEDING

The obvious functional connection between expression of GLPs and the response of plants to biotic and abiotic stress has grown rapidly over the last decade and there are now many examples of projects that attempt to combine genomic information on the GLP gene family with phenotype selection.

10.1. Marker Assisted Breeding

Candidate genes involved in both recognition (resistance gene analogs [RGAs]) and general plant defense (putative defense response [DR]) were used as molecular markers to test for association with resistance in rice to blast, bacterial blight (BB), sheath blight, and brown plant-hopper (BPH) (Ramalingam *et al.*, 2003). The 118 marker loci were either PCR-based RGA markers or restriction fragment length polymorphism (RFLP) markers that included RGAs or putative DR genes from rice, barley, and maize. Two GLPs (Accessions Y14203, X93171) were included in the test list of 20 candidate genes. On chromosome3, qBB3-2, a short BB QTL region accounting for 10.3% phenotypic variation, is closely associated with OxO; a portion of the same chromosomal region is associated with a QTL defined by percent disease leaf area. However, when a permutation test was conducted to determine the appropriate threshold, the association of qBB3-2 with OxO was not significant. Nonetheless, in other mapping populations,

OxO is found to provide a high level of quantitative resistance to fungal pathogens of wheat (Faris *et al*. 1999) and rice (Bin and Leung, *unpublished data*). For example, OxO on chromosome 3 is associated with BB and blast resistance (Jie and Makoto, 2004). One DR gene, OxO (accession number Y14203) maps within a BB resistance locus.

Although quantitative trait loci (QTL) underpin many desirable agronomic traits, their incorporation into crop plants through marker-assisted selection is limited by the low predictive value of markers on phenotypic performance. In one study relevant to the present review, candidate defense response (DR) genes were used to dissect quantitative resistance in rice using recombinant inbred (RI) and advanced backcross (BC) populations derived from a blast-resistant cultivar, Sanhuangzhan 2 (SHZ-2) (Liu *et al*., 2004). Based on DNA profiles of DR genes, RI lines were clustered into two groups corresponding to level of resistance. Five DR genes, encoding putative OxO, dehydrin, PR-1, chitinase, and 14-3-3 protein, accounted for 30.0, 23.0, 15.8, 6.7, and 5.5% of diseased leaf area (DLA) variation, respectively. Together, they accounted for 60.3% of the DLA variation and co-localized with resistance QTL identified by interval mapping. Advanced BC lines with four or five of these effective DR genes showed improved resistance under high disease pressure in field tests. These and other similar results (Wu *et al*., 2004; Leach *et al*., 2007; Manosalva, 2006; Raghavan *et al*., 2007) demonstrate that the use of natural variation in a few candidate genes, principally OxO, can solve a long-standing problem in rice production, and this approach has the potential to address other problems involving complex traits (Wisser *et al*., 2005) such as disease resistance in maize (Wang *et al*., 2007a), and drought tolerance in wheat (Kirigwi *et al*., 2007; Diab *et al*., 2008) and sunflower (Fusari *et al*., 2008).

In another plant breeding project, Pivorienė *et al*. (2008) employed the Inter-simple Sequence Repeat (ISSR) marker system to improve the mapping data of perennial ryegrass. This

method allows the detection of polymorphisms in inter-microsatellite loci without previous knowledge of a DNA sequence. One of the ISSRs, a 780 bp fragment amplified with (TG)8RT, showed identity to the *H. vulgare* subsp. *vulgare* GLP gene 4c (*GER4c*) and to the germin A (*GerA*) gene present within 190 bp and 140 bp segments, respectively. Such Inter-SSR mapping will provide useful information for gene targeting, quantitative trait loci mapping and marker-assisted selection in this crop, and in *Citrus sinensis* where similar results were reported (Shanker *et al*., 2007).

10.2. TRANSGENIC APPROACHES

An associated example of the use of GLP genes in combating biotic stress is that of introducing germin as a means of protecting transgenic plants against the toxic effects of oxalate secreting pathogens such as Sclerotinia (*Sclerotinia sclerotiorum*) (Chipps *et al*., 2005; Lu, 2003; Zhao *et al*., 2007b). In the first such example, expression of a barley OxO gene in oilseed rape provided resistance to oxalic acid (Thompson *et al*., 1995; Zou *et al*., 2007; Dong *et al*., 2008) and a similar strategy was shown subsequently to provide disease resistance in transgenic soybean (Donaldson *et al*., 2001; Cober *et al*., 2003), poplar (Liang *et al*., 2001; 2005), potato (Schneider *et al*., 2002), sunflower (Hu *et al*., 2003), peanut (Livingstone *et al*., 2005), tomato (Walz *et al*., 2007) and American chestnut (Polin *et al*., 2006; Merkle *et al*., 2007; Welch *et al*., 2007). For a review of such methods, please see Gilbert *et al*. (2006). Less success was achieved in a study involving a 2.3 kb fragment of the wheat *GstA1* promoter in combination with an intron-containing part of the wheat *WIR1a* gene to drive strong and constitutive expression of OxO in wheat leaves (Altpeter *et al*., 2005). Neither transient nor stable expression provided resistance in infection assays with *Blumeria graminis* f.sp. *tritici*.

