DISSERTATION

FUNCTIONAL CHARACTERIZATION OF GERMIN FAMILY GENES CONTRIBUTING TO BROAD-SPECTRUM, QUANTITATIVE DISEASE RESISTANCE IN RICE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY REBECCA M. DAVIDSON ENTITLED FUNCTIONAL CHARACTERIZATION OF GERMIN FAMILY GENES CONTRIBUTING TO BROAD-SPECTRUM, QUANTITATIVE DISEASE RESISTANCE IN RICE BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FUNCTIONAL CHARACTERIZATION OF GERMIN FAMILY GENES CONTRIBUTING TO BROAD-SPECTRUM, QUANTITATIVE DISEASE RESISTANCE IN RICE

Quantitative trait loci (QTL) are predicted to confer broad-spectrum and durable disease resistance. Practical application of disease resistance QTL in crop improvement programs has been hindered because we lack several levels of knowledge, including an understanding of (1) the genes contributing to the QTL-governed phenotype and (2) why certain alleles of those genes are more effective than others in conferring resistance. Two candidate defense response gene markers of the germin protein family, germin-like protein (GLP) and oxalate oxidase (OXO), co-localized with disease resistance QTL on chromosome (chr) 8 and 3, respectively, in several rice mapping populations. In this study, QTL-associated GLP and OXO genes were identified in the rice genome and their functions were tested with respect to biotic and abiotic stress responses. Paralogous multi-gene families underlie the physical QTL regions, with twelve *OsGLP* members on chr 8 and four *OsOXO* members on chr 3. Based on shared motifs in 5' regulatory regions and/or protein sequence similarities to cereal orthologues, rice *OsGLP* genes belong to two germin subfamily groups (GER3 and GER4), and *OsOXOs* belong to the GER1 group.

Conserved sequences for each gene family were used for gene silencing experiments by RNA-interference. As more *OsGLP* genes were silenced, the more susceptible the plants were to two distinct fungal pathogens, *Magnaporthe oryzae* (*Mo*) and *Rhizoctonia solani* (*Rs*). Similarly,

OsOXO-RNAi plants showed enhanced susceptibility to *Mo*, *Rs* and the broad host range pathogen, *Sclerotinia sclerotiorum*.

OsGLP alleles were compared in resistant (+chr8 QTL) and susceptible (-chr8 QTL) parental rice lines. Cultivar-specific combinations of *OsGLP* genes were constitutively expressed and transiently induced by both wounding and *Mo* infection. In agreement with the silencing data, expression profiles suggest that multiple members of the GER4 subfamily are involved in rice defense response. Transient induction occurred early in the infection process before fungal penetration of the plant cuticle, and differential expression between resistant and susceptible cultivars correlated with differential hydrogen peroxide accumulation after fungal infection and abiotic stresses.

Gene silencing data confirms the roles of *OsGLP* and *OsOXO* as contributors to broad-spectrum, basal disease resistance in rice. Studies of allelic diversity among rice varieties suggest that regulation of *OsGLP*s may explain the effectiveness of resistant alleles compared to susceptible. Germin family proteins are encoded by diverse gene families in rice and across plant taxa and are developmentally regulated. The germin subfamily members studied here have acquired functions in broad-spectrum defense responses and are important loci for crop improvement.

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CHAPTER 1

Introduction

1.1 Rice: An important agricultural crop and model monocot species

Rice is one of the most important food crops and is consumed by half the world's population daily [1]. Domesticated rice originated from the wild species *Oryza rufipogon* in two separate events around 5-10 thousand years ago. One occured in Africa and resulted in the regionally grown species ,*O. glabberima*. The another in Asia resulted the widely grown rice, *O. sativa* [2]. *O. sativa* species can be further subdivided into two subspecies, *japonica* and *indica*. Japonica varieties, also known as sticky rices, are grown in temperate climates and upland, high-elevation regions. By contrast, *indica* varieties are generally non-sticky and are grown in tropical climates at low elevations, and sometimes in flooded conditions. Rice is a unique grain crop because it is consumed almost exclusively by humans as opposed to use as animal feed.

Rice is a model species for monocot plants including cereals such as wheat, barley, oats, rye, maize and sorghum, because of its relatively small genome (approximately 370 million bases, Mb). It is about half the size of the sorghum genome (736 Mb), one-sixth of the maize genome (2500 Mb), and one-fiftieth of the wheat genome (15000 Mb) [3]. There are two fully sequenced genomes of rice including the reference *japonica* cultivar, Nipponbare [4, 5] and thereference *indica* cultivar, 93-11 [6]. Two high quality annotations of the Nipponbare genome [7-10] estimate 57,000 genes in rice. Genome-wide transcriptome datasets for multiple rice tissues and stress treatments are currently available [11]. There is remarkable collinearity

among cereal genomes in gene order and chromosome organization [12]. The rapid advances in rice functional genomics over recent years provide promise of knowledge transfer to related cereal crops for which there are no genome sequences [13]

Rice is grown and consumed mostly in Asia where the population is expected to grow by one-third in the next fifty years [14]. This will require an increase in rice production by 50% using, at best, the current acreage of agricultural land and ever decreasing amounts of irrigation water [1]. To accomplish this, rice plants that are more productive with lower inputs and less susceptible to environmental stresses than current varieties are needed. This will most likely be achieved by utilizing the natural variation that exists among rice varieties from diverse geographic regions and by exploiting genes from wild rice varieties [15, 16].

1.2 Fungal Diseases of Rice

In rice production, losses are mainly due to reductions in yield as a result of abiotic and biotic stresses. The major abiotic stresses of rice include salt, drought and submergence. There are a number of biotic stresses affecting rice including weeds, insect and animal pests and pathogens such as fungi, bacteria and viruses. Economic impacts of all biotic stresses include yield losses in the range of 20-50%, and losses to pathogens alone are in the range of 7-16% [17, 18]. The most important fungal diseases of rice include leaf and panicle blast caused by *Magnaporthe oryzae* (formerly known as *Magnaporthe grisea*), and sheath blight caused by *Rhizoctonia solani*. Both fungi have distinct infection strategies and disease phenotypes on susceptible hosts.

M. oryzae is a hemibiotrophic ascomycete fungus whose conidial spores spread by wind, rain or water [19]. Humidity and proper temperatures are required for attachment of the spore to the leaf surface [20]. The fungus forcibly enters the plant cuticle using a penetration peg that emerges from a specialized structure called an appresorium. Then, in its biotrophic phase, *Mo* is

disguised from plant recognition by a surrounding plant endomembrane that encases its hyphae as it grows through extracellular spaces and plasmodesmata [21]. Next, it enters a necrotrophic phase, and the growing hyphae spread throughout leaf tissue by penetrating the cell walls of adjacent cells [21]. *M. oryzae* causes characteristic blast symptoms on leaves and panicles that are diamond shaped necrotic lesions. The lesions often spread to kill entire leaf and/or flower structures resulting in reductions in grain yield.

R. solani, the causal agent of sheath blight, is a necrotrophic basidiomycete fungus with a broad host range. Isolates are classified by anastomosis groups (AG) and those that infect rice belong to the AG-1 group [22]. When rice fields are flooded, durable fungal structures called sclerotia dislodge from the inoculum source and float in the water. After fields are drained, sclerotia that are in contact with rice sheaths at the soil level infect the plant tissue. *R. solani* germinates and forms an infection cushion that differentiates into a specialized structure called a lobate appresorium [23]. The precise mechanism of entry by *R. solani* into the plant is unknown, but the fungus likely secretes cell wall-degrading enzymes as it grows upward on the sheath and causes necrosis. Several isolates of *R. solani*, including those in the AG-1 group, produce a host-specific toxic [24, 25]. The purified toxin causes similar disease symptoms as the fungal infection; however, it is not clear whether it is a pathogenicity factor required by the fungus to cause disease [24].

1.3 Plant immune responses to fungal pathogens

Plants are immobile organisms that cannot flee when confronted by pathogens. Therefore, they must alter the local environments at sites of infection using a complex and layered defense system [26]. Some defense mechanisms are specific to particular isolates of one microbial species while others provide broad-spectrum, general protection against multiple isolates of one species or toward diverse pathogen species. The first layers of defense are

preformed morphological barriers, e.g., the plant cuticle and other components that cover the plant surface. These preformed physical barriers are often factors in determining host and non-host specificities and can determine whether a fungal spore, for example, can successfully attach and penetrate into the tissue [20].

The next layers of plant defense are inducible responses that may be specific to a plant species or cultivar, but are non-specific in their pathogen targets [26]. These non-specific, inducible defenses can be triggered by the perception of conserved microbial features called pathogen-associated molecular patterns (PAMP), and subsequent defense responses are called PAMP-triggered immunity (PTI). Downstream responses of PTI include induction of pathogenesis related (PR) genes, production of reaction oxygen species (ROS) and deposition of callose in cell walls, all of which impede invading pathogens [27, 28]. The ability of plants to perceive PAMPs may dictate their level of basal disease resistance.

The most specific type of resistance is called effector triggered immunity (ETI). This resistance was recognized previously as gene-for-gene resistance [29] and occurs when pathogen-produced effector proteins, encoded by avirulence (avr) genes, are injected into the plant cell and are recognized by corresponding resistance proteins (R-gene products) in the host. The result is an incompatible interaction wherein disease progress is arrested by host defense responses that often include a hypersensitive response (HR) or programmed cell death. Many of the downstream defense responses of ETI are similar to those elicited by PTI, however, the kinetics of induction are different. In the absence of either the Avr protein or the corresponding R-protein, the result is a compatible, disease interaction.

1.4 Genetics of disease resistance in rice

Successful plant disease infection requires three factors: a virulent pathogen, a susceptible host and an environment suitable for disease [19], and any of these components can

be manipulated for disease control. For example, pathogen populations can be subdued by pesticide applications. This can be costly to farmers and may have negative environmental impacts. Additionally, excessive pesticide use puts selective pressure on pathogen populations toward pesticide resistance. Pathogen inoculum levels can also be decreased in the field by altering environments thorough crop rotations, sanitation, watering methods and low crop densities. These methods alone are unsatisfactory, however, because susceptible hosts will still become infected after any contact with virulent pathogens. In addition, genetically narrow monocultures grown continuously on large acreages are particularly vulnerable to low levels of inoculum [18]. An important tool in the disease management arsenal is to exploit the natural diversity in disease resistance/susceptibility that exists among plant varieties to develop disease resistant cultivars.

Traditionally, plant breeders have relied on single gene resistances to control rice diseases because they are easy to identify and track by segregation analyses. Nine resistance genes (R-genes) that control particular strains of *M. oryzae* have been cloned to date, and many more have been genetically mapped [30]. These have been largely unsuccessful with respect to longevity due to the diverse population structure of *M. oryzae* isolates in the field [18]. R-genes correspond to the ETI type of resistance which means that the resistance is often complete, but is only effective toward one or a few *M. oryzae* isolates. Once the targeted fungal isolate(s) evolve to avoid R-gene recognition, the R-gene is 'broken down' or ineffective against the new fungal population. The breakdown of R-genes in the field can occur in as little as 2 to 5 years after release which is a difficult problem because new cultivars take between 5 and 20 years to generate.

The situation for breeders trying to protect rice from sheath blight is even more critical, because there have been no sources of single gene resistance identified to control *R. solani* [18].

This is a common situation for necrotrophic pathogens as they are believed to exploit the hypersensitive cell death response by entering the plant through the dead cells [31].

An alternative to single gene resistance is quantitative resistance which is controlled by multiple genes or quantitative trait loci (QTL) contributing additive effects. Quantitative resistance is more difficult for the pathogen to overcome compared to a single R-gene resistance due to the multiple layers of partial resistance mechanisms. In addition, because each QTL encode a component of resistance, they are predicted to put limited selection pressure on pathogen populations [31]. Few disease resistance QTL have been cloned to date resulting in a great deal of speculation regarding the types of genes conferring partial resistance [32]. Postulations about mechanisms of QTL-based resistance include genes involved in morphology/development, basal defense or PTI, chemical warfare or signal transduction [31]. Another hypothesis is that QTL contain weak forms of R-genes. Knowing what genes confer partial resistance is highly advantageous to plant breeders. These genes are perfectly linked to phenotypic outcome, and can be directly targeted for development of molecular markers and utilized in marker assisted selection [33].

One strategy for uncovering genes functioning in QTL-based resistance is the candidate defense response (DR) gene approach [34]. Putative DR genes are believed to be downstream components in the defense pathway and they encode structural proteins, enzymes of secondary metabolism, enzymes directly involved in defense, or regulatory proteins [35] . Polymorphic candidate DR gene markers were used in rice, wheat and maize QTL mapping studies to test hypotheses that inducible downstream DR genes are associated with partial resistance to fungal pathogens conferred by QTL [36-40]. Indeed, many DR gene markers are significantly associated with reductions in disease symptoms among the three diverse crop species.

Genes of two previously described DR genes from barley, oxalate oxidase (OXO) and germin-like protein (GLP), were amplified and used as restriction fragment length polymorphism (RFLP) markers [36]. The GLP marker mapped to rice chromosome (chr) 8, and, in two mapping studies, co-localized with QTL intervals that contributed over 30% of the variation in rice blast diseased leaf area [37, 41]. This region corresponds to one of the top ten candidate QTL for broad spectrum rice blast resistance in a meta-analysis of rice disease QTL [30]. The OXO marker mapped to the end of chr 3 and was associated with a reduction in rice blast disease symptoms [38]. This chr 3 region corresponds to QTL for sheath blight and bacterial blight resistance [36]. Regions of overlapping disease QTL in rice have been investigated by various methods [30, 42]. However, only coarse genetic maps are available for predicting physical QTL coordinates. The physical QTLs regions predicted from these genetic maps range in size from 100 Kb to 3 Mb and contain hundreds of genes potentially responsible for the phenotypes observed. Assumptions that associated candidate DR genes, such as GLP and OXO, contribute to QTL-based resistance must be validated in rice with functional studies. Once accomplished, molecular pathologists will have confidence in predicting DR genes for use in breeding programs. More broadly, we can begin to unravel the complex interplay of multiple loci influencing broad-spectrum disease resistance.

1.5 Scope of dissertation

Proteins in the germin family, including GLP and OXO, have been studied in a variety of crop species, but have not been fully examined in rice. Both are DR genes in the closely related cereals, barley [43-45] and wheat [44, 46], and are genetically linked with QTL for disease resistance in rice. The overall goal of the study is to functionally validate these genes as contributors to QTL-based, fungal disease resistance in rice.

