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REVIEW

Germin-like proteins (GLPs) in cereal genomes: gene clustering and dynamic roles in plant defence

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Abstract The recent release of the genome sequences of a number of crop and model plant species has made it possible to define the genome organisation and functional characteristics of specific genes and gene families of agronomic importance. For instance, *Sorghum bicolor*, maize (*Zea mays*) and *Brachypodium distachyon* genome sequences along with the model grass species rice (*Oryza sativa*) enable the comparative analysis of genes involved in plant defence. Germin-like proteins (GLPs) are a small, functionally and taxonomically diverse class of cupin-domain containing proteins that have recently been shown to cluster in an area of rice chromosome 8. The genomic location of this gene cluster overlaps with a disease resistance QTL that provides defence against two rice fungal pathogens (*Magnaporthe oryzae* and *Rhizoctonia solani*). Studies showing the involvement of GLPs in basal host resistance against powdery mildew (*Blumeria graminis* ssp.) have also been reported in barley and wheat. In this mini-review, we compare the close proximity of GLPs in publicly available cereal crop genomes and discuss the contribution that these proteins, and their genome sequence organisation, play in plant defence.

Keywords Germin-like proteins (GLPs) · Disease resistance · Cereal genomes

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Introduction

Many genes and gene families in plants have been implicated in specific and quantitative responses to a range of stresses. In times of biotic stress, plants can produce several different types of defence responses and genetic mechanisms for recognising and responding to attack. Qualitative disease resistance to pathogens is mediated by a single resistance (R)-gene whose products can recognise pathogen effectors (Zimmermann et al. 2006; Jones and Dangl 2006). Disease resistance receptor genes (R-genes and host-pattern recognition receptor (HPRR) genes; Kou and Wang 2010) generally make proteins such as receptor-kinases (such as rice *Xa21* (Song et al. 1995) and barley *Rpg1* (Brueggeman et al. 2002)) and nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. NBS-LRR proteins have highly variable LRR domains which are primarily used for pathogen recognition (Yahiaoui et al. 2004; Caplan et al. 2008; McDowell and Simon 2008; Bhullar et al. 2009; Padmanabhan et al. 2009). Many R-genes have been isolated in crop species using map-based cloning techniques such as *Lr10* (Feuillet et al. 2003), *Lr1* (Cloutier et al. 2007), *Pm3* (Yahiaoui et al. 2004) and *Lr21* (Huang et al. 2003).

Other than receptor genes, defence-responsive or defence-related genes (acting as either activators or suppressors) can respond to pathogen attack through post-translational protein modification and variation of expression (Eulgem 2005; Benschop et al. 2007; Kou and Wang 2010). These race non-specific genes, along with HPRRs in combination, act to provide quantitative disease resistance at a quantitative trait locus (QTL). While possibly performing with multiple

defensive roles, these genes give an additive affect on the plant leading to a resistant phenotype (Jones and Dangl 2006). This quantitative type of resistance does not give a complete resistant phenotype compared to the single R-gene resistance, or so-called 'race-specific resistance' model, but often provides a more durable or broad-spectrum resistance to pathogens (Ayliffe et al. 2008).

Disease resistance QTLs, as well as candidate genes that are known to be involved in disease resistance, have been shown to cluster on particular chromosomal segments in rice (Wisser et al. 2005) and maize (Wisser et al. 2006). The consequence of having multiple genes in a closely linked genome region such as a QTL creates difficulties in studying their effects. However, the availability of whole genome sequences in multiple crop species, along with QTL mapping techniques, has allowed easier identification and analysis of genes linked to quantitative diseases resistance. Studies like those already carried out in rice (Wisser et al. 2006) have looked to fuse QTL and genome data to identify candidate genes for quantitative disease resistance.

Major and minor quantitative disease resistance genes have recently been identified in wheat and rice from a diverse variety of gene families. The wheat fungal resistance QTL, which contains the gene *Lr34* was identified on chromosome 7D and encodes an ATP binding cassette transporter from the pleiotropic drug resistance subfamily (Krattinger et al. 2009). *Lr34* is effective against diseases such as leaf rust, stripe rust and powdery mildew. A stripe rust resistance gene *Yr36*, conferring broad-spectrum resistance at high temperatures was identified on wheat chromosome 6B and encodes a kinase-START protein (Fu et al. 2009). A rice chromosome 4 QTL for the recessive *pi21* gene, conferring durable resistance to blast disease, was identified as a mutated proline-rich protein that contains a putative heavy metal-binding domain (Fukuoka et al. 2009). Other genes conferring minor disease resistance effects have also been identified in rice (Hu et al. 2008).

In this publication we review germin-like protein (GLP) gene clusters, one of which was recently identified in a study of a diseases resistant QTL on rice chromosome 8 (Manosalva et al. 2008). The germin-like proteins will be reviewed with regards to their physical protein structure, functional contribution to plant defence and taxonomic distribution. We also present Bioinformatics and comparative genomic analysis of cereal plant genomes, that have either been completed or are in draft stages of sequencing or annotation, and propose major germin-like protein clusters that may identify disease resistance loci.

Defining GLPs

Germins and GLPs were originally identified in wheat plants as a specific marker for the start of germination (Lane et al. 1993; Dunwell et al. 2008). These proteins generally code two exons and contain a 'cupin' protein domain (PF00190; Finn et al. 2008) at their C terminus (Fig. 1). The cupin protein domain family are a large, functionally diverse super-family (Dunwell and Gane 1998; Dunwell et al. 2000, 2004) that are distantly related to seed storage proteins such as vicilins (Gane et al. 1998). The cupin domain is named for the protein's classic jellyroll beta-barrel structural domain, with 'cupa' meaning small barrel in Latin (Dunwell et al. 2000).