Improved resistance to European corn borer (*Ostrinia nubilalis*) has been demonstrated in maize expressing wheat OxO (Ramputh *et al*., 2002; Mao *et al*., 2007), whereas in a study of the effect of salt tolerance in transgenic potato expressing a barley OxO, Turhan (2005) reported no conclusive results.

10.3. Promoter Analysis

All genes are regulated by their promoters. Tobacco Nectarin I is a GLP, with SOD activity and has been suggested to be involved in the production of H_2O_2 (Carter and Thornburg, 2002; Ren *et al*., 2007). Its promoter was analyzed by construction of full-length promoter region with chloramphenicol acetyl transferase (CAT) marker gene. It was observed that the deletion of 271 nucleotides from the 5' end of Nectarin I promoter altered the organ specificity; the CAT activity was not only observed in the nectary of transformed tobacco but also appeared in the petals. To evaluate whether promoter deletions would affect marker gene expression, the CAT gene was fused with two 5' promoter deletions and the shortest promoter was observed to be non-functional in the nectaries (Carter and Thornburg, 2002). Staiger *et al.* (1999) indicated that clock-responsive elements contributing to high-amplitude *AtGer*3 promoter oscillations largely reside between -299 and -967. In another related study (Fan *et al*,. 2005), the promoter of *Zm*GLP1 was analyzed by deletion studies in transgenic *Arabidopsis* to identify the regions responsible for activity and tissue specificity as well as the circadian rhythm. *Zm*GLP1 promoter activity was found in filaments and most of the green tissue i.e., rosette leaves, cauline leaves, stem, pedicels, sepals, stigmas, green carpel, and dissepiments while no activity was observed in roots of seedlings and mature plants. The tissue specificity was observed to change with different promoter deletions. Further, it was also observed that a positive and a negative regulatory

element responsible for circadian rhythm-responsiveness might be located within the regions -739 to -349 and -349 to -161, respectively. In another comprehensive wheat germin promoter (fused with GUS) analysis Berna and Bernier (1997; 1999) reported the induction of the promoter in transgenic tobacco treated with salt, heavy metals, aluminum and plant growth regulators, specifically auxin and gibberellin. Naqvi *et al.* (unpublished) have demonstrated the expression of one germin in *Arabidopsis* root tips and another in whole root except root tip, by cloning a β-glucuronidase (GUS) gene under the control of the respective homologous promoters. Recently Mahmood *et al.* (2007) reported five common regions of different sizes (CR1-CR5) in the *Os*RGLP2 promoter, which are repeated at 3-5 other locations in a 30 Kb region of *Oryza sativa* chromosome 8 in which this *Os*RGLP2 gene is located. Interestingly it was found that all the genes driven by promoters harboring these common regions are GLPs/putative germins.

Also relevant in this context is the study of *Pinus caribea* promoter GUS fusions in tobacco Bright Yellow 2 (BY-2) cells (Mathieu *et al*., 2003). The *PcGER1* promoter sequence was cloned upstream of the GUS reporter gene and transferred to BY-2 cells via *Agrobacterium tumefaciens*-mediated transformation. Optimal growth, maximum cell-wall yield and *PcGER1* promoter activity were observed in the presence of 2,4-D and BA at day 4, the end of the exponential growth phase where 70–75% cells have a 2C DNA content. Analysis of promoter activity during the cell cycle in an aphidicoline-synchronized culture suggested that the expression is maximal in G1 cells.

A more complex genetic approach showed that the *MYB11*, *MYB12* and *MYB111* genes share significant structural similarity and represent subgroup 7 (SG7) of the *Arabidopsis thaliana* R2R3-MYB gene family. They all display very similar target gene specificity, principally for

several genes of flavonoid biosynthesis. To determine the global regulatory potential of these three transcription factors, a combination of genetic, functional genomics and metabolite analysis approaches was used (Stracke *et al*., 2007). Screening by transcriptome analysis identified the At4g14630 (GLP9) gene as a SG7 MYB candidate target.