The goals of my dissertation research are:

1) To expedite identification of candidate genes underlying QTL for crop improvement traits (Chapter 2 and Appendix I) by combining multiple data sources including gene and protein sequences, gene expression, single nucleotide polymorphism (SNP) and QTL data. My focus was a bioinformatic study of germin family proteins across diverse genera of plants to predict candidate DR genes in rice (Chapter 2). My approach is to perform phylogenetic reconstruction and functional analyses of all 40 rice germin family proteins along with 49 characterized germin proteins from other plant taxa. I also suggest putative roles for small RNAs in regulating GLP gene expression and show physical locations of germin family proteins relative to disease resistance QTL.
2) To functionally analyze the allelic diversity of 12 rice GLP gene family members that co-localize with major effect disease resistance QTL on chr 8. I accomplish this through gene silencing studies (Appendix II), gene and promoter sequence analyses and gene expression profiling after disease and abiotic stress treatments (Chapter 3). Moreover, I study the subcellular localization of rice GLP proteins and compare the regulation of GLP genes to other early physiological stress responses.
3) To test for the involvement of a cluster of four rice OXO genes in broad-spectrum, QTL-based disease resistance using a gene silencing approach and advanced breeding materials (Chapter 4). I study the correlation between OXO gene silencing and increased susceptibility to fungal pathogens using populations of transgenic lines carrying constructs designed to silence all family members by RNA interference (RNAi)., Further, I confirm the role of OXO in QTL-based resistance by studying co-segregation of disease phenotypes with genotypes in advanced backcross lines.

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CHAPTER 2

Germins: a diverse protein family important for crop improvement

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Abbreviations: GLP, germin-like protein; OXO, oxalate oxidase; SOD, superoxide dismutase; chromosome, chr; QTL, quantitative trait loci

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R. Davidson performed bioinformatics analyses of germin family gene/protein expression, small RNAs putatively targeting germin family genes, and germins relative to quantitative trait loci. R. Davidson wrote the manuscript and constructed the figures. P. Reeves contributed to the phylogenetic analysis and interpretation and P. Manosalva contributed to the small RNA and disease sections.

ABSTRACT

The germin protein family is comprised of two main subgroups in plants, oxalate oxidases (OXO) and germin-like proteins (GLP). These proteins are implicated in a variety of plant processes including germination, development, pollen formation, and response to abiotic and biotic stress. Here, we examine the phylogenetic relationships and functional diversity of the germin gene family across diverse genera, and then focus on rice (*Oryza sativa*) as a model. In general, germin genes are expressed in all tissue types and are induced by biotic and/or abiotic stresses. In rice, many of the stress induced genes physically co-localize with quantitative trait loci (QTL) for disease resistance, and emerging evidence suggests that small RNAs may regulate their transcript abundance. We analyze the extensive sequence, gene expression and functional data for germin family proteins and relate them to QTL map locations to predict additional candidate germin genes for future crop improvement.

Keywords: Germin-like protein, oxalate oxidase, superoxide dismutase, biotic and abiotic stress, quantitative disease resistance

1. Introduction

The germin family comprises a group of proteins belonging to the cupin superfamily. All germins contain the characteristic germin motif that gives rise to a jellyroll β-barrel structure involved in metal binding [1, 2]. The term "germin" was coined after a family member was initially identified as a specific marker for germination in wheat embryos; that member was later identified as a homohexamer glycoprotein with oxalate oxidase activity [3-5]. Proteins with an average of 50% identity to this wheat germin, that also contained the germin motif, were found in several plants other than cereals and were designated germin-like proteins (GLPs) [1, 6]. Most share biochemical attributes such as resistance to extreme heat and denaturing agents, homo-polymer formation, glycosylation and cell wall localization, though they differ in their tissue specificities and enzyme activities [3, 7-10]. Germin family proteins have been widely studied in crop plants due to their diverse roles in important plant processes [1]. We focus this review on two major groups within the germin protein family, oxalate oxidases (OXO) and germin-like proteins (GLPs).

OXOs, also referred to in the literature as "true germins", possess oxalate oxidase activity and are the products of a highly homogenous group of gene homologues present only in true cereals [3, 11-13]. Proteins with oxalate oxidase activity have also been discovered in beet, spinach, banana and sorghum, however, the limited evidence suggests they are distinct proteins from the germin-type OXOs [14-16]. Potential substrates for the OXO enzyme are endogenously produced oxalic acid (oxalate) and calcium oxalate, an insoluble crystal form stored in plant vacuoles and cell walls [17]. Oxalate is also a known pathogenicity factor produced by *Sclerotinia* fungi [18]. Hydrogen peroxide (H_2O_2) is one of the two products of the reaction catalyzed by OXO and has been implicated in signaling during abiotic and biotic stresses [4, 17, 19]. *OXO* gene expression is detected in both epidermal and mesophyll tissues [20]. The OXO proteins contain

N-terminal secretion signals and are extracted from soluble and cell wall plant protein fractions, suggesting that these proteins are secreted into the apoplast [9, 21, 22]. Germins with OXO activity are found only in cereals, suggesting that they may play unique roles in calcium regulation, oxalate metabolism, and response to pathogenesis.

GLPs are encoded by a heterogeneous group of genes present in many land plants including monocots, dicots, gymnosperms and moss. The diversity of possible functions and enzymatic activities for GLPs is reflected in the many synonyms for names found in the literature, including oxalate oxidase-like proteins, nectarins, rhicadhesin-like receptors and ADP glucose pyrophosphatase/phosphodiesterase (AGPPase) [1, 7, 11, 21, 23, 24]. GLP is a term referring to all germin motif-containing proteins with unknown enzyme activity or those that do not possess oxalate oxidase activity [1]. Many GLPs, including those from moss (*Barbula unguiculata; Bu)*, barley (*Hordeum vulgare; Hv*), wheat (*Triticum aestivum, Ta; Triticum monococcum,Tm*), tobacco (*Nicotiana sp.; Na* or *Np*), pea (*Pisum sativum; Ps*), azalea (*Rhododendron mucronatum; Rm*) and grape (*Vitis vinifera; Vv)* possess superoxide dismutase (SOD) activity [24-29]. SOD converts superoxide anions into H_2O_2. One GLP from barley does not possess OXO or SOD activity, but, instead, has ADP glucose pyrophosphatase/phosphodiesterase activity and catalyzes the hydrolytic breakdown of activated monosaccharides [7, 20]. GLP transcripts were detected primarily in epidermal tissues. Native and recombinant GLP proteins were isolated from cell wall fractions of protein extracts [20, 27, 29, 30]. Like OXO proteins, GLPs also contain N-terminal secretion signals, and the targeting of these proteins to the cell wall and extracellular matrix [30] is consistent with their putative roles in cell wall expansion and response to environmental stress [8, 10, 22, 29, 31, 32].

Enzyme activity and gene expression data suggest that both *OXO* and *GLP* genes and their encoded proteins play roles in plant defense responses. Barley and wheat *OXO* gene

expression and enzyme activity were induced in response to powdery mildew infection [9, 33]. A family of *OXO* genes from ryegrass is associated with wound-induced H_2O_2 accumulation [12]. In rice (*Oryza sativa, Os*), there is a strong genetic association between a cluster of four *OXO* genes and rice blast resistance [34]. Cultivar-specific induction of one *OsOXO* gene was observed after infection with *Magnaporthe oryzae,* the rice blast pathogen [11]. Rice *GLP* genes, including a tandemly duplicated cluster of seven members, are differentially induced in rice leaves after inoculation with *M. oryzae*, and contribute collectively to disease resistance against two very different fungal pathogens, *M. oryzae* and *Rhizoctonia solani* [35]. *GLP* induction after fungal infection has also been observed in barley and wheat leaf tissue [20, 22, 26, 33], barley spikelets [36], and grape leaves and fruits [29]. Finally, oxalic acid produced by *Sclerotinia* fungi suppresses the oxidative burst in host plants and promotes pathogen ingress and disease [37]. Resistance to *Sclerotinia*-induced diseases was enhanced by over-expressing monocot *OXO* alleles in dicot crop plants such as peanut, sunflower, tobacco and rapeseed [38-41]. Because *OXO* and *GLP* genes play important roles in general defense against a wide variety of fungal pathogens, they are desirable candidates for utilization in crop improvement programs.

OXOs and GLPs are encoded in large gene families in barley, wheat, rice, maize, ryegrass, grape and *Arabidopsis thaliana* [11-13, 29, 42, 43]. Given the potential importance of these genes to disease resistance and crop improvement, this review aims to elucidate commonalities in functional attributes of rice germins and well-studied germins from other crop plant species. First, we describe phylogenetic similarities among 90 germin family proteins from 17 plant species with regards to developmental deployment, stress-response and catalytic activity. Next, focusing on rice, we use genome-wide expression data to explore germin gene expression profiles during diverse stress responses. By combining information on the physical location of germin genes relative to disease resistance QTL and differences in their stress-

response profiles, we predict the utility of these genes for crop improvement. The predictive power was further improved by adding comparisons of functionally characterized germins across crop species, including cereals such as barley, wheat and maize.

2. Developmental regulation of germin expression

2.1 Diversity of germin gene expression among tissue types

Germins have been studied in many plant species in a multitude of contexts. A meta analysis of transcript data for all 41 rice genes plus an additional 48 genes from 17 other species from 19 published studies was performed to explore the diversity of germin gene expression in crop plants with respect to tissue specificity and stress responsiveness (Fig. 1; Tables 1 and 2). Two additional data sources were used for the rice germins, the Massively Parallel Signature Sequencing (MPSS) Rice Expression Atlas (http://mpss.udel.edu/rice/; University of Delaware, [44] and the Rice Genome Annotation Digital Northern database (http://rice.plantbiology.msu.edu/dnav.shtml; Michigan State University, [45]. The MPSS database allows genome-wide quantitative assessment of mRNA transcripts or small RNAs by identifying short sequence signatures that can be used to estimate relative gene expression. The MPSS signatures uniquely identify >95% of all rice genes in 80 normalized MPSS transcript libraries encompassing various tissue types and stress treatments [44]. Most of the transcript data were derived from the reference rice cultivar, Nipponbare. Among all MPSS libraries, expression levels were above five transcripts per million (TPM) for 27 of 41 rice germins. The Rice Genome Annotation Digital Northern database is an annotated, curated collection of rice ESTs from many cultivars [45]. Using their Rice Gene Expression Anatomy Viewer, EST abundances, by gene locus identifier (Table 2), were obtained for four tissue types for 26 of 41 rice germins. There was no evidence of transcription for six rice germins in either database.

Among the 89 germin family genes considered, transcripts were observed for genes under non-stress conditions in all major plant organs including seeds, flowers, leaves and roots (Fig. 1). Most germin genes (74%) are expressed in leaf tissue including cotyledons, shoots, young and mature leaves, and somewhat fewer are expressed in root tissue (61%) including young and mature roots. Relatively fewer germins are expressed in flowers (29%) and seeds (45%). Most germins are constitutively expressed in more than one tissue type. Four genes (*AtGer2*, *VvGLP5*, Os03g59010 and *OsOXO1*) were expressed exclusively in reproductive tissues, and two (*AtGLP5* and Os03g08150) were expressed only in leaf tissue.

The diversity of germin family gene expression among plant tissue types is consistent with their potential roles in tissue expansion and growth, from seed to flower. One third of rice germins, including members in all major phylogenetic groups, are expressed in developing and/or germinating seeds. This corresponds to the early discoveries and naming of germins as markers of seed germination [1]. Although initially discovered in cereals, germins are expressed in developing siliques of *A. thaliana* [32], the skin and pulp of grapes [29] as well as expanding cotton (*Gossypium hirsutum, Gh*) fibers [46]. Germins are expressed in expanding shoots of developing seedlings, including young whorl leaves of maize [47], cotyledons of *Pharbitis nil* (*Pn,* [48]), and young leaves of rice, barley and grape [20, 29]. Germin genes are expressed in mature leaf tissue of most plants, and also in root tissue of rice, barley [20], grape [29], pepper (*Capsicum annum, Ca*, [31]), *Nicotiana attenuate* (*Na,* [49]), pea (*Ps,* [27]) and alfalfa (*Medicago truncatula, Mt,* [50]). Finally, only 13 rice germins are expressed in floral tissues including mature pollen, ovary/mature stigma and immature panicles, and two are flower specific (Os03g59010, *OsOXO1*). Five of the 13 are related to nectarin 1, encoded by *NpNEC1,* from *Nicotiana sp.* nectarin 1 produces H_2O_2 in the nectar of tobacco flowers (Fig. 1, brown clade)[24].

The unique combinations of germins expressed among rice tissue types suggest that particular suites of genes contribute to distinct developmental processes.

2.2 Can phylogenetics predict functional roles of germin family proteins?

Phylogenetic relationships were analyzed for the germins discussed above to determine if functional roles are conserved among germin gene lineages [51-53](Fig. 1; Tables 1 and 2). The tree suggests a complex evolutionary history with recent gene duplications. Thus, it is difficult to identify orthologs of particular rice germins in other species. However, subfamilies of germin genes are described for rice and barley [20, 35]. Within subfamilies, which we expand to include germin sequences from additional taxa (Fig. 1), relationships among gene lineages are roughly congruent with established phylogenetic relationships among taxa. For example, relationships among gene lineages in the GER1 subfamily (Fig. 1, green clade) correspond closely with phylogenetic relationships among rice, ryegrass, barley, and wheat [54, 55].

There is suggestion that tissue specific expression patterns are conserved within broadly defined subfamilies. Members of the GER3 subfamily (Fig. 1, red clade) are expressed in all tissue types in both rice and barley, with only a few exceptions [20, 35]. Similarly, expression of GER4 subfamily genes from barley, rice and wheat (Fig. 1, purple clade) is largely restricted to vegetative tissue. However expression patterns are highly variable within the majority of clades. This suggests that, over evolutionary time, germins with similar or identical catalytic properties were recruited for new uses via deployment to different tissues or altered time of expression. The four predicted rice oxalate oxidases of the GER1 subfamily (Fig. 1, green clade) are an excellent example. *OsOXO1* is only expressed in flowers, *OsOXO2* is not expressed in any of the four tissues assayed, *OsOXO3* is expressed in leaves and roots, and *OsOXO4* is expressed in leaves, roots and seeds. Only *OsOXO4* is induced by stress (Fig. 2, [11]). Diversity in expression

patterns may reflect divergence in regulatory sequences and cis-acting elements that direct expression to specific tissues [56].