Each member of the protein family contains two amino acid sequence motifs, described as the 'germin box' (Lane et al. 1991; Dunwell and Gane 1998; Dunwell 1998; Yamahara et al. 1999). Motif 1-G(x)5HxH(x)3,4E(x)6G and Motif 2-G(x)5PxG(x)

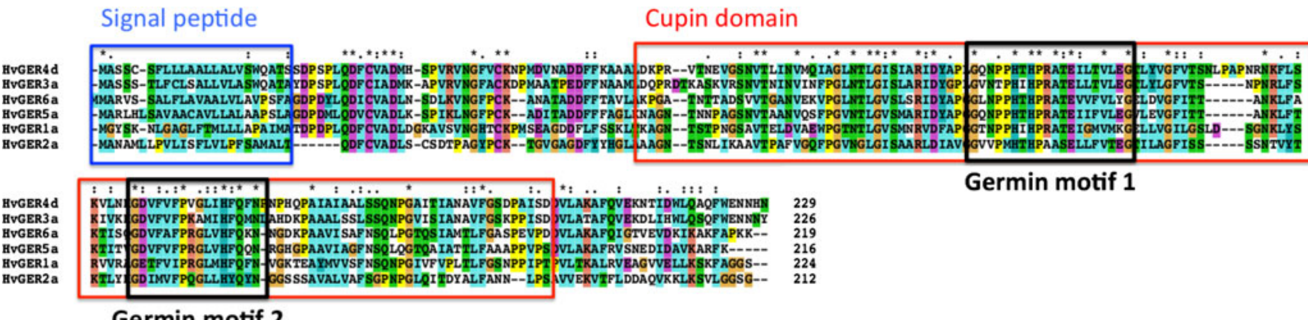

Fig. 1 Multiple sequence alignment of the barley germin amino-acid sequences that characterise the GER families in cereals (Druka et al. 2002; Zimmermann et al. 2006; Manosalva et al. 2008). Annotated on the figure is the signal peptide that is found in most GLPs (*blue box*), cupin domain (*red box*) and the two defining germin motifs (*black boxes*). The asterisks above the sequence indicate amino acid conservation over all six HvGER families

2H(x)3N are both contained in the classic jellyroll beta-barrel structural domain. Crystallographic evidence carried out on a barley germin protein confirmed that six germin proteins (which each bind a single manganese-ion) make up an extremely stable hexamer protein structure (Woo et al. 2000). Each germin protein 'monomer' binds to another creating a hexamer structure made from a 'trimer of dimers' (Fig. 2). Manganese ions are bound by ligands similar to manganese superoxide dismutase (SOD), with enzyme activity in germin and GLPs confirmed with biochemical evidence (Yamahara et al. 1999; Carter and Thornburg 2000; Woo et al. 2000). Each germin monomer is comprised of an irregular N-terminal extension, the beta-barrel and a C-terminal sequence containing three alpha-helices (Woo et al. 2000). Interestingly the irregular N-terminal domain shape is conserved in many GLPs. In total the hexamer contains about 1,200 amino acids with an approximate molecular mass of 130 kDa (Lane 2002). This is contrasted by the discovery of a single copy GLP from rice that has SOD-activity in its dimeric form (Banerjee and Maiti 2010).

GLPs and other cupin domain-containing proteins are a functionally and taxonomically diverse family of proteins (Table 1). Germins and GLPs are single cupin domain proteins (or monocupins) along with proteins such as microbial phosphonmannose isomerases, AraC-type transcriptional regulators and oxalate oxidase (OXO) enzymes in plants, while bicupins (with two cupin domains) are found in proteins such as seed storage proteins and oxalate decarboxylases (Khuri et al. 2001). The bicupin globular storage proteins such as legumins (11S) and euvicilins (7S) have also been identified as major food allergens being found in such plants as soybean, peanut, walnut and lentil (Mills et al. 2002). Multiple epitopes have been mapped onto their complex multi-dimer structures, which are often associated with these high thermotolerance proteins (Mills et al. 2002; Xiang et al. 2002; Dunwell et al. 2004). The clustering of proteins in a structure-based phylogeny study of bicupin-containing proteins revealed that N- and C-terminal cupin domains had evolved independently of each other (Agarwal et al. 2009). Sequence-based phylogenetic analyses of all cupin-domain-containing proteins in rice can clearly separate monucupins and bicopins, despite >90% amino acid and nucleotide identity Carrillo et al. (2009).

The high sequence conservation of germins and GLPs over multiple plant species presents difficulties in classification (Dunwell and Gane 1998). Structural characteristics of these proteins offer a more robust system of classification due to the clustering of proteins with conserved functions (Agarwal et al. 2009). The well-conserved homogeneous group of so-called 'true germins' are those found to have OXO activity and are almost exclusively found within cereal plant species (Lane et al. 1991; Woo et al. 2000; Bernier and Berna 2001; Carrillo et al. 2009; Davidson et al. 2009). The heterogeneous GLP group of proteins have a far wider taxonomic range in plants and include germin-motif