11. MEDICAL AND COMMERCIAL IMPORTANCE

Several members of the GLP family of proteins, particularly OxO, have considerable commercial significance. These various applications have been reviewed previously (Dunwell *et al*., 2000) and will not be repeated here. One method of assessing this topic is by inspection of the various patent databases (Dunwell, 2005a), a valuable source of information that relates to protection of intellectual property, and a summary of some US patents in this field are given in Table 2. Of equal importance is the role of GLPs in the medical context and this is described in the following sections.

11.1 GLPs and Allergy

It is now well known that many plant-derived members of the cupin superfamily have allergenic properties (Breiteneder and Mills, 2005; Radauer and Breiteneder, 2006; 2007). Although the peanut allergen Ara h 1 (Kang *et al*., 2007) and related globulins are the most important subgroup in this context (Dunwell, 2005b), germin and other GLPs are now being increasingly recognized as significant allergens. Jensen-Jarolim *et al.* (2002) studied germins and GLPs produced in transformed tobacco to estimate their impact for IgE-binding to these allergens, examined their capabilities to cross link via IgE, the high affinity IgE receptor, and reported that germin and GLPs are plant glycoproteins with allergenic properties. Model antigens

used in their study were wheat germin and *Atger*3. There is accumulating evidence for the participation of glycans in IgE epitopes recognition (Garcia-Casado *et al.,* 1996). Ahrazem *et al.* (2006) have reported a 24-kDa purified allergen, designated Cit s 1, from orange and identified it as a germin-like glycoprotein. It was subsequently established (Crespo *et al.* 2006; Ebo *et al*., 2007) that Cit s 1 is a major allergen found in oranges and most recently Poltl *et al.* (2007) revealed the molecular characterization of Cit s 1 which explains the immunological cross-reactive properties of Cit s 1 as well as its equivocal nature as a clinically relevant allergen. Recently the first case was reported of a peanut allergic patient who exhibited cosensitivity to citrus seeds and who had experienced anaphylaxis to lemon soap (Glaspole *et al*., 2007).

11.2 Germin and Medical Diagnostics

An important challenge faced in medicine is the need for efficient and cost effective diagnostic techniques, and one of the GLP proteins, OxO is already used in this context. Barley OxO (98% identical to wheat germin) is used in kits to assay levels of oxalate in blood plasma and urine (Porowski *et al*., 2008), a process required in the treatment of patients with kidney stones (a crystallized form of calcium oxalate) (Marengo and Romani, 2008). Because of the importance of these diagnostic protocols, there is a continuous effort to improve the accuracy and efficiency of the assay (Fiorito *et al*., 2005; Bhambi and Pundir, 2007; Capra *et al*., 2007; Kumar *et al*., 2008; Pundir *et al*., 2008), and to develop more efficient heterologous expression systems for production of the purified enzyme, either in bacteria (Cassland *et al*., 2004) or yeast (Pan *et al*., 2007).

12. CONCLUDING COMMENTS

The various summaries provided in this review have provided a background understanding of one of the most ubiquitous of plant gene families, the expression of which is intimately linked to many diverse responses to the environment. Amongst the analyses relevant to this review is a recent study of the respiratory burst oxidase in which it was concluded that the production of reactive oxygen intermediates (ROI) is among the earliest temporal events following pathogen recognition in plants (Torres and Dangl, 2005). Initially, ROI were thought to be cell-death executioners. Emerging evidence, however, suggests a broader role for ROI as signals that mediate responses to infection, the abiotic environment, developmental cues, and programmed cell death in different cell types. In this context, it is interesting to consider results from using a T-DNA knockout of the Arabidopsis homologue of the tomato disease resistance gene Asc. The *asc* gene renders plants sensitive to programmed cell death (PCD) triggered by the fungal AAL toxin. To obtain more insights into the nature of AAL-toxin-induced cell death and to identify genes of potential importance for PCD, transcription profiling of AAL-toxin-induced cell death in this knockout was conducted using an oligonucleotide array representing 21,500 Arabidopsis genes (Gechev *et al*., 2004). Genes responsive to reactive oxygen species and ethylene were among the earliest to be upregulated, suggesting that an oxidative burst and production of ethylene played a role in the activation of the cell death. This suggestion was corroborated by induction of several genes encoding ROS-generating proteins, including a respiratory burst oxidase and germin-OxO.