GLPs from seven species, including the moss *Barbula unguiculata*, have SOD activity [20, 24-29]. These sequences occur in the GER4 and GER5/6 subfamilies as well as two additional major clades in the gene tree (Fig. 1). SOD activity was observed in divergent germin lineages including the base of the gene tree that was determined by a relaxed molecular clock method. Moreover, we can infer that a germin with SOD activity existed in the common ancestor of bryophytes and angiosperms. Hence, SOD activity is an ancient gene function within the germins that has persisted at least since the early diversification of land plants. The precise ancestral function of the germin gene products, however, cannot be definitively concluded. A distinct clade of genes, tentatively named here as GER2, is another early germin lineage. The catalytic activity of only one GER2 gene (*HvGLP1*) is known, and it has AGPPase activity [7]. Therefore, the possibility that AGPPase is the ancestral catalytic activity within the germins cannot be excluded.

Two proteins in the GER1 subfamily (Fig. 1, green clade) have OXO activity which is restricted to this major clade. OXO activity has only been experimentally demonstrated for the wheat and barley proteins [12, 57, 58]. However, the relationships among gene lineages within the GER1 subfamily reflect phylogenetic relationships among the taxa from which they were derived. Thus, it is reasonable to predict that other genes within the GER1 lineage will also exhibit OXO activity. Germins with oxalate oxidase activity have thus far only been found in true cereals suggesting a single origin of this catalytic activity at an early point during the diversification of cereals from other monocots. Based upon the gene tree, OXO activity appears to be a derived function that has evolved from ancestral genes with SOD activity, presumably following gene duplication.

The sugarbeet germin *BvGER165* is intriguing in that it is speculated to encode a protein with OXO activity [59]. Expression of *BvGER165* correlated with increased OXO activity and H_2O_2 production in stressed seedling tissue, though the protein was not purified and the specific enzyme activity has not been confirmed [59]. *BvGER165* would represent the first germin with OXO activity from a dicot if ultimately shown to possess OXO activity. Curiously, the terminal branch length of *BvGER165* in the gene tree was much longer than neighboring branches (Fig. 1, not shown) suggesting the possibility of accelerated evolution in this sugarbeet gene, consistent with parallel acquisition of a novel oxalate oxidase catalytic function.

BvGER165 belongs to the GER2 subfamily of *GLPs* (Fig. 1, pink clade). The related proteins GhGLP1 and HvGER2a tested negative for OXO and SOD activities [10, 20]. Another protein in this subgroup, barley HvGLP1, has a distinct AGPPase enzyme activity [7]. This barley protein was first identified in cell wall fractions of seedling tissue and was noted for its insolubility after stress inducing treatments [8]. Transient silencing of the related gene (*HvGER2a*) did not result in loss of resistance to powdery mildew infection [20]. Evidence from the GER2 subfamily, although limited, suggests that it is distinct from other germin subfamilies in both enzymatic function and possibly its role in the defense response.

3. Germins and broad spectrum disease resistance

3.1 Germins and stress responses in monocots and dicots

Germin gene expression is consistent with participation of the encoded enzymes in stress responses. Indeed, two-thirds of the germins in our analysis were induced by a variety of biotic (fungal, bacterial and viral infections, herbivore damage, mycorrhizal association) and abiotic (mechanical wounding, excess salt, drought, cold, iron and atmospheric nitrogen dioxide) stresses (Figs. 1 and 2).

OXO genes of the GER1 subfamily were responsive to stress induced by fungal pathogens, wounding and elevated osmotic conditions (Fig. 1, green clade). Barley *(HvGER1)* and wheat (*gf2.8*) *OXOs* are important for resistance to *Blumeria graminis,* and both *OXOs* have been expressed in dicots such as peanut, sunflower and oilseed rape to enhance resistance against *Sclerotinia* pathogens [9, 33, 38, 39, 41]]. One of a cluster of four rice *OsOXOs* was induced by the rice blast fungus, *M. oryzae* (*Mo*) [11], and the same gene showed induction by the bacterial blight pathogen (*Xanthamonas oryzae* pv. *oryzae, Xoo)* and salt stress in MPSS expression libraries. Wounding and other abiotic stresses induced rice (*Os*) and ryegrass (*Lp*) *OXOs* [12] (Figs. 1 and 2).

GLP genes from all major clades are induced by biotic or abiotic stresses. Three subgroups contain genes associated with broad-spectrum fungal disease resistance in grape, barley and rice (Fig. 1, blue, red and purple clades). In grape, several *VvGLPs* were differentially induced by three pathogens, *Erysiphe necator, Plasmopara viticola* and *Botrytis cinerea* [29]. Both barley *HvGER3* and *HvGER4* genes were induced in leaf tissue by *Blumeria graminis* f. sp. *hordei* [20], and *HvGER3* was upregulated in spikelets by *Fusarium graminaerum* [36]. Transient silencing of *HvGER4* genes resulted in loss of resistance to *B. graminis*, but silencing of *HvGER3* did not [20]. Silencing of closely related rice *OsGLPs* in the GER4 subfamilies conferred loss of resistance to *M. oryzae* and *R. solani* [35]. The GER4 family members are likely involved in basal defense responses based on their non-specific pathogen induction and the broad-spectrum loss of resistance in silenced mutants.

The other major groups of GLPs have different pathogen specificities and responses to abiotic stress (Fig. 1, light blue and brown). For example, pepper *CaGLP* was induced in leaf tissue by *tobacco mosaic virus* (*TMV*) and in resistant and susceptible interactions with *X. campestris* pv. *vesicatoria* (*Xcv;* [31]. Root specific *GLPs* in these subgroups include *MtGLP1*, a

gene induced by mycorrhizal infection, *PsGLP*, a pea gene detected in root nodules, and *NaGLP*, a gene important for protection against the herbivore, *Manduca sexta* [27, 49, 50]. As many as 13 rice *GLPs* are induced by abiotic stresses including drought, cold and salt. Similarly, rhododendron *GLPs*, *RmGLP1* and *RmGLP2*, were upregulated after treatment with the environmental pollutant nitrogen dioxide [28]. Finally, a wild wheat gene, *TmGLP*, in the GER4 subfamily, was induced by excess iron application; the authors speculate that the H_2O_2 produced by oxidative enzymes regulates cell wall iron homeostasis after pathogen attack [60].

Half (21 of 41) of all rice germins are induced by stress [44, 61](Fig. 2). Most were induced by both biotic and abiotic factors, though seven genes were induced only after pathogen infection. Rice germins were induced in both resistant and susceptible interactions suggesting that they are involved in basal defense rather than resistance gene specific responses. The expression of some GER4 subfamily members contributing to broad spectrum fungal disease resistance in leaf tissue [35] is suppressed in roots after abiotic stress (Fig. 2).

The literature is mixed on the contribution of germins to the oxidative burst that occurs shortly after elicitation of stress [17, 62]. OXO proteins consume oxalate influencing local pH [17]. SOD-type GLPs may regulate apoplastic superoxide production by membrane associated NADPH oxidases which are activated after biotic infection [63]. Both OXO and SOD reactions produce the active oxygen species, H_2O_2. This accumulation of H_2O_2 in the extracellular matrix may participate in cell wall remodeling processes, including signaling, cell wall polymer cross-linking, and cell expansion and elongation [17, 64]. Currently, only correlative evidence for OXO and/or GLP contribution to stress-induced H_2O_2 accumulation is available, and the evidence is contradictory. For example, in ryegrass, an increase in wound-dependent OXO enzyme activity was temporally correlated with increasing oxalate concentrations and H_2O_2 accumulation [12]. In barley, induction of *HvGLP4* (*HvGER4*) was correlated with papillae formation (cell wall

remodeling) after *B. graminae* infection [22]. However, when *HvGLP4* was transiently silenced, there was no change in H_2O_2 production in neighboring cells after pathogen infection [26]. The interpretation of these studies is complicated by the known induction or presence of several other germins as well as other active oxygen generating enzymes.

3.2 Potential for regulation of rice GLP expression by small RNA molecules

Eukaryotic organisms produce endogenous small RNA molecules (approximately 21-24 nucleotides) of two general types, microRNA (miRNA) and short interfering RNA (siRNA) [65]. Small RNAs are non-coding regulatory RNA molecules that control gene expression by mediating RNA degradation, translational inhibition, or chromatin modification [65]. These small RNAs have important roles in many aspects of gene regulation in plants, controlling developmental processes, signal transduction, protein degradation, and response to abiotic and biotic stress [66]. Small RNAs regulate the expression of tandemly arrayed, multigene family members [67]. For example, regulation of a cluster of highly related resistance genes in *Arabidopsis* by small RNAs was predicted to play an important role in optimizing plant defense response to pathogen attack [68, 69]. Many rice germin family members are tandemly arrayed on the chromosomes [11], including members of GER3 and GER4, and several have complex expression patterns (Fig. 1 and 2). Therefore, we explore the possibility that small RNAs regulate rice germins.

A scan of six small RNA libraries at Rice MPSS (http://mpss.udel.edu/rice/; [44]) revealed 30 unique small RNA signatures that map to predicted coding sequences and 5' untranslated regions (UTR) of rice germin genes (Table 3). One unique small RNA sequence putatively targets a GLP on chr 1, and 10 unique small RNAs target three tandemly arrayed, highly related GLP genes on chr 2. Nineteen small RNAs putatively target 10 genes in the GER3 and GER4 subfamilies and three related genes on chr 12; all of which are highly similar and tandemly arrayed.

An interesting example of possible small RNA regulation of germins is found in rice stem tissue (Fig. 3). Of the five germin subfamilies previously categorized [35], genes in three (GER2, GER5 and GER6) are expressed in non-stressed stem tissue while no transcripts from genes in the GER3 and GER4 subfamilies were detected. Interestingly, the corresponding stem small RNA library contains small RNAs with putative targets in the GER3 and GER4 subfamilies that are involved plant defense responses after fungal infection [35]. These observations are consistent with a hypothesis that small RNAs down regulate the expression of this subset of germins in non-stressed stem tissues.

The potential targeting of GER3 and GER4 subfamily members on chr 8 by small RNAs is of particular interest because of their roles in QTL-governed resistance [35]. The apparent down-regulation of rice GER3 and GER4 genes by small RNAs in non-stressed rice tissue may reflect coordinated regulation of genes involved in defense (Figure 3). Consistent with this are the presence of the small RNAs corresponding to GER3 and GER4 family members in the control seedlings and their absence in the seedlings after blast infection (Table 3). Such regulation may optimize defense responses by reducing the fitness costs to the plant. For example, inducible rather than constitutive responses may minimize energy requirements and enhance the capacity for response to pathogen attack.

Abscisic acid (ABA), an important plant hormone, is implicated in determining the outcome of interactions between many plants and their pathogens. Increased concentrations of ABA within *Arabidopsis* leaves correlates with susceptibility to an avirulent strain of a bacterial pathogen [70]. Conversely, the ABA signaling pathway is required for plant resistance to a necrotrophic fungus [71]. After ABA treatment of rice, subfamily specific changes occur in small RNA populations that putatively target genes in the GER3 and GER4 subfamilies (Table 3). Small RNAs targeting GER3 increase after ABA treatment, while those targeting GER4 disappear,

suggesting that these gene family members are oppositely regulated by this hormone. Rice GER4 genes are predicted to play a general role during defense responses since they are effective against two fungal pathogens using different infection strategies (hemibiotrophic vs necrotrophic) [35]. The differences in regulation of these two subfamilies by ABA and pathogen treatment provide testable hypotheses regarding possible overlaps in regulation of plant defenses.

3.3 Germin associations with quantitative trait loci in rice and other cereal crops

Polymorphic markers from barley *HvOxOa (HvGER1*, germin with OXO activity*)* and *HvOXOLP* (*HvGER4*, germin with SOD activity) were used as candidate genes in multiple rice QTL mapping experiments and were associated with reductions in disease [34, 72, 73]. The *HvGER1* probe co-localized with a cluster of four *OsOXO* genes on rice chr 3, and the *HvGER4* probe associated to a cluster of *OsGLPs* on rice chr 8. The rice germin subfamilies were functionally characterized and found to contribute to broad spectrum fungal disease resistance ([11, 35] and Chapter 4). *HvGER4* co-localized with barley QTL for fungal resistance [74], and the barley markers flanking this QTL were physically mapped to rice chr 8 [35]. The same barley marker, *HvOXOLP*, co-localized to a wheat QTL for resistance against *Pyrenophora tritici-repentis*, the causal agent of tan spot [75]. Furthermore, in maize, the OXO probe (*HvGER1*) mapped to five maize chromosomes, and the GLP probe (*HvGER4*) mapped to four chromosomes [43]. As in rice, barley and wheat, both germin markers co-localized to known maize QTL for broad-spectrum disease resistance, including resistance to *Cercospora spp.*, the causal agent of gray leaf spot, and *Exserohilum turcicum*, the causal agent of northern corn leaf blight [43]. Given the above examples of germin genes co-localizing with disease resistance QTL and the functional validation of several in rice and barley disease resistance, we propose that combining gene

location, expression profile, and QTL associations will identify additional candidate germin genes for disease resistance.

In assessing candidate genes and their roles in disease resistance, it is critical to consider the number of genes under the QTL that may contribute to the phenotype. For example, three-fourths of rice germins (31/41 = 75.6%) are present in tandemly duplicated clusters of two or more members. The genes in each cluster may contribute collectively to a phenotype, such as disease resistance [35]. The number of genes in the cluster may correlate with the effect (more genes, more resistance). A chr 8 *OsGLP* gene cluster of 12 genes was originally identified under a major effect QTL interval (log of odds (LOD) score > 7) for rice blast resistance. The QTL explained over 30% of the variation for diseased leaf area in five independent locations [73]. Conversely, another cluster of four genes, the chr 3 *OsOXOs* that co-localized to a minor effect QTL region for blast resistance and explained only 11% of variation in disease [34], and would be expected to contribute less resistance than the larger cluster of *OsGLPs* on chr 8.

Molecular marker data from 19 published rice QTL mapping studies were mapped onto the rice genome along with the physical locations of all germin genes to predict additional rice germins that may contribute to complex, multi-gene resistance (Fig.4; Table 2, 4). The cluster of *OsGLPs* on chr 8 co-localized to the originally targeted blast resistance QTL [73] and another rice blast QTL in a different mapping population [76]. For both studies, the chr 8 QTL regions contributed over 30% of the phenotypic variation in different mapping populations [73, 76]. In addition, a sheath blight QTL [77] co-localized with the genes, consistent with the loss of resistance in the silenced mutants to both the blast and sheath blight pathogens [35]. Four clustered *OsOXOs* on chr 3 co-localized with multiple rice blast QTL [34, 78], two sheath blight QTL [79, 80] and a bacterial blight QTL [72]. QTL in this chr 3 region contributed around 10% of phenotypic variation, with the exception of one sheath blight QTL, which contributed 26%

[34],[77, 79]. The addition of the expression data for the germins to the alignment adds another dimension to the analysis, and increases confidence in the predictions. For example, some family members that were confirmed to co-localize with chr 8 and 3 QTL were induced after pathogen challenge (Fig. 2).