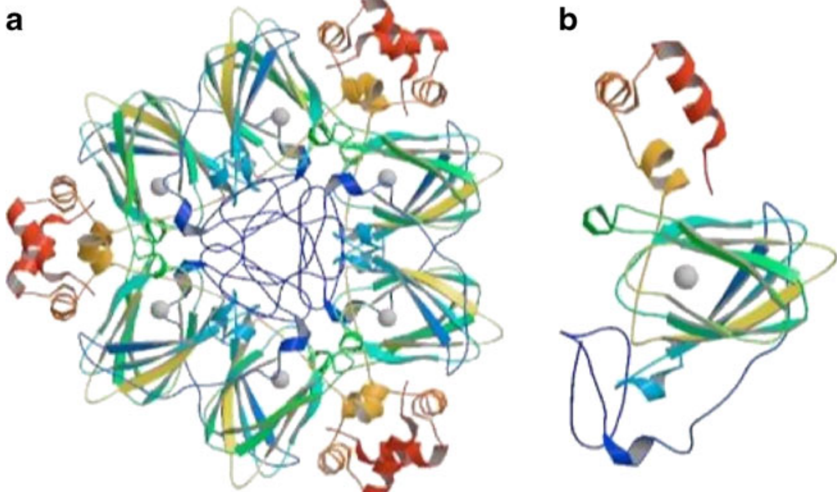

Fig. 2 The hexameric 3D-protein structure of the barley 130 kDa germin protein (**a**) made up of six individual germin monomer proteins (**b**) that each bind one manganese ion each (grey spheres in the middle of **b**). The germin monomer is made up of an alpha-helical C-terminal domain (coloured in red), a barrel structure made up of multiple beta-sheets (*green* and *yellow*) and an irregular N-terminal extension (*blue*). The protein structure on this barley germin hexamer (PDB Accession: 1FI2) was determined by X-ray crystallisation (Woo et al. 2000) and the figures were taken from the RCSB protein data bank (PDB; Kouranov et al. 2006)

Table 1 Summary of major groups of cupin domain-containing proteins

Protein	Type	Organisms	Literature cited
Type II—Phosphomannose isomerases (PMIs)	Monocupin	Microbial	Dunwell and Gane (1998)
HTH Transcription factors	Monocupin	Microbial	Aravind and Koonin (1999)
AraC-type transcriptional regulators	Monocupin	Microbial	Gallegos et al. (1997); Soisson et al. (1997a, b)
Gentisate 1,2-dioxygenases (GDOs)	Monocupin	Microbial	Dunwell et al. (2000)
Oxalate decarboxylases	Bicupin	Microbial	Tanner and Bornemann (2000)
7S and 11S seed storage proteins (legumins, globulins, triticin, glutelin and euvicilins)	Bicupin	Plants	Bäumlein et al. (1995); Adachi et al. (2001)
Germins	Monocupin	Plants, Fungi	Lane et al. (1993)
Germin-like proteins (GLPs)	Monocupin	Plants, Fungi	Dumas et al. (1993)

containing proteins with an average of 50% amino acid sequence identity that either do not possess OXO activity or have not yet been assigned an enzymatic function (Dumas et al. 1993; Carter et al. 1998; Dunwell et al. 2008).

Multiple studies have examined the role of germins and GLPs in a wide variety of plant systems such as Arabidopsis (Membré et al. 1997; Carter et al. 1998), woody azalea (*Rhododendron mucronatum*; Kondo et al. 2008), conifers (Mathieu et al. 2006), soybean (Klink et al. 2007), grapevine (Ficke et al. 2002; Cramer et al. 2007; Godfrey et al. 2007) and Medicago (Doll et al. 2003; Soares et al. 2009). The majority of germin and GLP research has centred on cereal plants, especially rice, barley, maize and wheat, where the protein was first discovered. Six germin subfamilies (GER1-6) were characterised in Druka et al. (2002) with varying enzymatic activities e.g. OXO action in GER1 ('true germin' proteins) and SOD activity in GER2 (Banerjee and Maiti 2010), GER4 and GER5 sub-families. The GER1 subfamily has also been shown to be important for germination and early plant development in plants (Federico et al. 2006). Also, OsGLP1 downregulated transgenic plants in rice (a member of the GER2 subfamily) were shown to induce dwarfism, change cell morphology, cause dramatic increases in sheath blight and blast fungal diseases (Banerjee and Maiti 2010).

Plant defence mechanisms of GLPs

GLPs have been studied in a wide variety of systems and implicated as plant cell defenders in many species, conditions and diseases. These proteins have been shown to be highly resistant to proteases, heat, sodium dodecyl sulphate (SDS) and extreme pH (Lane et al. 1993; Lane 1994; Wei et al. 1998; Carter et al. 1998; Membré et al. 2000; Mahmood et al. 2010). Significant GLPs and/or OXO expression have been shown in environmental conditions such as salt stress (Hurkman et al. 1991, 1994; Cramer et al. 2007), aluminium stress (Houde and Diallo 2008) and drought stress (Ke et al. 2009). These genes are also expressed when attacked by fungal pathogens (Schweizer et al. 1999; Donaldson et al. 2001; Liang et al. 2001; Ficke et al. 2002; Zimmermann et al. 2006; Manosalva et al. 2008), bacteria and viruses (Park et al. 2003) and Erysiphe nectar infections (Godfrey et al. 2007). They are expressed in a diverse range of tissues and can act against parasites like nematodes (Knecht et al. 2010) and insects (Ramputh et al. 2002; Lou and Baldwin 2006).