At a practical level, the overall features of germin and the GLP gene family are important and significant for plant breeders (Wang *et al*., 2007c), biochemists and molecular biologists working in the field of stress tolerance. More work is required in order to understand the increasing list of biochemical functions being assigned to the products of this gene family, an

understanding that may also be utilized in the production of genetically modified crops resistant to biotic and abiotic stresses (Dunwell, 2000). Further, there is a real incentive for an investigation of the evolutionary process in which members of germin and GLP family underwent a rapid duplication and diversification that coincided with the evolution of land plants.

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Tables

TABLE 1
GLP derived SAGE tags identified in libraries from mature leaves and immature seed of rice (data modified from Gibbings *et al*., 2003)

No.	TC[a]	Location[b]	Sequence	GLP No.	Tag count	
					Seed	Leaf
1	298368	3	GCTACAGCAT	7	0	1
2	305106	8	CACCTGATGC	16	8	0
3	309308	8	ATATGCTAAA	3 (fragment)	3	0
4	321372	8	GAATATGTTG	3 (fragment)	7	0
5	321848	1	CAAAATGTAG	2	0	1
6	332166	8	TTACTATGGA	16	2	0
7	356070	8	ACAAGCCAGC	1	0	5

[a]TIGR tentative concensus number [b] Chromosome

TABLE 2

Summary of US Patents that include reference to germins

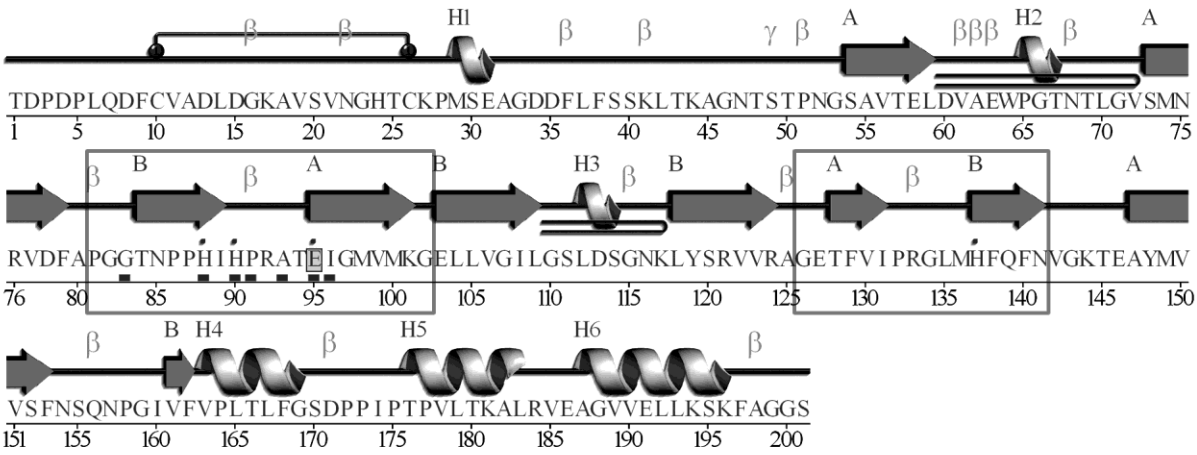
Patent No.	Date	Inventors	Subject
7,271,314	2007	Hirochika and Okamoto	Genes in tobacco
7,253,341	2007	Wang, W. *et al*.	Protease resistant proteins
7,109,033	2006	Harper *et al*.	Stress regulation
7,094,952	2006	Pagniez *et al*.	Transgenic plants
6,441,275	2002	Bidney *et al*.	Pathogen resistance
6,235,530	2001	Freyssinet and Sailland	Oxalate oxidase
5,866,778	1999	Hartman *et al*.	Oxalate oxidase

Figure legends.

FIG 1. Sequence and structure of the archetypal germin, oxalate oxidase from barley (PDB code 1FI2). Primary peptide sequence and secondary structure, adapted from the PDBsum database (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) entry for 1FI2. The dots above the residues H88, H90, E95, and H137 identify the four residues acting as ligands for the single active site manganese ion. The two boxed regions represent the two conserved cupin motifs, each spanning two beta strands (broad arrows) and separated by the intermotif region with two strands and one loop.

B. Quaternary structure showing the homohexameric assembly of six monomers, each containing a manganese ion (small sphere).

A


TDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMN
RVDFAPGGTNPPHIHPRATEIGMVMKGELLVGILGSLDSGNKLYSRVVRAGETFVIPRGLMHFQFNVGKTEAYMV
VSFNSQNPGIVFVPLTLFGSDPPIPTPVLTKALRVEAGVVELLKSKFAGGS

B