Six other rice germins from the analysis may be good candidates for sources of resistance because they (1) co-localize with one or more disease resistance QTL; (2) are induced after pathogen or abiotic stress; and (3) are phylogenetically related to defense response germins. For example, a *GLP* on chr 1 (Os01g72990) is physically located under QTL for sheath rot and yellow mottle virus [81] [82]. This GLP was induced in leaves by drought stress and *M. oryzae* infection in MPSS libraries, and is closely related to the barley *HvGER5* genes that were induced by infection with *Blumeria graminis* [20]. A *GLP* on chr 3 (Os03g58980) is within a minor QTL for resistance to brown plant hopper [83], and is induced by infection with *M. oryzae* and *Xanthomonas oryzae* pv. *oryzae* as well as drought stress. A chr 11 *GLP* (Os11g33110) co-localizes with several rice blast QTL [34, 76, 84], and is induced by *X. oryzae* pv. *oryzae* and cold stress. An intriguing *GLP* on chr 2 is located under a sheath blight QTL [79] and is induced by cold stress and infection with *M. oryzae* and *X. oryzae* pv. *oryzae*. Lastly, two *GLP*s on chr 5 (Os05g10830 and Os05g19670) that exhibit stress induction are within a large rice blast QTL [76]. We propose that polymorphic markers in these genes could be successful in locating QTL for disease resistance.

4. Conclusions and Prospects

The germins are a functionally diverse protein family, with at least three different enzyme activities. Germins are encoded by large gene families with distinct subgroups which is consistent with their functional diversity. Most germin genes are expressed in multiple tissue types including seed, flowers, leaves and roots. There is evidence of cell type specificity among

germin transcripts as some *GLPs* are restricted to epidermal cells and *OXOs* are present in epidermal and mesophyll cells. The presence of germin-box containing proteins in multiple species from bryophytes to angiosperms suggests that they have conserved roles in plant developmental processes.

There are 41 predicted germin genes in rice distributed on nine chromosomes. Six of these may be non-functional given the lack of gene transcript evidence in multiple genome wide expression databases. Over half of the rice germins are induced by abiotic and biotic stresses. Our data suggests multiple gene duplication events in rice, and that many of these paralogs have been retained in the genome. This is likely due to subtle changes in temporal and spatial expression patterns after duplication that provide distinct and useful functions for each paralog.

Natural variation in germin alleles is currently being utilized in breeding programs to improve disease resistance. The use of the chr 8 GLP as a molecular marker in rice has resulted in cultivar improvement that is a broad-spectrum, durable type of resistance [35]. Thus, rapid identification of additional candidate genes for disease resistance within the germin gene family will be useful for future crop improvement efforts. The prediction that germins with OXO and SOD activities are most likely to be involved in disease resistance is based upon phylogenetic relationships in the reconstructed gene tree. Though germin-type OXOs have only been identified in cereals, GLPs are involved in defense responses in both monocots and dicots, including responses to bacteria, virus, fungi and herbivores.

The comprehensive data available for rice, including a well-annotated genome sequence, robust genome-wide expression data and extensive published QTL data provided us the platform to identify additional candidate germin genes for disease resistance. In addition, the comparative analysis allows us to predict candidate germin gene lineages with possible relevance to disease resistance in other taxa. This approach of layering genomic and functional

data will become more powerful as an increasing number of crop genomes are sequenced and gene functions are determined. Rice germins previously confirmed to contribute to disease resistance were shown in our analysis to co-localize with numerous additional QTL from different mapping populations. Furthermore, based on co-localization with QTL, expression profiles, and relationships to other defense related germins, we predict six additional rice germins as promising candidates for being defense response genes. The importance of germins is not exclusive to rice; there are intriguing similarities across taxa with respect to particular subfamily members (GER4, in particular) and contributions to broad-spectrum disease resistance. The future use of germins to enhance disease resistance will be facilitated by using bioinformatics to identify candidates and connect them across plant species. More broadly, we propose that application of this comprehensive analysis will expedite identification of other types of candidate genes underlying QTL for various traits useful for crop improvement.

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Table 1. Accession numbers of germin family gene and protein[a] sequences used in phylogenetic analyses.

Gene Name	Accession No.[b]	Plant Species	Reference
AtGER1/AtGLP1	U75206	*Arabidopsis thaliana*	[32, 85]
AtGER2	X91957	*Arabidopsis thaliana*	[32]
AtGER3/AtGLP3b	ATU75195	*Arabidopsis thaliana*	[32, 85]
BuGLP	AB036797	*Barbula unguiculata*	[25]
BvGER165	AF310016	*Beta vulgaris*	[59]
CaGLP	AY391748	*Capsicum annuum*	[31]
GhGLP1	AF116537	*Gossypium hirsutum*	[46]
HvGER1a	DQ647619	*Hordeum vulgare*	[20]
HvGER1b	BI949639	*Hordeum vulgare*	[20][c]
HvGER1c	BY847725	*Hordeum vulgare*	[20][c]
HvGER1d	BG366014	*Hordeum vulgare*	[20][c]
HvGER2a	DQ647620	*Hordeum vulgare*	[20]
HvGER2b	BY837622	*Hordeum vulgare*	[20][c]
HvGER3a	DQ647621	*Hordeum vulgare*	[20]
HvGER3b	n/a	*Hordeum vulgare*	[20][c]
HvGER3c	DN178806	*Hordeum vulgare*	[20][c]
HvGER4a	CD056200	*Hordeum vulgare*	[20][c]
HvGER4b	n/a	*Hordeum vulgare*	[20][c]
HvGER4c	DQ647622	*Hordeum vulgare*	[20]
HvGER4d	DQ647623	*Hordeum vulgare*	[20]
HvGER4e	BQ762852	*Hordeum vulgare*	[20][c]
HvGER5a	DQ647624	*Hordeum vulgare*	[20]
HvGER5b	AV923347	*Hordeum vulgare*	[20][c]
HvGER6a	DQ647625	*Hordeum vulgare*	[20]
HvGLP1	Y15962	*Hordeum vulgare*	[8]
LpOXO1	AJ291825	*Lolium perenne*	[12]
LpOXO2	AJ492380	*Lolium perenne*	[12]
LpOXO3	AJ504848	*Lolium perenne*	[12]
LpOXO4	AJ492381	*Lolium perenne*	[12]
MtGLP1	AY184807	*Medicago truncatula*	[50]
NaGLP	AY436749	*Nicotiana attenuata*	[49]

NpNEC1	**AF132671**	*Nicotiana plumbaginifolia*	[24]
PnGLP	**D45425**	*Ipomoea nil*	[48]
PsGer1	**AJ250832**	*Pisum sativum*	[27]
RmGLP1	**AB272079**	*Rhododendron mucronatum*	[28]
RmGLP2	**AB272080**	*Rhododendron mucronatum*	[28]
Ta_gf2.8	**M63223**	*Triticum aestivum*	[13]
Ta_gf3.8	**M63224**	*Triticum aestivum*	[13]
TaGLP2a	**AJ237942**	*Triticum aestivum*	[9]
TmGLP4	**AY650052**	*Triticum monococcum*	[60]
VvGLP1	**EF064171**	*Vitis vinifera*	[29]
VvGLP2	**DQ673106**	*Vitis vinifera*	[29]
VvGLP3	**AY298727**	*Vitis vinifera*	[29]
VvGLP4	**EF064172**	*Vitis vinifera*	[29]
VvGLP5	**EF064173**	*Vitis vinifera*	[29]
VvGLP6	**EF064174**	*Vitis vinifera*	[29]
VvGLP7	**EF064175**	*Vitis vinifera*	[29]
ZmGLP1	**AAQ95582**	*Zea mays*	[47]

[a]Protein sequences were predicted from genomic DNA sequences using FGENESH HMM gene structure prediction program. http://linux1.softberry.com/berry.phtml

[b] Gene sequences were acquired from the NCBI sequence database http://www.ncbi.nlm.nih.gov/. Germin sequences from crop plants with published gene and/or protein expression data were included in the analysis.

[c] Designated barley (*Hv*) sequences were extracted from the TGI database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=Barley) and analyzed by [20, 35].

Table 2. Rice germin family genes, locus identifiers[a], and chromosomal coordinates.

Locus ID	Chromosome	5' Coordinate	3' Coordinate
LOC_Os01g18170	1	10166094	10164664
LOC_Os01g50900	1	29558747	29559638
LOC_Os01g72290	1	42241533	42242785
LOC_Os01g72300	1	42244715	42245955
LOC_Os02g29000	2	17160127	17159318
LOC_Os02g29010	2	17161547	17166870
LOC_Os02g29020	2	17169352	17170023
LOC_Os02g32980	2	19592747	19591621
LOC_Os03g08150	3	4136722	4137613
LOC_Os03g44880	3	25269898	25271593
LOC_Os03g48750	3	27729269	27728259
LOC_Os03g48760	3	27733754	27733065
LOC_Os03g48770	3	27738087	27737226
LOC_Os03g48780	3	27741221	27740193
LOC_Os03g58980	3	33525399	33526261
LOC_Os03g59010	3	33530129	33531009
LOC_Os04g52720	4	31176126	31173818
LOC_Os05g10830	5	5965397	5966166
LOC_Os05g19670	5	11446464	11447387
LOC_Os08g08920	8	5180531	5181354
LOC_Os08g08960	8	5202041	5203225
LOC_Os08g08970	8	5215873	5216960
LOC_Os08g08980	8	5222478	5223690
LOC_Os08g08990	8	5227424	5228454
LOC_Os08g09000	8	5232663	5233804
LOC_Os08g09010	8	5236151	5237313
LOC_Os08g09020	8	5242322	5243488
LOC_Os08g09040	8	5247942	5248886
LOC_Os08g09060	8	5253808	5254949
LOC_Os08g09080	8	5257901	5259045
LOC_Os08g13440	8	7989374	7988056
LOC_Os08g35750	8	22421190	22420215

LOC_Os08g35760	8	22426255	22425051
LOC_Os09g39510	9	22695664	22694639
LOC_Os09g39520	9	22697908	22697264
LOC_Os09g39530	9	22700478	22699440
LOC_Os11g33110	11	19084161	19084993
LOC_Os12g05840	12	2688828	2687775
LOC_Os12g05860	12	2692513	2691487
LOC_Os12g05870	12	2696216	2695409
LOC_Os12g05880	12	2699188	2698387

[a]Rice germin family protein sequences were acquired from the MSU Rice Genome Annotation Database http://rice.plantbiology.msu.edu/ [45].

Table 3. Number of small RNAs that potentially target specific rice GLPs or multiple genes within germin (GER) subfamilies[a] in units of transcripts per million.

Putative gene targets[b]	Unique siRNA sequences[c]	Germinating seedling control	Germinating seedling after *Mo*[d]	Control for ABA	ABA treated[e]
GER3	9	2	0	0	16
GER4	10	5	0	11	0
Os01g50900	1	22	0	0	0
Os02g29000	2	4	0	0	0
Os02g29010	7	7	0	13	56
Os02g29020[f]	1	0	0	0	0
Totals	**30**	**40**	**0**	**24**	**72**

[a] Small RNA libraries were from MPSS data at [44].

[b] Putative gene targets of small RNA based on sequence identity. Small RNAs putatively target individual genes or multiple genes within a germin (GER) subfamily.

[c] Number of unique small RNA sequences with identity to putative targets.

[d] RNA samples were obtained 24 hours post inoculation with the rice blast pathogen, *M. oryzae* (*Mo*).

[e] RNA samples were obtained from seedling tissue 24 hours post application with abscisic acid (ABA).

[f] This small RNA sequence was detected only in the stem small RNA library.

Table 4. Estimated physical coordinates of molecular markers and QTL intervals in disease resistance mapping studies

QTL Paper Reference, Parental cultivars, Rice Disease, Causal Organism[a]	Chr	Flanking Markers[b]		Estimated Coordinates[c]	
		5'	3'	5'	3'
Alam et al [83]	1	AMY1B	RZ276	14442432	14603974
IR64 x Azucena	2	RG157	RZ318	19865083	24565710
brown planthopper, *NI*	3	RG191	RZ678	5709784	7327744
	4	RG143	RG620	33630028	34663799
	4	RZ590	RG163	32668236	*
	6	RZ144	RZ667	6718648	6928404
Albar et al [82]	1	RZ730	C112	35265873	43281154
IR64 x Azucena	2	RZ318	RG95	24565710	*
rice yellow mottle virus	4	RG190	RG788	8595185	*
	9	RZ228	RG667	18513777	20481607
	12	R617	Y6854R	7477327	13429316
Chen et al [74]	1	C161	R753	196624	1887955
Zhenshan 97 x Minghui 63	1	RM259	RM243	7443429	7968524
rice blast, *Mg*	1	RG532	RM259	5759761	7443429
	1	G393	R2201	31373895	*
	1	RM212	C547	33381385	40391516
	1	C547	C2340	40391516	34798491
	1	C2340	C86	34798491	38041145
	2	RM213	RM208	34652316	35135783
	3	RZ403	R19	23044452	24551002
	7	RG528	RG128	1570817	2315990
	7	RG128	C1023	2315990	7231613
	7	RM234	R1789	25471987	26527687
	8	RG333	RM25	4100172	4372099
	9	RM201	C472	20174289	14594969
	9	RM257	RM242	17719660	18810067
	9	RG570	RG667	19946740	20481607
	9	RM215		21189110	
	9	R1952		2260721	
Fukouka et al [86]	4	G271		20156485	
Nipponbare x Owarihatamochi	9	G103		10799952	
rice blast, *Mg*	12	C625		11058522	

Han et al [87]	5	C624	C246	21351430	27057523
Zhenshan 97 x Minghui 63	5	C246	RM26	27057523	27321395
sheath blight, *Rs*	9	C472	R2638	14594969	17835012
	9	RM257	RM242	17719660	18810067
Huang et al [88]	3	R1925	R2443	35385979	31892032
B5 x Minghui 63, brown planthopper, *Nl*	4	C820	R288	6882835	8198506
Li et al [77]	3	RG348	RG944	1430443	3219536
Lemont x Tequing	4	RG143	RG214	33630028	31881866
sheath blight, *Rs*	8	RG20	RG1034	2029021	16241105
	12	RZ397	RG214A	5758288	*
Li et al [89]	2	RG520	RZ476A	35662091	*
Lemont x Tequing	3	C515	RG348	698124	1430443
bacterial blight, *Xoo*	4	RG214	Ph	31881866	*
	4	RZ69	RG190	11217892	8595185
	8	G104	G1314A	8917705	*
	9	RZ404	RG451	22194746	*
	10	C16	RG1064F	20840254	*
Liu et al [73]	2	RM262	RM5789	20790000	22380000
SHZ-2 x LTH	7	RM3404	RM3826	20110000	20810000
rice blast, *Mg*	8	RM310	RM3215	5020000	8560000
	10	RM8207	RM3311	9550000	10360000
Liu et al [79]	1	RM1361	RM104	39407759	40494732
Jasmine 85 x Lemont	2	RM424	RM5427	11389704	21519085
sheath blight, *Rs*	2	RM112	RM250	32013785	*
	3	RM16	RM426	23082184	27544733
	3	RM5626	RM426	24820470	27544733
	3	RM514	RM85	35227302	*
	5	RM507	RM7349	80784	3225473
	6	RM435	RM190	537327	1764638
	9	RM409	RM257	14372062	17719660
	9	RM215	RM245	21189110	*
Pinson et al [90]	1	RG532		5759761	
Lemont x Teqing	3	RG348		1430443	
sheath blight, *Rs*	3	RZ474		25084678	