So far germins and GLPs have been shown to function by using three main enzyme actions. Studies originally from barley revealed that germins have an OXO enzyme action (Lane et al. 1993; Dumas et al. 1993). OXOs act by catalysing the production of CO_2 and H_2O_2 (Requena and Bornemann 1999), the latter being present and implicated in plant defence (Wojtaszek 1997a, b). As mentioned above, SOD activity has been identified in previous studies on germins (Woo et al. 2000) and GLPs (Yamahara et al. 1999) and they work through the conversion of toxic reactive oxygen species (ROS) to H_2O_2 (Kukavica et al. 2005). Additionally, a barley GLP was identified to have a different ADP glucose pyrophosphatase/phosphodiesterase (AGPPase) enzyme activity (Bernier and Berna 2001; Mahmood et al. 2010), which may help to control metabolic flow towards starch, cell wall polysaccharides, glycoproteins and glycolipids in plants (Rodríguez-López et al. 2001).

A previous study into germin isoforms (Lane et al. 1992) identified that approximately 40% of germins in wheat embryonic tissue are cell wall-associated and important for plant development. To date, all GLPs analysed possess N-terminal secretory signals, which further indicates cell wall/extracellular matrix targeting (Lane et al. 1992; Heintzen et al. 1994; Lane 1994; Bernier and Berna 2001; Zimmermann et al. 2006). Other cell wall localisation studies have also confirmed this cell surface association (Heintzen et al. 1994; Davidson et al. 2010). A cell-wall-associated GLP in

rice was also shown to be located in higher abundance in sub-epidermal cells (Banerjee and Maiti 2010). It is believed that GLPs might also possess a structural role in cross-linking the cell wall after a pathogen attack (Christensen et al. 2004).

Most studies investigating the physiological cell defence mechanisms of GLPs have focused on the expression and defence mechanisms of GLPs in the apoplast (Felle et al. 2005), with their cell-wall interaction enabling early response to pathogens (Davidson et al. 2010). GLPs have been shown to increase in expression within plant cells after infection (causing the production of ROS such as H_20_2) and form an ‘oxidative burst’ response (Wojtaszek 1997a; Averyanov 2009). H_20_2 is also involved in cell signalling in the apoplast. A transgenic study in sugar beet indicated that expression of BvGLP-1 constitutively activates the expression of other defence-related proteins and downregulates others (Knecht et al. 2010). Transgenic ectopic expression studies in Soybean (Donaldson et al. 2001) and gene silencing in epidermal cells of *Nicotiana attenuata* (Lou and Baldwin 2006) also indicated the expression of GLP-encoding genes in cell wall regions proximal to the pathogen attack or wounding site.

GLP gene clusters in currently available cereal genome sequences

Two studies of GLP expression in barley (Zimmermann et al. 2006) and a QTL on chromosome 8 of rice (Manosalva et al. 2008) have shown the complex nature of GLP genes in cereal genomes and confirmed their involvement in broad-level disease resistance. Both studies analysed plant interaction with fungal pathogens such as powdery mildew (*Blumeria graminis f* sp. *hordei*) in barley and rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctania solani*) diseases in rice. Pre-genome sequence analyses carried out on each rice chromosome in Wisser et al. (2005) also indicated that the chromosome 8 disease resistance QTL was indeed a ‘hotspot’ for different disease resistance genes. Developmental transcript analysis of barley tissues showed a broad range of expression of five germin sub-families previously outlined in Druka et al. (2002). Gene silencing through RNA interference showed that transient silencing of GER3 and GER5 sub-families in barley and transient over-expression of GER4 and GER5 protected epidermal cells from powdery mildew attack. The silencing of GER4 led to high susceptibility to pathogen attack (Zimmermann et al. 2006).

Similar results were found in rice (Manosalva et al. 2008) except a fully sequenced rice genome enabled the authors to identify genes involved in a rice blast disease associated QTL. Eight GLPs were identified to form the QTL on chromosome 8 in a tightly linked cluster and after RNA silencing of each GLP gene in the cluster, the susceptibility to the two rice diseases mentioned above increased. Like in Zimmermann et al. (2006), the GER4 subfamily was identified to contribute most to diseases resistance.

The recent studies identifying GLP gene clusters in both the rice chromosome 8 QTL region studied in Manosalva et al. (2008) and a sequenced region in barley (Himmelbach et al. 2010) indicated a strong connection between both GLP clusters and disease resistance phenotypes. To identify any more GLP clusters the publicly accessible genome sequences of *Brachypodium distachyon*, rice (*Oryza sativa*), *Sorghum bicolor* and maize (*Zea mays*) were assayed to identify GLP gene locations. One rice GLP (OsGLP8-1) from the chr8 QTL region (Manosalva et al. 2008) was used to query the JGI Phytozome comparative genomics resource (http://www.phytozome.net/). The OsGLP8-1 belonged to the ‘hypothetical grass post-duplication gene’ family (Cluster 22740912), which encompasses all the rice chromosome 8 GLPs from the QTL region defined in (Manosalva et al. 2008).

The chromosomal locations of all genes within the family were identified and defined as a cluster if three or more GLPs were found in close proximity (a region with less than 100 kb between each gene member). Genes were renamed according to their position on each chromosome, mirroring the nomenclature used to describe the rice GLP genes in Manosalva et al. (2008) e.g. OsGLP8-1 is the first gene family member on rice chromosome 8 (Table 2). One *Sorghum bicolor* GLP did not have a designated chromosome and was therefore named SbGLP-unk. All genes contained within the gene family contained a cupin domain (Pfam domains PF07883 and PF00190) except for BdGLP3-7 which contains a F-BOX domain (PF00646). The size of BdGLP3-7 is almost double the size of all other genes in the family and it is likely that this is due to an insertion within the cupin domain. Five GLPs found on chromosome 10 in maize were not classified as a cluster (ZmGLP10-1 to ZmGLP10-5) as they were spaced over 800 kb. The size and number of GLPs within each cluster are detailed in Table 3.