	4	RZ590		32668236	
	6	RZ508		30945227	
	8	G104		8917705	
	9	RZ404		22194746	
	10	RG561		21518696	
Ramalingam et al [72]	2	RG181	XLRFRI2	27488270	*
IR64 x Azucena	3	OXO	CDO337	28539529	*
brown planthopper, *Nl*	3	RG191	RZ678	5709784	7327744
bacterial blight, *Xoo*	4	RZ675	RG163	24131148	*
	5	RZ70	XLRFRA1	24241592	*
	7	RG477	NLRIN121	6778514	*
Sirithunya et al [78]	3	RM16	RM168	23082184	28047759
CT9993-5-M x Khao Dawk Mali 105	5	C597	RM122	238174	289101
leaf and neck blast, *Pg*	6	R2171	RG64	8062746	9549516
	6	C1478	R2171	6822190	8062746
	7	G20P	RM2	17525116	15969160
	7	RM10	OSR22	22135223	*
	7	R3089	G20P	18334208	17525116
	9	R1687	RG553	8346089	9216376
Srinivasachary et al [81]	1	RG140	RG612	5093011	*
C039 x Moroberekan	1	RZ276	CDO920	14603974	19926001
sheath rot, *So*	1	RG109	RG236	38525259	42296614
	2	RG102	RG73	27483091	31106412
	5	RG573	RG360	21488330	*
	6	RG172	RG192	24072058	*
	7	RZ272	RG511	4657304	5131861
	8	RG20	RG333	2029021	4100172
Tabien et al [91]	1	RZ14		40015287	
Lemont x Tequing	2	RG520		35662091	
rice blast, *Mg*	3	RZ474		25084678	
	5	CDSR49		22497768	
	12	RZ397		5758288	
Wang et al [76]	1	RZ276	RZ744	14603974	20926224
CO39 x Moroberekan	1	RG140	RG612	5093011	*
rice blast, *Mg*	2	RG102		27483091	
	3	RG104A	RG348	463840	1430443

	4	RG214		31881866	
	5	RG182	RG573	6112140	21488330
	6	RZ744	WAXY	20926224	1764623
	6	RG64	RG172	9549516	24072058
	7	RG351	CDO553	29413782	*
	8	RG333	RZ562	4100172	5415950
	11	RG103		20301909	
	11	RG16		18143751	
	12	RG574		1594835	
	12	RG869B		7731471	
	12	RG9		18050023	
Wu et al [34]	2	RM250		32774365	
Moroberekan x Vandana	3	RM168		28047759	
rice blast, *Mg*	9	RM215		21189110	
	11	RM21		18973973	
Zenbayashi et al [84]	11	RM209	G4001	17771755	22781184
Norin x Chubu, rice blast, *Mg*					
Zou et al [80]	2	G243	RM29	11750492	*
Lemont x Jasmine 85	2	RG171	RM29	17590791	*
sheath blight, *Rs*	3	R250	C746	25071688	27084381
	7	RG30	RG477	12785785	6778514
	9	C397	G103	12289952	10799952
	9	RG570	C356	19946740	18525201
	11	G44	RG118	9934607	4413928

[a]Disease-causing organisms used in phenotypic assays of mapping populations: *Nilaparvata lugens* (*Nl*), *Magnaporthe oryzae also called M. grisea* (*Mg*) and *Pyricularia grisea* (*Pg*), *Rhizoctonia solani* (*Rs*), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), *Sarocladium oryzae* (*So*)

[b]Polymorphic molecular markers associated with reductions in measured disease symptoms. Marker data are reported as QTL intervals with 5' and 3' flanking markers, or as single markers that are significantly associated with phenotype.

[c]The estimated physical coordinates of molecular markers relative to the rice genome were acquired at Gramene ([92], http://www.gramene.org/) and MSU Rice Genome Annotation [45], http://rice.plantbiology.msu.edu/index.shtml) databases. Coordinates were also estimated by performing BLASTN of primers and other associated sequences against the reference japonica rice genome at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Asterisks indicate molecular markers that could not be physically mapped to the rice genome. Entire QTL intervals and single markers that could not be physically mapped are not shown.

Figure 1. Phylogenetic relationships and gene expression evidence for germins in rice and 17 other plant species. Amino acid sequences were aligned using Dialign-TX [51] and the gene tree estimated using Bayesian maximum likelihood [52]. The root position was inferred using a relaxed molecular clock [53]. Clade support shown as posterior probabilities at nodes. Rice (Os) germin expression data were obtained from MPSS rice expression libraries [44] and the MSU rice digital northern database [45]. Expression data from other species are from published literature. For the heatmap summary, transcripts were considered present if bands were visible in published gel images. Gene expression in four tissue categories refers to non-inducing conditions, and stress-induced expression includes gene induction above basal expression levels. Gene expression is indicated as present (purple), absent (yellow) or no data (grey) in seeds (Sd), flowers (Fl), leaves (Lv), roots, (Rt) or stress induced (biotic or abiotic; Str). Symbols next to gene names indicate verified enzyme activity; oxalate oxidase (circle), superoxide dismutase (triangle) and ADP glucose pyrophosphatase/phosphodiesterase (cross).

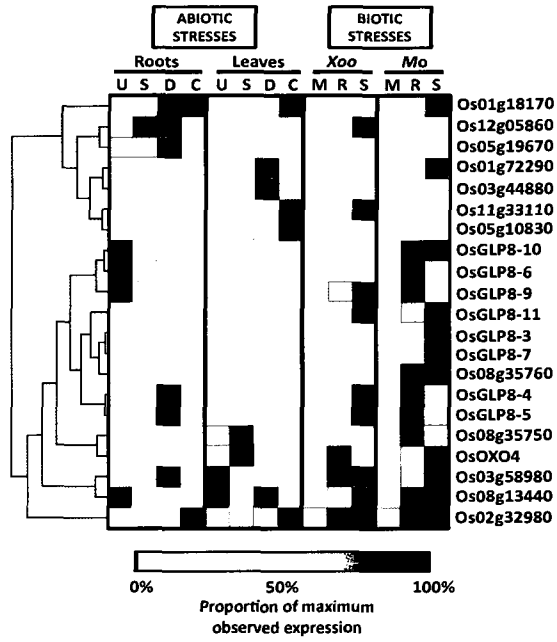

Figure 2. Rice germin gene induction after abiotic and biotic stresses. Genome-wide transcript data from 34 MPSS stress libraries [44] showed that 21 rice germin genes were induced by abiotic or biotic stresses or both. Abiotic stresses included salt (S), drought (D) and cold (C) treatments in roots or young leaves compared to untreated (U) controls. Biotic disease treatments included resistant (R) and susceptible (S) interactions with *Xanthomonas oryzae pv. oryzae* (*Xoo*) and *Magnaporthe oryzae* (*Mo*) compared to mock (M) inoculations. Proportions of maximum observed expression values, by gene, for each of four stress catetories (black boxes; root/abiotic, leaves/abiotic, *Xoo*, *Mo*) are shown in the heat map. Clustering of genes was performed by hierarchal clustering based on induction patterns using MeV [61].

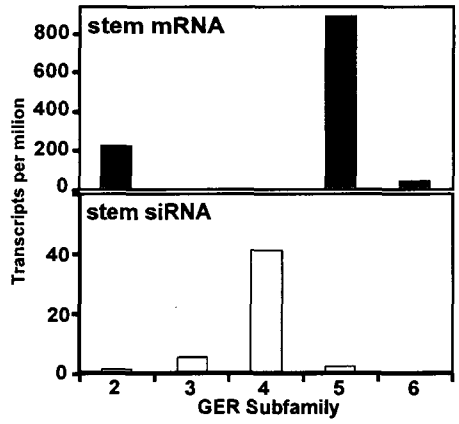

Figure 3. Small RNA regulation of GLPs in rice stem tissue. MPSS mRNA and small RNA (siRNA) signature data corresponding to GLPs belonging to GER subfamilies described in Manosalva et al. [35] are shown as transcripts per million. The stem siRNA transcript population includes four unique small RNA signatures that putatively target GER3 subfamily members, and four others that target GER4 subfamily members.

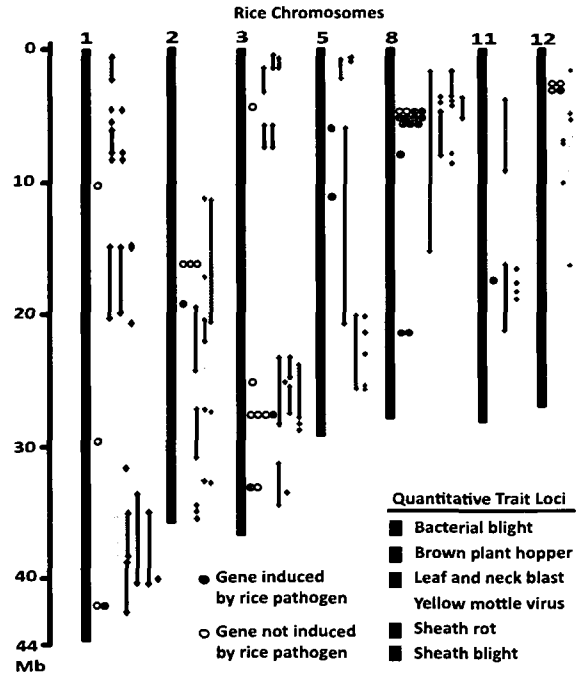

Figure 4. Rice germin co-localization with quantitative trait loci. Both *GLP*s and *OXO*s physically co-localize with quantitative trait loci for resistance against biotic pathogens. Molecular marker data from 20 published QTL mapping studies were physically mapped on the rice genome. Individual points show single marker associations with disease phenotypes, and line segments show QTL intervals. Chromosomal positions of germin genes that are induced (filled circles) or not induced (open circles) by either *X. oryzae pv. oryzae* or *M. oryzae i*n MPSS expression libraries are indicated next to the chromosomes. One *GLP* on chr 4 and a cluster of three *GLP*s on chr 9 are not shown because they are not associated with QTL in these mapping studies.

CHAPTER 3

Allelic diversity of germin-like proteins in rice and relationships to early stress responses

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R. Davidson performed promoter analyses, cloning and sequencing, expression profiling and subcellular localization of germin-like proteins (GLP). J. Snelling, an undergraduate fellow, assisted with hydrogen peroxide staining experiments. P. Manosalva, C. Vera Cruz, H. Leung and J. Leach contributed to conceptual design of experiments.

ABSTRACT

Germin-like protein (GLP) markers were associated with quantitative trait loci (QTL) for resistance to the rice blast pathogen, *Magnaporthe oryzae* in multiple rice (*Oryza sativa*) mapping populations. Twelve paralogous *OsGLP* gene family members are located within the physical QTL region on chromosome 8, and gene silencing studies suggest that they contribute collectively to the resistance phenotype. We compared sequence and expression profiles of *OsGLP* alleles in two resistant and two susceptible parental rice lines to find functional polymorphisms that correlated with the resistant phenotype. Based on coding and promoter sequences, the genes belong to two germin subfamily groups (GER3 and GER4). *OsGLP* members from both subfamilies were constitutively expressed and developmentally regulated in all cultivars. Transient induction above constitutive levels was observed for some *OsGLP*s, especially GER4 subfamily members, at early time points after *M. oryzae* infection and mechanical wounding. Differential expression of some family members between resistant and susceptible cultivars corresponded with differential hydrogen peroxide (H_2O_2) accumulation after the same stimuli. OsGLP of both GER subfamilies localized to the plant cell wall. The protein location and early gene induction suggest that *OsGLPs* protect rice leaves at early stages of infection before fungal penetration and subsequent ingress. Our data suggest that regulation of *OsGLP* genes defines resistant versus susceptible phenotypes.

INTRODUCTION

Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive rice (*Oryza sativa*) diseases [1]. Genetic resistance controlled by single genes has been widely used to control rice blast, but the resistance is often short-lived due to changes in the fungal population structure [2]. Disease resistance governed by quantitative trait loci (QTL), or many genes with small effects, however, is predicted to be durable partially because it exhibits no specificity to a given pathogen group or race, and in some cases, confers resistance to multiple types of pathogens [3, 4]. There is good evidence that QTL might control broad-spectrum genetic resistance in some rice populations. A germin-like protein (GLP) gene marker that was associated with a QTL on rice chromosome (chr) 8 conferred resistance against rice blast in multiple rice mapping populations across diverse environments [5-7]. Twelve paralogous *OsGLP* genes were identified within the physical QTL region [8]. Members of the *OsGLP* family were shown to contribute collectively to disease resistance because gene silencing of several genes confers susceptibility to two distinct fungal pathogens, *M. oryzae* and *Rhizoctonia solani* (*Rs*), the sheath blight pathogen [8]. The susceptible phenotypes of the *OsGLP*-silenced mutants confirm roles for some gene family members, particularly those in the GER4 subfamily, in broad-spectrum disease resistance [8].

The rice chr 8 *OsGLP*s belong to two subfamilies of germins, the GER3 and GER4 (Chapter 2) [8]. These subfamilies were also described in barley (*Hordeum vulgare*, *Hv*), wheat (*Triticum aestivum*, *Ta*), grapevine (*Vitus viniferous*, *Vv*) and

Arabidopsis thaliana (*At*) [9-12]. GLP genes in these subfamilies are developmentally regulated and constitutively expressed in multiple tissue types including leaves, roots and flowers (Chapter 2) [13]. Many of these genes are induced by fungal and bacterial infections, and some produce proteins with superoxide dismutase (SOD) activity (Chapter 2)[10, 11, 14]. GLP gene induction was observed in rice after infection with *M. oryzae* [8](Chapter 2), in barley and wheat leaves after infection with the powdery mildew pathogen [9, 14, 15], in barley spikelets after infection with *Fusarium graminearum* [16] and in grapevine leaves and fruit after infection with *Erysiphe necator* [11]. Transcripts of wheat and barley GER3 and GER4 genes accumulated predominantly in epidermal cells compared to mesophyll cells as early as 6 hours post inoculation (hpi) [9, 10, 15]. Interestingly, *HvGER4* were also induced by exogenous hydrogen peroxide (H_2O_2) application and the non-host, penetrating soybean fungus, *Phakopspora pachyrhizi* [9]. Consistent with gene silencing results in rice [8], transient silencing of barley *HvGER4d* resulted in hyper-susceptibility to *Blumeria graminis,* though silencing of *HvGER3a* did not [9]. Genes in both subfamilies show induction by pathogen infection, however, gene silencing of only GER4 subfamily members results in increased susceptibility.

The proposed mechanism by which GLPs provide broad spectrum resistance relates to their SOD activity and subcellular localizations in cell walls [12, 14, 15]. GLP enzymes with SOD activity convert the superoxide anion into H_2O_2 [17]. Hydrogen peroxide is a player in early plant defense responses and has potential roles in cell wall reinforcement and papillae formation [10, 18], as a signaling

molecule for downstream defense responses [19, 20] and as a toxic defense compound against invading microbes [21]. Cell wall localizations of GLPs have been documented in barley and wheat [10, 15], but have not been studied in rice.

We have shown that *OsGLP* in the GER4 subfamily function in non-specific resistance to two fungal pathogens [8]. The molecular basis for differences in the involvement of specific *OsGLP*s in QTL-based resistance, however, is still unknown. This includes a lack of understanding of the mechanisms of resistance in cultivars with differing disease phenotypes. The goals of the study are to understand the differences among cultivar-specific *OsGLP* alleles and to functionally link sequence polymorphisms observed in QTL mapping to differential defense responses. Differences between alleles underlying QTL can vary from single nucleotide substitutions to large insertions or deletions between parental cultivars [22]. Some differences may affect coding sequences while others may occur in upstream regulatory regions and influence gene expression. We predicted defense-related *OsGLP* family members by comparing the *OsGLP* promoters from the two reference rice genomes, cv. Nipponbare (ssp. *japonica*) [23] and cv. 93-11 (ssp. *indica*) [24]. Then, *OsGLP* alleles of two rice blast resistant (both ssp. *indica*) and two susceptible rice cultivars (both ssp. *japonica*) were studied for coding sequence polymorphisms and differential gene expression, both developmentally and after biotic and abiotic stresses. Furthermore, we confirm the subcellular localization of GER3 and 4 subfamily members and study H_2O_2 peroxide accumulation in QTL-based resistant and susceptible interactions with *M. oryzae*. This information can guide breeding

efforts to accumulate desirable alleles from resistant donors into locally adapted cultivars.

MATERIALS AND METHODS

Plant and Fungal Growth, Inoculation/Wounding Methods

Rice cultivars, Azucena (subsp. *japonica*), Lijiangxin-tuan-heigu (LTH; subsp. *japonica*), IR64 (subsp. *indica*) and Sanhuangzhan 2 (SHZ-2; subsp. *indica*) were grown in a Bacto soilless media (Michigan Peat Co., Houston, TX) for 21 days and fertilized once a week with 5 g/L NH_4SO_4. Plants were grown with a photoperiod of 16 h light/8 h dark in a growth chamber and alternating day/night temperatures of 28°/26°C and 80% RH.

Plants were inoculated with Philippine *M. oryzae* isolate PO6-6 that exhibits broad virulence to rice genotypes and is routinely used for the evaluation of quantitative resistance to blast [5, 8, 25]. Cultures were grown on oatmeal agar media under constant light at 26°C for 21 days, and plants were inoculated with 5 x 10^5 spores/ml at 20 psi using an artist's air brush [26]. Mock inoculation consisted of a spray with gelatin solution.

For the wounding experiments, LTH and SHZ-2 were grown for 21 days. The two youngest fully expanded leaves were pierced with a needle at 1 cm intervals from the tip downward for 8 cm.

Plant Harvest, RNA Isolation and RT-PCR

For developmental expression assays, tissue was harvested from the three most fully expanded leaves of 21-day-old plants and pooled from six plants per

cultivar. For expression experiments after *M. oryzae* inoculation, tissue was harvested by combining the three most fully expanded leaves pooled from two plants per cultivar. In the wounding experiments, the two youngest, wounded leaves were pooled from two plants per cultivar.

Samples were ground in liquid nitrogen, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). RNA was treated with one unit of DNase (Promega, Madison, WI) per μg total RNA. Single-strand cDNA was synthesized from 2 ug of total RNA using Superscript III reverse transcriptase (RT; Invitrogen, Carlsbad, CA) and 50 pmol oligod$T_{(20)}$ primer. Gene-specific primers (10 pmol of each primer) were used in reverse transcriptase (RT-) PCR for 35 cycles at the appropriate annealing temperature [8]. Total RNA was measured with a spectrophotometer, and *18s rRNA* or *EF1*-alpha were used as reference genes [27] to ensure equal RNA loading among samples.

Expression profiling by RT-PCR was performed from leaf tissue of three independent plant inoculation/wounding experiments. PCR products were fractionated on 0.8% agarose gels (w/v) and stained with ethidium bromide. Stained gels were visualized and digitally photographed using the Syngene Gene Genius Bioimaging System, and gel band intensities were quantified using Gene Tools Gel Analysis software (Syngene, Frederick, MD). Band intensity values were normalized by subtracting the signal of the negative control in a given gel. Intensities for each gene/treatment/cultivar combination were averaged over biological replicates (n=3).

Cloning of *OsGLP* alleles and Phylogenetic Analysis

The 12 *OsGLP* were PCR amplified from genomic DNA of four cultivars (Azucena, LTH, IR64 and SHZ-2) and cloned into pGEM-T (Promega) using primers and annealing temperature described in [8]. Genes were sequenced from plasmids at a minimum of 4x coverage. Protein predictions were performed using FGENESH (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). An *Arabidopsis thaliana* germin-like protein sequence was used as an outgroup. For phylogenetic analysis, genomic sequences of putative *OsGLP* genes were aligned using the ClustalW2 algorithm [28]. The final tree represents a random tree from a heuristic Maximum parsimony search. Bootstrap support values were calculated from 1000 replicates with 1000 trees saved each replicate.

Promoter Sequencing and Motif Analysis

Because sequence differences were observed in the 5' regulatory regions of *OsGLP8-6* between the two reference genomes (*japonica* vs. *indica*), the regions were amplified from each of the four cultivars with primers designed 639 and 85 bp upstream from start codon (Figure 4B, 4C; GLP6proF2, 5' cgttcaattttctaagccagattgtg 3' and oxo6ProR 5' CTTCCCATCAGAGAAAGATAGCAG 3'). The sequences were cloned into pGEM-T (Promega) and sequenced at 4x coverage.

Putative regulatory sequences that are 5' to *OsGLP* genes (1 Kb upstream from the start codon) for the 12 gene family members were extracted from the Nipponbare genome sequence, Build 4.0, using a custom perl script. The putative promoter sequences for the 12 *OsGLP* genes and the *OsGLP8-6* insertion sequence

(858 bp, only present in *indica* sequences) were analyzed as a training set (N=13) for statistically overrepresented sequence motifs using the MEME algorithm [29]; motif size = 2-11 bp, 15 motifs, +/- strand, pval <0.001). The 15 most significant motif sequences were searched for known plant-associated cis elements using PLACE Signal Scan [30]. Perfect match cis-elements within motif sequences were counted for all putative regulatory sequences in the training set (Table 2).

Construct design, transient protein expression, GFP microscopy and immunodetection

OsGLP8-6 and *OsGLP8-12* genes from cultivar SHZ-2 were amplified from p-GEM-T plasmids using the primers (GLP6_prot_F, 5' CACCATGGCTTCACCCTCTTCCCT 3', GLP6_prot_R, 5' GTAGTGATTGTTCTCCCAGAAC 3', GLP12_prot_F, 5'CACCATGGCCTCCTCTTCCTTATTTC 3', and GLP12_prot_R, 5' GTAGTTGTTCTCCCAGAACTGAG 3') and were ligated into the entry vectors (pENTR-D TOPO; Invitrogen, Carlsbad, CA). Positive entry clones were digested into two fragments using *Mlu*I and were purified using sodium acetate precipitation. Plasmid fragments containing GLP genes were recombined into the GFP protein fusion plasmid, pEarleyGate103 [31] using LR Clonase (Invitrogen, Carlsbad, CA). After amplification in *E. coli*, purified plasmids were transformed into *Agrobacterium tumafaciens* strain EHA105. Transformed *A. tumafaciens* strains containing one of three constructs (OsGLP6+C'GFP, OsGLP12+C'GFP and OsGF14e+C'GFP) were grown in 20 ml cultures according to [32] and were infiltrated into leaves of *Nicotiana*

benthimiana. Epidermal peels of transformed *N. benthimiana* leaves were imaged at 200X magnification with a compound epi-fluorescent microscope.

GFP-fusion proteins were visualized by immunodetection. *N. benthimiana* leaves were ground in Laemmli buffer (BioRad, Hercules, CA) 4 days post inoculation. Crude protein extracts were boiled for 5 min, and separated on 12% SDS PAGE gels. Proteins were transferred to a nylon membrane and were probed with anti-GFP and anti-rabbit antibodies. Antibodies were detected with West Pico Chemi-luminescent Substrate (Thermo Scientific).

Hydrogen peroxide (H_2O_2) staining

H_2O_2 was visualized in leaf tissue using 3, 3'-diaminobenzidine (DAB) as described [33]. Twenty-one day old plants of cultivars LTH and SHZ-2 were spray inoculated with *M. oryzae* P06-6. Leaves were collected at three time points after inoculation and then stained with DAB. Leaves from SHZ-2 were subjected to a mock spray treatment or mechanical wounding with a needle, were harvested 24 h post treatment, and then stained with DAB. Leaves were cleared of chlorophyll in 95% ethanol and digital images were taken under a dissecting microscope.

RESULTS

A germin-like protein gene family is located within the chr 8 QTL region

Twelve germin-like protein genes were predicted in a 2.8 Mb region within the proposed rice blast QTL region on rice chromosome 8 (Figure 1). Eleven family members are clustered in a short 80 Kb section from approximately 5.18 to 5.26 Mb, and the twelfth is located downstream at approximately 7.99 Mb. Six *OsGLP* were

sequenced and described previously (Table 1). Alignments of gene and protein sequences from the Nipponbare reference genome (TIGR Pseudomolecules, Build 4.0; [23] indicate that the chr 8 *OsGLP* are highly similar to each other (data not shown). Pairwise comparisons of genomic sequences range in identity from 47% to 88% and corresponding comparisons of inferred amino acid sequences range from 70% to 98%. The gene family members are predicted to be transcribed from the positive strand with the exception of *OsGLP8-12.* All putative proteins have predicted N-terminal signal sequences of 22-25 amino acids and are predicted to be secreted extracellularly.

Phylogenetic reconstruction of *OsGLP* alleles among five rice cultivars including those from the reference cv. Nipponbare shows that the chr 8 *OsGLP* cluster contains genes in two germin subfamily groups, GER3 and GER4 (Figure 2; [8]. The GER3 subfamily includes *OsGLP8-1, 8-2, 8-3* and *8-12.* The GER4 subfamily includes seven members, *OsGLP8-5* to *8-11;* these are tandemly arranged as a cluster within 30 Kb and have pair wise amino acid identities in the range of 90%-98%.

Motifs in 5' regulatory regions are shared among *OsGLP* subfamily members

Initial bioinformatic comparisons of putative *OsGLP* promoter sequences from the two rice reference genomes, cv. Nipponbare (ssp. *japonica*) and cv. 93-11 (ssp. *indica*) revealed an 858 bp insertion sequence in the *indica* version (Figure 4B, 4C). The insertion fragment is located 590 bp upstream from the initiation codon (Figure 4B). The two resistant *indica* cultivars, IR64 and SHZ-2 contained the

insertion while the susceptible cultivars Azucena and LTH, like the *japonica* Nipponbare, did not (Figure 4C).

Shared motif analysis was performed using the 12 putative *OsGLP* promoter sequences from the Nipponbare reference genome sequence. The insertion sequence in the *indica* version of the putative promoter of *OsGLP8-6* was included in the training set (N=13) to look for additional cis-elements. The 15 most statistically overrepresented motifs among the thirteen 1 Kb upstream sequences were identified by the MEME algorithm. Each of the 15 motifs was identified in many *OsGLP* members (Table 3). Motif sequences were analyzed for known plant cis-elements and totals were summarized for each putative promoter (Table 2). Only exact match cis-elements were counted, though sequence degeneracy was observed across motif sequence alignments (Table 3). These are not the only putative cis-elements in the 5' regulatory regions of *OsGLP* genes, but rather, they represent motifs that are shared among family members.

Known plant associated cis-elements were identified within overrepresented motifs (Table 2). TATA-box sequences (TATATAA); [34] which are present just upstream of initiation codons were identified between 77-152 bp upstream from the ATG for the 12 *OsGLP* genes, but not in the *OsGLP8-6* insertion sequence. The W-BOX motif [(T)TGAC(Y)], a cis-element associated with rapid induction by wounding or fungal elicitors [35, 36], was present in multiple copies in all of the 5' regulatory regions and the *OsGLP8-6* insertion sequence. More W-BOX elements were discovered in GER4 family members compared to GER3. A pollen-specific cis-

element (GTGA) [37] and an endosperm specific element (AAAG) [38] were observed in all but two *OsGLP* regulatory regions, and higher copy numbers were also present in GER4 compared with GER3.

OsGLP alleles show sequence diversity among rice cultivars

Gene sequences of all 12 *OsGLP* alleles from two resistant and two susceptible cultivars were analyzed for sequence variation. Single nucleotide polymorphisms (SNPs) were present in one or more cultivars for all genes as noted by tree branching within gene clades among cultivars (Figure 2). SNPs predicted to result in non-synonymous codon changes were observed in one or more cultivars for all genes except *OsGLP8-6* (Table 4). There were no nucleotide and/or codon substitutions predicted to change protein function that correlated with resistant and susceptible genotypes. Two *OsGLP* alleles from the most resistant cultivar, SHZ-2, contained SNPs conferring premature stops; *OsGLP8-1* had a one base pair deletion resulting in a frameshift mutation and *OsGLP8-11* had a nucleotide substitution encoding a premature stop codon.