Six clusters were identified in the four genomes and contained 56 out of the 65 genes (86%) found within the gene family, indicating that GLPs within this family are likely to be contained in clusters. Syntenic genome segment analysis from whole genome analyses (Salse

Table 2 All genes found in the hypothetical gene (#22740912) family (Phytozome 5 families; http://www.phytozome.net/) that were used to query GLP clustering in completely sequenced post-duplication grass genomes

Genome	Chromosome	Chromosome position	Cluster name	Annotated name[a]
Brachypodium distachyon	2	18393515–18394672	BdGLP2-1	Bradi2g21010.1
	3	13504258–13505260	BdGLP3-1	Bradi3g15190.1
		13507473–13508475	BdGLP3-2	Bradi3g15200.1
		13511002–13512125	BdGLP3-3	Bradi3g15210.1
		13513339–13514481	BdGLP3-4	Bradi3g15220.1
		13525866–13526904	BdGLP3-5	Bradi3g15240.1
		15477075–15477893	BdGLP3-6	Bradi3g17310.1
		15480755–15484032	BdGLP3-7	Bradi3g17320.1
		15517276–15518266	BdGLP3-8	Bradi3g17330.1
Oryza sativa	8	5185878–5186701	OsGLP8-1	LOC_Os08g08920.1
		5207388–5208572	OsGLP8-2	LOC_Os08g08960.1
		5221217–5222314	OsGLP8-3	LOC_Os08g08970.1
		5227825–5229037	OsGLP8-4	LOC_Os08g08980.1
		5232771–5233801	OsGLP8-5	LOC_Os08g08990.1
		5238002–5239151	OsGLP8-6	LOC_Os08g09000.1
		5241498–5242660	OsGLP8-7	LOC_Os08g09010.1
		5247669–5248835	OsGLP8-8	LOC_Os08g09020.1
		5253289–5254233	OsGLP8-9	LOC_Os08g09040.1
		5259155–5260302	OsGLP8-10	LOC_Os08g09060.1
		5263250–5264392	OsGLP8-11	LOC_Os08g09080.1
		7993396–7994721	OsGLP8-12	LOC_Os08g13440.1
	12	2687758–2688828	OsGLP12-1	LOC_Os12g05840.1
		2691487–2692513	OsGLP12-2	LOC_Os12g05860.1
		2695409–2696216	OsGLP12-3	LOC_Os12g05870.1
		2698177–2699274	OsGLP12-4	LOC_Os12g05880.1
Sorghum bicolor	6	2974644-2975922	SbGLP6-1	Sb06g001690.1
	7	7102087–7103153	SbGLP7-1	Sb07g005240.1
		7160130–7161537	SbGLP7-2	Sb07g005250.1
		7239661–7240653	SbGLP7-3	Sb07g005260.1
		7251112–7252308	SbGLP7-4	Sb07g005270.1
		7259660–7260808	SbGLP7-5	Sb07g005280.1
		7306801–7307936	SbGLP7-6	Sb07g005290.1
		7315270–7316197	SbGLP7-7	Sb07g005300.1
		7409465–7410307	SbGLP7-8	Sb07g005310.1
		7419332–7420175	SbGLP7-9	Sb07g005320.1
		7426073–7426916	SbGLP7-10	Sb07g005330.1
		7435638–7436234	SbGLP7-11	Sb07g005340.1
		7453736–7454803	SbGLP7-12	Sb07g005350.1
	?	?	SbGLP-unk	Sb0737s002010.1
Zea mays	2	102590903–102592174	ZmGLP2-1	GRMZM2G045809_P01
	3	28196791–28197411	ZmGLP3-1	AC190772.4_FGP011
		28210385–28211585	ZmGLP3-2	GRMZM2G030772_P02
		28242531–28243821	ZmGLP3-3	GRMZM2G149714_P01
		28261457–28262833	ZmGLP3-4	GRMZM2G093622_P01
		28271569–28273041	ZmGLP3-5	GRMZM2G093606_P01
		28304728–28305909	ZmGLP3-6	GRMZM2G093554_P01
		28326084–28327223	ZmGLP3-7	GRMZM2G157364_P01
		28331328–28332493	ZmGLP3-8	GRMZM2G157298_P01
		28354736–28355846	ZmGLP3-9	GRMZM2G148026_P01
		28361093–28362101	ZmGLP3-10	GRMZM2G148014_P01
		28410680–28411226	ZmGLP3-11	GRMZM2G155506_P01
		28468821–28470086	ZmGLP3-12	GRMZM2G165839_P01
		28476442–28477847	ZmGLP3-13	GRMZM2G074443_P01
		28486541–28487446	ZmGLP3-14	GRMZM2G105940_P01
		28491220–28492488	ZmGLP3-15	GRMZM2G093076_P01
		28515885–28517050	ZmGLP3-16	GRMZM2G072965_P01
		28560860–28561920	ZmGLP3-17	GRMZM2G176798_P01
		28566314–28576866	ZmGLP3-18	GRMZM2G471355_P01

Table 2 (continued)

Genome	Chromosome	Chromosome position	Cluster name	Annotated name[a]
		28591509–28592973	ZmGLP3-19	GRMZM2G170829_P01
		28598452–28599606	ZmGLP3-20	GRMZM2G170857_P01
	10	42520607–42521751	ZmGLP10-1	GRMZM2G178817_P01
		42648230–42649497	ZmGLP10-2	GRMZM2G071390_P01
		43077044–43078521	ZmGLP10-3	GRMZM2G049930_P01
		43234112–43235435	ZmGLP10-4	GRMZM2G012530_P01
		43263304–43264488	ZmGLP10-5	GRMZM2G087111_P01

[a] Annotated name in respective genome annotation databases contained within the http://www.phytozome.net comparative genomics site

et al. 2008; Schnable et al. 2009; Initiative et al. 2010) indicate that five out of the six clusters are located on an ancestral genome segment, the same segment that contains the chromosome 8 GLP cluster in rice (Manosalva et al. 2008).