Expression of _OsGLP_ genes is developmentally regulated

To examine the constitutive and developmental expression of *OsGLP* genes, expression was monitored using gene-specific primers for all 12 genes in three positions of non-stressed leaves in three cultivars: Azucena, IR64 and SHZ-2 (Table 5). Transcripts for *OsGLP8-1, 8- 2* and 8-*10,* were not detected in any of the three leaf positions tested in any cultivar. The two resistant cultivars showed constitutive expression of *OsGLP8-3* in all leaf positions, while the susceptible variety showed no

expression. Conversely, *OsGLP8-9* was expressed only in Azucena and not in IR64 and SHZ-2. Some *OsGLP* family members exhibited developmental regulation of gene expression among leaf positions.

OsGLP genes are transiently induced in response to _M. oryzae_ and wounding

To determine if differential expression of *OsGLP* family members correlates with disease phenotypes, gene expression was tested by RT-PCR on a time course (0, 12, 24, and 48 h post inoculation, hpi) after *M. oryzae* infection (Figure 3A). As observed in developmental studies, transcripts of *OsGLP8-1* and 8-*2*, were not detected in any cultivar under any treatment or time point. Transcripts of GER3 subfamily members, *OsGLP8-3* and *OsGLP8-4,* were observed in untreated tissue in SHZ-2 and IR64, respectively, but not in the other cultivars (Figure 3A). *OsGLP8-12* was more highly expressed in untreated tissue of Azucena and LTH compared to IR64 and SHZ-2. Transient induction of *OsGLP8-12* was observed at 12 and 24 hpi in three of four cultivars.

GER4 subfamily genes were clearly induced in response to *M. oryzae* infection in all cultivars (Figure 3A). *OsGLP8-5* and *8-7* were constitutively expressed and transiently induced by *M. oryzae* infection in all cultivars. There were low baseline levels of *OsGLP8-8, 8-9* and *8-10* and all were transiently induced at 12 and 24 hpi. Basal transcript levels among cultivars differed for *OsGLP8-11,* but elevated expression was observed at 12 and 24 hpi. As predicted, transcripts of *OsGLP8-11* were not observed from SHZ-2 presumably due to the nonsense mutation in the gene sequence. SHZ-2 plants had a higher constitutive level of *OsGLP8-6* transcripts

compared to other cultivars (Figure 4A). This gene was induced at 12 and 24 h in all lines, but the relative induction levels were greater in resistant compared to susceptible cultivars.

Overall, *OsGLP* expression between resistant and susceptible alleles differs at two time points: 0 h and 48 hpi (Figure 3A). The resistant cultivars constitutively express different combinations of *OsGLP* genes compared to the susceptible. For the GER3 subfamily, the indica varieties utilize *OsGLP8-3* and *OsGLP8-4* while the japonicas express *OsGLP8-12*. The most resistant cultivar, SHZ-2, is the only one to constitutively express *OsGLP8-6* and also has the fewest number of genes induced at 48 hpi.

Most of the *OsGLP* genes were also induced by the mock spray treatment (not shown), though to lower levels than the *M. oryzae* inoculated. To test whether induction of *OsGLP* genes by the mock treatment is consistent with response to wounding, a subset of *OsGLP* genes was tested by RT-PCR in leaf tissue after pressurized water spray and mechanical wounding (Figure 3B). Of the five genes tested, all were induced by both treatments except for *OsGLP8-11* which was not observed from SHZ-2. Overall, these results show that *OsGLP* genes are induced by biotic and abiotic stimuli.

OsGLP proteins are localized in the plant cell wall

Fusion proteins of OsGLP8-6 and OsGLP8-12 with C' terminal green fluorescent proteins (GFP) were visualized by transient expression in the heterologous system, *N. benthimiana* (*Nb*; Figure 5A). Both OsGLP fusion proteins

showed spotted patterns at cell perimeters and across cell surfaces. The fusion proteins also associated with subcellular organelles including vesicles. The punctate, fluorescent patterns observed for OsGLP fusion proteins were distinct from the diffuse fluorescence observed for the cytoplasmic localized OsGF14e (Figure 5A). Visualizations of OsGLP8-6 and 8-12 fluorescent proteins in membrane-bound vesicles suggest that they are secreted to the cell wall.

Denatured monomer subunits of OsGLP fusion proteins were observed by immunoblot at the expected size of 55 KDa (OsGLP predicted size = 24KDa) compared to the control GFP protein of 26 KDa (Figure 5B). Two bands were observed for OsGLP8-12+C'GFP proteins suggesting multiple isoforms of this protein.

Resistant varieties produce H_2O_2 in early responses to rice blast infection

H_2O_2 was visualized in leaf tissue after rice blast inoculation by oxidation of 3,3'-diaminobenzidine (DAB) which results in a dark brown color (Figure 6A). The SHZ-2 leaves showed a few DAB stained spots as early as 12 hpi with *M. oryzae* while the LTH leaves did not. Leaves of SHZ-2 also showed a higher number of DAB stained spots at 24 and 48 hpi compared to LTH. Mock inoculated plants sprayed with gelatin solution displayed fewer and fainter spots than the *M. oryzae*-inoculated leaves suggesting that the *M. oryzae* spores themselves induce H_2O_2 production.

To test for H_2O_2 production after abiotic stimulation, leaves of SHZ-2 were subjected to a water spray treatment and mechanical wounding with a needle. H_2O_2 accumulated in faint spots after the water spray compared to the untreated, control leaf. There was also H_2O_2 accumulation in areas directly surrounding the needle

wounds at 24 h post treatment. These results show that rice leaves produce H_2O_2 after a variety of stimuli including wounding and pathogen infection.

DISCUSSION

We previously demonstrated that the more chr 8 *OsGLP* genes are silenced in a transgenic plant, the more susceptible the plant is to *M. oryzae* and *R. solani* [8]. This suggested that the multiple *OsGLP* among the 12 genes within the QTL region contribute collectively to broad-spectrum disease resistance. Our goals for this study were to identify functional polymorphism(s) in *OsGLP* alleles among rice blast resistant donor cultivars compared to susceptible cultivars. The result is a story of duplicated genes in two germin subfamilies with complex, cultivar-specific expression patterns. Biotic stress inducible expression confirms that multiple *OsGLP*s are pathogenesis related (PR) proteins in rice as in other crop species [39]. The early temporal induction of *OsGLPs* and subcellular localizations suggest that they play roles in early layers of plant defenses in the cell wall. The timing of differential expression observed between resistant and susceptible cultivars corresponds with differences in early physiological defense responses. We conclude that *OsGLP* gene regulation is the main advantage in resistant cultivars as opposed to loss of function mutations in coding sequences of susceptible cultivars.

The 12 *OsGLP* genes within the QTL region (Figure 1) represent two germin subfamilies, GER3 and GER4 (Figure 2). Members of these groups have been identified from multiple taxa (Chapter 1)[8]. Phylogenetic analyses indicate that both subfamilies were derived from a common ancestor, and that gene duplication

events occurred after separation of subfamily progenitors. The most recent duplication events occurred in the GER4 subfamily based on their close physical proximity to one another and similarities in gene and promoter sequences (Table 2 and 3). Allelic diversity occurs among *OsGLP* genes within a single cultivar, and among cultivars for a given *OsGLP* gene. Therefore, the potential number of allelic combinations of *OsGLP*s present among rice germplasm is very high.

We analyzed the variation in coding sequences among the alleles from the various cultivars. Most sequence variations correlated to subspecies pedigrees (*japonica* vs. *indica*), so we focused on mutations that putatively cause major-effect functional changes. Of all putative amino acid (aa) substitutions and insertion/deletions identified (Table 4), none were predicted in conserved functional domains such as active sites or N-terminal signal sequences. The most resistant cultivar, SHZ-2, had nonsense mutations in two genes (*OsGLP8-1* and *OsGLP8-11*), consistent with lack of visible transcripts. These mutations were not present in the moderately resistant cultivar, IR64, and gene silencing of *OsGLP8-11* conferred some loss of resistance in the cultivar, Kitaake [8]. It is unlikely that the loss of these genes provides an advantage to SHZ-2.

Analyses of 5' regulatory regions suggested that GER4 subfamily members are most important for defense as was predicted from gene silencing studies (Table 2)[8]. Gene expression profiling shows that many *OsGLP* of the GER4 subfamily are induced after challenge with *M. oryzae* in all cultivars, though one GER3 (*OsGLP8-12)* is also induced. In barley, *HvGER4* transcripts accumulated rapidly, as early as 3 hpi,

which correlated to the timing of papilla formation [9, 15]. We also observed induction of *OsGLP* early in defense responses at 12 hpi (Figure 3A). This induction occurs before penetration of the *M. oryzae* fungus into the cell wall which occurs between 18 and 24 hpi [40, 41]. The genes also showed sustained induction through 48 hpi indicating that they remain present throughout pathogenesis. Unexpectedly, many *OsGLP* were also induced by the mock spray treatment. The comparative induction after mock spray and mechanical wounding (Figure 3B) suggests that gene induction may, in part, be due to physical stimulation by the fungus [42]. The presence of multiple W-BOX transcription factor binding site motifs in *OsGLP* promoters supports this idea. WRKY transcription factors are rapidly induced by pathogen elicitors and in response to wounding and were shown to physically bind to W-BOX motifs [35]; [36]. The GER4 subfamily member promoters contained the highest number of W-BOX motifs, consistent with their visible induction by wounding and *M. oryzae*. In addition, some GER4 genes were induced by inoculation with *Xanthomonas oryzae* pv *oryzae* genome wide expression profiling experiments (Chapter 2).

Two obvious differences were observed between *OsGLP* expression patterns of resistant and susceptible plants. The first includes constitutive expression and transient induction of *OsGLP8-6* (Figure 4). The coding region of this gene was highly conserved suggesting that it may be functionally important for all cultivars (Table 4). The promoter insertion sequences identified in the two indica cultivars likely influence the differential expression patterns, especially with the addition of two W-

BOX motifs to the existing three copies. WRKY proteins bind cooperatively to clusters of W-BOX motifs [43]. Thus, we hypothesize that enhanced expression of *OsGLP8-6* in resistant cultivars is conferred through this alternative promoter. Though this particular polymorphism has not been tested in the QTL mapping population derived from SHZ-2 x LTH, associations of SNPs in the introns of *OsGLP8-6* and *OsGLP8-8* with disease phenotypes have been validated [44]. The second difference was observed in expression of *OsGLP8-12* which was more highly expressed in susceptible versus resistant cultivars (Figure 3A). Members of this subfamily (GER3) were not important for disease resistance in rice or barley [8, 9]. In fact, transient silencing of *HvGER3* resulted in hypersusceptibility to *B. graminis* [9]. It is plausible that this gene negatively regulates constitutive expression of GER4 family members which would be a disadvantage for the susceptible cultivars.

OsGLP genes are clearly involved in plant defenses, and they also show developmental regulation (Table 5). This is consistent with the previously identified rice chr 8 *OsGLP* (Table 1) which were identified in cDNA libraries from tissues including panicles, shoots, seeds and roots [45]. Developmentally-related cis-elements identified in *OsGLP* promoters support these observations (Table 2) [37, 38]. The cell wall localization of GLPs in rice and other species substantiates their roles as mediators of cell wall expansion [12], and also suggests that they may reinforce the cell wall upon stress induction [46].

Highly related orthologs of *OsGLP* have SOD activity that converts unstable and transient superoxide anions into H_2O [14] [11, 17]. Superoxide is produced

quickly in the apoplast following recognition of pathogen elicitors by NAPDH oxidases and peroxidases [18]. It was recently shown that *M. oryzae* requires fungal-produced superoxide for successful penetration of plant cuticles [47]. If rice *OsGLP* also exhibit SOD activity in the cell wall as predicted, then they likely produce some or all of the H_2O_2 produced after stress stimuli. Indeed, we observed H_2O_2 accumulation after the same stimuli that induced *OsGLP* genes (Figure 3 and 6). The earlier accumulation of H_2O_2 after infection with *M. oryzae* in the resistant cultivar, SHZ-2, compared to the susceptible, LTH, indicates that timing of defense response is key to the resistance strategy.

The evidence presented here, as well as that from other monocot and dicot species, shows the contribution of GLP to disease resistance QTL and the complexity of the gene family member regulation. Our data shows that *OsGLP* genes, particularly GER4 subfamily members, are induced by pathogens in all cultivars, but that temporal expression patterns differed. Corresponding with differential *OsGLP* expression among cultivars was differential accumulation of H_2O_2 after pathogen infection. Taken together, we conclude that *OsGLP* play roles in early defense responses by protecting plants at or near fungal penetration sites. Identifying sources of broad spectrum resistance and the underlying genetic components is promising for plant breeders who have found current sources of qualitative resistance unsatisfactory [2, 3] This knowledge facilitates the introgression of donor alleles into adapted cultivars.

Table 1. Previously described *OsGLP* genes on rice chromosome 8

Previous Name	GenBank Acc. No.[a]	TIGR[b] Locus ID	Gene Description	New Gene Name
GER3[c]	AF032973	LOC_Os08g08960	cDNA from panicle at ripening	*OsGLP8-2*
GLP16[e]	AF042489	LOC_Os08g08960	cDNA from immature seed	*OsGLP8-2*
GER2[c]	AF032972	LOC_Os08g08970	cDNA from etiolated shoot	*OsGLP8-3*
GER1[c]	AF032971	LOC_Os08g08980	cDNA from etiolated shoot	*OsGLP8-4*
GER6[c]	AF032976	LOC_Os08g09010	cDNA from green shoot	*OsGLP8-7*
RGLP2[d]	DQ414400	LOC_Os08g09060	promoter region from indica	*OsGLP8-10*
RGLP2[e]	AF141879	LOC_Os08g09060	cDNA from rice root	*OsGLP8-10*
RGLP1[e]	AF141880	LOC_Os08g09080	cDNA from rice root	*OsGLP8-11*

[a]GenBank gene sequence database (http://www.ncbi.nlm.nih.gov)

[b]MSU Rice Genome Annotation Database (http://www.tigr.org/tdb/e2k1/osa1/)

[c][45]

[d][48]

[e]unpublished

Table 2: Numbers of plant cis-elements in statistically overrepresented motifs[a] among *OsGLP* gene family 5' regulatory regions[b]

Cis-Element	*OsGLP8* Promoters												6 insert.[c]	Functional Association
	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***		
AAAG	1	2	0	1	2	3	3	3	3	3	0	1	1	endosperm specific [38]
GTGA	1	1	1	0	1	2	3	3	3	2	1	0	1	pollen specific [37]
TATATAA	1	1	1	1	1	1	1	1	1	1	1	1	0	gene transcription [34]
(T)TGACY	2	1	2	2	1	3	3	4	4	3	2	2	2	early plant defense/ wounding [35]; [36]

[a]The 15 most overrepresented motifs (size=2-12 bp) among training set sequences (n=13) were recognized by the MEME algorithm [29]. Known plant associated cis-elements in motif sequences were identified using PLACE signal scan [30]. The numbers of exact match cis-elements are presented in the table.