All of the 65 genes involved in the above analyses belong to the GER3 and GER4 germin subfamilies as characterised by previous studies in barley (Druka et al. 2002; Zimmermann et al. 2006). As shown on Fig. 3, genes from a species-specific cluster were usually grouped together indicating that one or more genes were duplicated multiple times at that region. Duplication of the GER4 sub-family members in the chromosome 8 QTL genes (OsGLP8-1 to OsGLP8-12) was confirmed in Davidson et al. (2010) based on their proximity to each other and similarities within the sequence and putative regulatory sequences. However, genes from a chromosomal gene cluster in the same species were not exclusively from the same GER subfamily (e.g. out of the 11 genes in the rice chromosome 8 cluster, five were grouped with the GER3 subfamily and seven grouped with GER4). This suggests diversification of duplicated genes within the loci, as was also suggested in Manosalva et al. (2008).

The expressed GER3 and GER4 subfamilies of GLPs contributed the most to resistance against powdery mildew in wheat and barley (Christensen et al. 2004; Zimmermann et al. 2006) and both powdery mildew and blast fungus in rice (Manosalva et al. 2008). The cluster of eight functional GLP genes on barley chromosome 4H were all identified to be from the GER4 sub-family (Himmelbach et al. 2010). This barley GLP cluster did not show synteny to the other GLP clusters in *Sorghum*, *Brachypodium*, rice and maize, however its presence on an ancestral genome segment could not be verified (Salse et al. 2008).

The high sequence similarity between family members in close proximity suggests functional redundancy of GLP proteins (Zimmermann et al. 2006). This is one possible explanation for the clustering of GLPs in cereal genomes. It was suggested in Manosalva et al. (2008) that as GLP genes are amplified through duplication, the protein family diversifies (Kafri et al. 2006) and evolves new functional or structural roles. Agarwal et al. (2009) suggested that the functional diversity of cupin domain-containing proteins is believed to be due to variations in the length of α-strands and greater conformation freedom in folds holding metal binding residues.

The acquisition of new functional roles through duplication was highlighted in rice where the chromosome 8 GLP cluster contributed far more to resistance

Table 3 Clustering of the genes in the 'hypothetical grass post-duplication gene' family (Cluster 22740912) from the Joint Genome Institute (JGI)/Center for Integrative Genomics (CIG) comparative genomics website (http://www.phytozome.net)

Species	Genes	Number of gene clusters	GLP clusters		
			Chromosome	Number of genes	Approximate cluster size
Brachypodium distachyon	9	3	**Chr 3**	**5**	**21 kb**
			Chr 3	3	41 kb[a]
Oryza sativa	16	2	**Chr 8**	**12**	**80 kb**[b]
			Chr 12	4	12 kb
Sorghum bicolor	14	2	**Chr 7**	**12**	**353 kb**
Zea mays	26	3	**Chr 4**	**20**	**405 kb**
	65	10		52	

The bold type chromosome of the major germin clusters indicate the clusters found on syntenic genome segments between the cereal species (i.e.**Bd3/Os8/Sb7/Zm4**)

[a] There are two GLP clusters in close proximity on Bd3

[b] Eleven of the 12 rice chr8 genes are found in a 80 kb cluster, however found the gene OsGLP8-12 (located over 2 Mb away) to be within the QTL boundary

SbGLP7-3
SbGLP7-10
SbGLP7-8
ZmGLP4-20
ZmGLP4-1
ZmGLP4-17
ZmGLP4-7
ZmGLP4-9
ZmGLP4-4
ZmGLP4-18
ZmGLP4-12
ZmGLP4-15
ZmGLP4-13
ZmGLP4-8
ZmGLP4-5
ZmGLP4-6
ZmGLP4-16
ZmGLP4-19
ZmGLP4-3
ZmGLP4-2
SbGLP7-6
SbGLP7-4
SbGLP7-11
SbGLP-unk
ZmGLP4-1 1
BdGLP3-6
ZmGLP4-10
ZmGLP4-14
OsGLP12-4
SbGLP7-9
SbGLP7-2
SbGLP7-5
SbGLP7-7
SbGLP7-12
ZmGLP10-5
ZmGLP10-4
ZmGLP10-1
ZmGLP10-3
ZmGLP10-2
SbGLP7-1
OsGLP12-2
OsGLP12-1
OsGLP12-3
OsGLP8-6
OsGLP8-9
OsGLP8-8
OsGLP8-10
OsGLP8-7
OsGLP8-5
OsGLP8-11
BdGLP3-2
BdGLP3-3
BdGLP3-1
BdGLP3-5
HvGER4d
BdGLP3-8
BdGLP3-7
BdGLP3-4
OsGLP8-12
OsGLP8-3
OsGLP8-4
SbGLP6-1
ZmGLP2-1
BdGLP2-1
HvGER3a
OsGLP8-2
OsGLP8-1
HvGER1a
HvGER5a
HvGER6a
AT1G02335
HvGER2a

GER4

GER3

Fig. 3 Maximum Parisomy phylogenetic tree (100 bootstraps with sequence order re-arranging over 100 data sets) of the genes in the 'hypothetical grass post-duplication gene' family (Cluster 22740912) from the Joint Genome Institute (JGI)/Center for Integrative Genomics (CIG) comparative genomics website (http://www.phytozome.net). The six barley germin genes (HvGER1a, HvGER2a, HvGER3a, HvGER4d, HvGER5a and HvGER6a) that characterise the subfamily classification (Druka et al. 2002; Zimmermann et al. 2006; Manosalva et al. 2008) are boxed and a GLP from *Arabidopsis thaliana* (At1g02335) were used as comparison (*boxed* sequences). All other genes contained within the family were coloured according to their species with *Sorgum* in *green*, *Brachypodium* in *orange*, rice in *blue* and maize in *red*. Low bootstrap values are coloured *red* on the tree branches

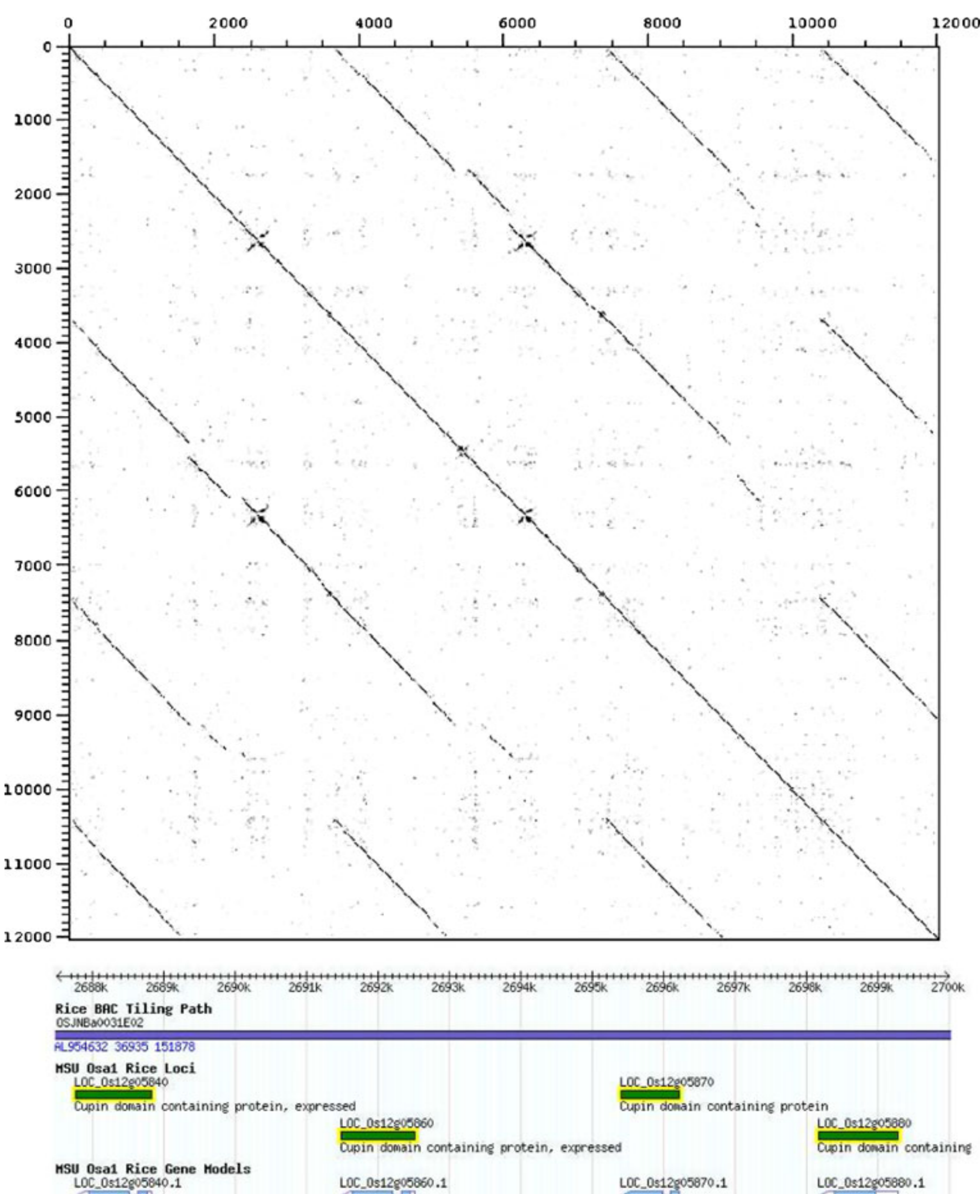

Fig. 4 A self dot-plot of a 12 kb sequence on rice chromosome 12 (2, 687, 500-2, 699, 500 bp) that contains four GLP genes. Located below the figure is the corresponding sequence annotation from the MSU (formerly TIGR) rice genome annotation database (Ouyang et al. 2007). The four GLP genes are coloured in *green* and highlighted in *yellow* and the *red bars* indicate repeats from the Oryza repeat database (Ouyang and Buell 2004)

than the smaller chromosome 12 cluster that contained only four genes (Manosalva et al. 2008). This four-gene cluster over 12 kb shows a duplicated nucleotide sequence pattern (Fig. 4), with the duplications likely to be recent and less likely to have evolved new functional attributes. Tandem duplication of genes from the closely related OXO cupin subclasses has also been reported (Carrillo et al. 2009).

The most recent GLP cluster identified in barley defined an interesting cyclic gene 'birth and death' scenario for the GLP genes within a cluster (Himmelbach et al. 2010) instead of a typical 'pseudogenisation' affect on multiple gene duplicates at a single loci (Ohno 1970). Mutations within regulatory elements showed to enhance transcript dosage and functional redundancy by pathogen-induced promoter activity. Conserved regulatory elements in GLP promoter sequences have also been shown to be responsive to environmental stresses and growth factors (Mahmood et al. 2010). The evolutionary driving force behind localised gene expansion, suggested in Himmelbach et al. (2010), was the enhancement of transcript dosage encoding functionally redundant proteins in a robust manner rather than the diversification of local genes paralogs.

The syntenic GLP cluster region on maize chromosome 4 also contains multiple QTLs for diseases like ear and stalk rot, common smut, common rust, southern rust, gray leaf spot and northern corn leaf

blight (Wisser et al. 2006). Rice GLPs and OXO gene clusters were also shown to co-localise with multiple published QTLs, with the exception of the four-gene chromosome 12 gene cluster (Davidson et al. 2009). These regions outlined in Davidson et al. (2009) are associated with phenotypes such as bacterial blight, brown plant hopper, leaf and neck blast, yellow mottle virus, sheath rot and sheath blight.

GLP/OXO gene clusters were identified to be located in the boundaries of multiple QTLs on chromosome 8 (Manosalva et al. 2008) and chromosome 3 (Liu et al. 2009; Ramalingam et al. 2003; Sirithunya et al. 2002; Wu et al. 2004; Zou et al. 2000; Tabien et al. 2002; Pinson et al. 2005; Chen et al. 2003). The rice chromosome 3 multiple QTL region is syntenic to maize chromosomes 1 (distal region of both arms), 5 (distal short arm) and 9 (distal long arm; from Salse et al. (2008), all of which contain multiple published QTLs (Wisser et al. 2006). These regions, along with regions where single GLP/OXO genes are located in multiple QTL regions, are good candidates for future disease resistance (Davidson et al. 2009). And despite large amounts of segmental genome variation through duplications, chromosome fusions, and translocations since originating from their ancestral genome 90 MYA, there is a high degree of synteny between ancestral genome segments in cereal genomes (Salse et al. 2008). So by combining metagenome analysis with GLP cluster information, research from model organisms such as rice and maize may be transferred to organisms whose genomes are historically difficult and complex to sequence (Gill et al. 2004; Schulte et al. 2009).

Wheat and barley are two such genomes which are difficult to sequence due to a high proportion of repetitive DNA. GLPs contained within the GLP clusters located on Sorghum chromosome 7 and maize chromosome 4 are all separated by large stretches of repetitive DNA, most notably LTR Retrotransposons, which increase the length (and numbers of genes) of the GLP clusters in both species (355 kb in Sorghum and 405kb in maize; Table 2) compared to the syntenic rice and Brachypodium clusters. Transposable elements (TEs), found in high copy numbers within plant genomes such as wheat, maize and barley, may also be responsible for duplications of GLPs. TE-mediated gene sequence movement has been shown in maize by CACTA DNA Transposons (Li et al. 2009) and *Helitron* elements (Lai et al. 2005). Localised gene duplications of GLPs in TE-rich sequence has also been identified on a disease resistance loci in wheat (J. Breen and R. Mago, unpublished data).

The presence of TEs and smRNAs around GLP clusters may also affect the regulation of each gene within the GLP cluster. It would be speculative to suggest that an epigenetic affect, such as the affect of tandem repeats at the paramutation loci in maize (Chandler and Alleman 2008), may play a role in localised gene duplications of GLPs. Such a mechanism may account for the clustering of GLPs in cereals and the affect of QTLs to provide durable plant resistance to pathogen attack. Small RNA regulation has already been shown to play important roles in disease resistance gene clusters in *Arabidopsis thaliana* (Yi and Richards 2007). Davidson et al. (2009) scanned small RNA libraries and found instances of regulation of duplicated GLP genes. Within the GLP cluster at the QTL region on chromosome 8 (Manosalva et al. 2008), an insertion within the promoter sequence of OsGLP8-6 (a GER4 family member) enhanced expression in two resistant rice (ssp. indica) cultivars, while higher expression of OsGLP8-12 (a GER3 family member) was identified in susceptible cultivars (Davidson et al. 2010). The authors in Davidson et al. (2010), along with small RNA data in Davidson et al. (2009), suggest regulation through both small RNAs and regulatory domain variation are responsible for the variation of expression in the important GER3 and GER4 subfamilies in certain tissues.

Conclusion

Studies into QTLs conferring durable and/or broad-spectrum disease resistance phenotypes in cereals are an important step in crop improvement. A recent publication identifying a genomic GLP cluster linked to a disease resistance QTL in rice, along with other studies identifying their defensive actions, have highlighted the importance of this gene family. The large increase in sequence data from plant genomic sequencing projects, especially important cereal crops, has allowed researchers to identify candidate disease resistance hotspots and merge with current QTL mapping data.

In this mini-review we consider that GLP genes are involved in plant defence and reviewed recent literature that discusses possible mechanisms of non-race specific disease resistance. GLP clusters (three or more GLPs found in close proximity to each other) are common in cereal genome sequences and they could provide new opportunities to increase non-race specific disease resistance in crop species. The four major syntenic GLP clusters in maize, *Sorghum*, *Brachypodium* and rice are also interesting models in comparing how gene loci evolve and respond to pathogens. The interplay between localised gene duplications, small RNAs,

TEs and regulatory elements in promoter sequences could interact to affect GLP expression and its ability to defend plants cells using a broad-level and durable resistance mechanism.

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