[b]1000 bp sequences upstream of ATG initiation codons for *OsGLP8-1* to *8-12* were extracted from the Nipponbare reference genome sequence, TIGR Pseudomolecules, V.4.

[c]The indica-specific *OsGLP8-6* insertion sequence (858 bp) was cloned and sequenced from cultivars IR64 & SHZ-2 and was included in the motif search training set.

Table 3: Overrepresented motif sequences identified by the MEME algorithm from *OsGLP* promoters (1kb upstream from initiation codon)

Motif	*OsGLP8* Promoter	Strand	Location	Motif Sequence Alignment
1	LOC_Os08g09060	+	-118	C C A A A G C A T C A C
	LOC_Os08g09040	+	-102	C C A A A G C A T C A C
	LOC_Os08g09020	+	-128	C C A A A G C A T C A C
	LOC_Os08g09010	+	-107	C C A A A G C A T C A C
	LOC_Os08g08920	+	-54	C C A A A G C A T C A C
	LOC_Os08g08980	+	-59	C C A A A G C A T C T C
	LOC_Os08g09000	+	-79	C C A A A C C A T C A C
	LOC_Os08g08960	+	-51	T C A A A G C A T C A C
	LOC_Os08g13440	+	-86	C C A A A G C A T C T T
	LOC_Os08g08990	+	-149	C C A A A G C C A C A C
	LOC_Os08g08970	+	-757	C C T C A G C A T C G C
	LOC_Os08g09080	+	-77	C A A A T G C A T C T C
2	LOC_Os08g09060	+	-145	C T A T A T A A G C A C
	LOC_Os08g09010	+	-134	C T A T A T A A G C A C
	LOC_Os08g08990	+	-130	C T A T A T A A G C A C
	LOC_Os08g08960	+	-80	C T A T A T A A G C A C
	LOC_Os08g09040	+	-129	C T A T A T A A A C A C
	LOC_Os08g09020	+	-155	C T A T A T A A A C A C
	LOC_Os08g09000	+	-106	C T A T A T A A A C A C
	LOC_Os08g08920	+	-81	C T A T A T A A A C A C
	LOC_Os08g13440	+	-115	C T A T A A A A T C A C
	LOC_Os08g09080	+	-103	C T A T A T A A G C G C
	LOC_Os08g08980	+	-88	C T A T A A A A T C A C
	LOC_Os08g08970	+	-90	C T A T A A A A T C A C
	GLP8-6_insertion	+	-357	C T G T A G C A C C A C
3	LOC_Os08g09080	-	-178	G A A C A A G T C A A
	LOC_Os08g09060	-	-192	G A A C A A G T C A A
	LOC_Os08g09040	-	-175	G A A C A A G T C A A
	LOC_Os08g09020	-	-199	G A A C A A G T C A A
	LOC_Os08g09010	-	-181	G A A C A A G T C A A
	LOC_Os08g09000	**+**	**-799**	G A A C A A G T C A A
	LOC_Os08g08990	-	-181	G A A C A A G T C A A
	LOC_Os08g08980	-	-175	G A A C A A G T C A A
	LOC_Os08g08920	-	-146	G A A C A A G T C A T
	LOC_Os08g13440	-	-184	G A T C A A G T T A A
	LOC_Os08g08970	-	-178	G A T T A A G T C A A
	LOC_Os08g08960	-	-509	G A A C C A A T C A A
	GLP8-6_insertion	-	-799	G A C C A C G T C A G
4	LOC_Os08g09060	+	-251	C C T G C A G G C A G C
	LOC_Os08g09010	+	-242	C C T G C A G G C A G C
	LOC_Os08g09000	+	-195	C C T G C A G G C T G C

	LOC_Os08g08990	+	-233	CCTGCAGGCTGC
	LOC_Os08g09040	+	-234	CCTGCAAGCAGC
	LOC_Os08g09020	+	-260	CCTGCAGGCAAC
	LOC_Os08g08970	-	-366	CCTGCAGTCAGC
	GLP8-6_insertion	-	-590	CATGCAGGCATC
	LOC_Os08g13440	-	-660	CCAGCCGGCAGA
	LOC_Os08g09080	-	-157	CATGCGAGCAAC
	LOC_Os08g08960	-	-112	CATGCAGTTTGC
	LOC_Os08g08980	+	-440	CCAGCCAGATGC
5	LOC_Os08g09060	+	-230	ACCATTTCAGTC
	LOC_Os08g09040	+	-213	ACCATTTCAGTC
	LOC_Os08g09010	+	-221	ACCATTTCAGTC
	LOC_Os08g09080	+	-226	ACCATTTCAATC
	LOC_Os08g08990	+	-219	ACCATTTCAATC
	LOC_Os08g08920	+	-179	ACCATTTCAATC
	LOC_Os08g09000	+	-181	AACATTTCAGTC
	LOC_Os08g09020	+	-239	ACCATTTCACTC
	LOC_Os08g13440	+	-228	TACTTTTCAATC
	LOC_Os08g08980	+	-216	TACTTTTCAATC
	LOC_Os08g08970	+	-218	TACTTTTCGATC
6	LOC_Os08g09060	+	-166	CACGCTGCCGCG
	LOC_Os08g09010	+	-155	CACGCTGCCGCG
	LOC_Os08g09000	+	-126	CACGCTGCCGCG
	LOC_Os08g08970	+	-782	CACTCTGGCGCG
	LOC_Os08g09040	+	-149	CACGCTGGCGTG
	LOC_Os08g09020	+	-175	TACGCTGCCGCA
	LOC_Os08g13440	-	-877	CACTCTCTCGCG
7	LOC_Os08g09060	+	-103	ACGATAAACACA
	LOC_Os08g09020	+	-113	ACGATAAACACA
	LOC_Os08g09010	+	-92	ACGATAAACACA
	LOC_Os08g08970	+	-37	ACGATAAACACA
	LOC_Os08g13440	+	-60	ACGATAACCACA
	LOC_Os08g09040	+	-87	GCGATAAACACA
	LOC_Os08g08960	+	-35	ACCATAAACACA
	LOC_Os08g09000	+	-64	AAGAGAAACACA
	LOC_Os08g09080	+	-456	ACGATGAACGCA
	LOC_Os08g08920	-	-901	ACGAGAAACATA
	LOC_Os08g08990	+	-410	AAGATGTACACA
	LOC_Os08g08980	+	-382	AAGATATACATA
	GLP8-6_insertion	+	-197	AATCTAAACACA
8	GLP8-6_insertion	+	-971	GTGAGGGGTGAC
	LOC_Os08g09020	+	-520	GTGAGGGGTGAC
	LOC_Os08g09010	+	-844	GTGAGCGGTGAC
	LOC_Os08g09000	+	-647	GTGAGGGGTGAC
	LOC_Os08g09040	+	-499	GTGAGAGGTGAC
	LOC_Os08g13440	-	**-148**	GAGCGTGGTGAC

	LOC_Os08g08990	-	**-964**	GTGAGCGGCTAG
	LOC_Os08g08960	+	-457	GTTAGTGGTTAC
	LOC_Os08g08970	-	**-140**	TTGCGTGGCGAC
	LOC_Os08g09060	+	-626	GTGATCAGTGAC
9	LOC_Os08g09060	+	-490	AAAGGTTGGAAA
	LOC_Os08g09020	+	-463	AAAGGTTGGAAA
	LOC_Os08g09010	+	-464	AAAGGTTGGAAA
	LOC_Os08g09000	-	**-507**	AAAAGTTGGAAA
	GLP8-6_insertion	-	-997	AGGTTGGAAC
	LOC_Os08g09040	+	-445	AAAGGTCGGAAA
	LOC_Os08g08990	+	-464	AAAGGATGGAAA
	LOC_Os08g13440	+	-424	AAAAATTGGAAA
	LOC_Os08g08980	+	-632	AAACGTTGGATA
	LOC_Os08g08960	-	-412	AAAGATTCGAAA
	LOC_Os08g08970	+	-974	AAAAGTTGCACA
	LOC_Os08g09080	+	-352	AAACGTTTTAAA
	LOC_Os08g08920	+	-502	ATGGGTTGTAAA
10	LOC_Os08g09040	+	-463	GGTGACCAAGT
	LOC_Os08g09020	+	-480	GGTGACCAAGT
	LOC_Os08g09010	+	-481	GGTGACCAAGT
	LOC_Os08g09060	+	-508	GGCGACCAAGT
	LOC_Os08g08920	-	-845	TCTGACCAAGC
	LOC_Os08g09080	-	-210	TGTGACCAATT
	LOC_Os08g08980	-	-195	GCTGACCACGC
	LOC_Os08g09000	+	-586	GGTGGCCGAGT
	LOC_Os08g08990	+	-822	TGAGACCAAGT
	GLP8-6_insertion	+	-667	GGCAACCAAGT
	LOC_Os08g13440	-	-209	GCTGACCAAAA
	LOC_Os08g08970	-	-204	GCTGACCAAAA
	LOC_Os08g08960	-	-712	GATGACCAATT
11	LOC_Os08g09060	+	-310	CGACCACGTCAG
	LOC_Os08g09040	+	-291	CGACCACGTCAG
	LOC_Os08g09020	+	-317	CGACCACGTCAG
	LOC_Os08g09010	+	-286	CGACCACGTCTG
	LOC_Os08g08970	+	-744	CCGCCACGTCGG
	LOC_Os08g13440	+	-134	CGACGACCTCTG
12	LOC_Os08g09060	+	-213	GCTGACATCAGT
	LOC_Os08g09040	+	-196	GCTGACATCAGT
	LOC_Os08g09020	+	-220	GCTGACATCAGT
	LOC_Os08g09000	+	-169	GCTGACATCAGT
	LOC_Os08g09010	+	-204	GCTGACACCAGT
	LOC_Os08g08990	+	-205	GCTGACATCATC
13	LOC_Os08g09060	+	-265	GCTGAAAAGTTC
	LOC_Os08g09020	+	-274	GCTGAAAAGTTC
	LOC_Os08g09010	+	-256	GCTGAAAAGTTC

	LOC_Os08g09000	+	-226	GCTGAAAAGTTC
	LOC_Os08g08990	+	-254	GCTGAAGAGTTC
	LOC_Os08g09040	+	-248	GCTGAAAAGATC
14	LOC_Os08g13440	-	-26	AGTTAGCTAGC
	LOC_Os08g09060	+	-88	AGTTAGCTAGC
	LOC_Os08g09010	+	-73	AGCTAGCTAGC
	LOC_Os08g09080	-	-199	AGTCAGCTAGC
	LOC_Os08g09040	+	-72	AGTTAGCCAGC
	LOC_Os08g09020	+	-98	AGTTAGCTAGA
	LOC_Os08g08960	-	-799	ATTTAGCTAGC
	LOC_Os08g08990	-	-70	AGCTAGCTAGA
	LOC_Os08g08970	+	-237	GTCTAGCTAGC
	LOC_Os08g09000	+	-43	AGTTAGCTTCC
15	LOC_Os08g09060	+	-525	TTTGATCCCAAG
	LOC_Os08g09020	+	-497	TTTGATCCCAAG
	LOC_Os08g09040	+	-480	TTTGGTCCCAAG
	LOC_Os08g08960	+	-788	TTTGATCCCATG
	LOC_Os08g08970	-	-576	TTTGGTCGCAAG
	GLP8-6_insertion	-	-623	CTTGATGCCAAG
	LOC_Os08g09010	-	-656	TTTTAACCCAAG
	LOC_Os08g08920	-	-586	TTTTATGCCAAG
	LOC_Os08g13440	+	-173	TTTGCACGCAAG
	LOC_Os08g08990	+	-841	TTTGGTGCAATG
	LOC_Os08g09080	-	-631	ATTGATCCTACG
	LOC_Os08g09000	-	-862	ATTGTTCCAAAG
	LOC_Os08g08980	+	-106	TTTGAACCAACC

Table 4: Nucleotide (SNP) and amino acid (aa) substitutions[a] among *OsGLP* alleles from four rice cultivars

		Rice Cultivars					
		Azucena		IR64		SHZ-2	
OsGLP	**TIGR Locus ID**	**SNPs**	**aa subs**	**SNPs**	**aa subs**	**SNPs**	**aa subs**
8-1	LOC_Os08g08920	3	1	4	2	1[d]	-
8-2	LOC_Os08g08960	1	0	3	1[b]	2	0
8-3	LOC_Os08g08970	0	0	5	1	4	2
8-4	LOC_Os08g08980	1	1	2	1	5	1
8-5	LOC_Os08g08990	1	0	7	1[c]	3	0
8-6	LOC_Os08g09000	0	0	2	0	0	0
8-7	LOC_Os08g09010						
8-8	LOC_Os08g09020	0	0	11	5	3	2
8-9	LOC_Os08g09040	0	0	2	1	0	0
8-10	LOC_Os08g09060	2	0	4	2	0	0
8-11	LOC_Os08g09080	0	0	0	0	18[e]	-
8-12	LOC_Os08g13440	2	2	5	1	4	1

[a]All reported nucleotide and amino acid substitutions are in reference to allele sequences from the temperate japonica cultivar, LTH.

[b]3bp deletion / 1 amino acid deletion

[c]3bp insertion/ 1 amino acid insertion

[d]1bp deletion / frameshift mutation / premature termination codon

[e]nuceotide substitution / premature termination codon

Table 5. Developmental expression[a] of *OsGLP* genes in leaves of 21-day old rice plants

	Leaf Position[b]								
	Azucena (-QTL)			IR64 (+QTL)			SHZ-2 (+QTL)		
Gene ↓	1	2	3	1	2	3	1	2	3
OsGLP8-1	-	-	-	-	-	-	-	-	-
OsGLP8-2	-	-	-	-	-	-	-	-	-
OsGLP8-3	-	-	-	+	+	+	+	+	+
OsGLP8-4	+	+	-	+	+	+	+	-	+
OsGLP8-5	+	+	+	+	+	+	+	+	+
OsGLP8-6	-	+	-	-	-	+	+	-	+
OsGLP8-7	+	+	+	+	+	+	+	+	+
OsGLP8-8	-	+	-	-	-	-	-	-	+
OsGLP8-9	+	+	-	-	-	-	-	-	-
OsGLP8-10	-	-	-	-	-	-	-	-	-
OsGLP8-11	+	+	+	+	+	+	-	-	-
OsGLP8-12	+	+	+	+	+	+	+	+	+

[a] (+) indicates presence of band in RT-PCR after 35 cycles

[b] Three leaf positions include youngest emerging leaf (1), 2nd youngest, expanded leaf (2) and 3rd youngest, mature leaf (3).

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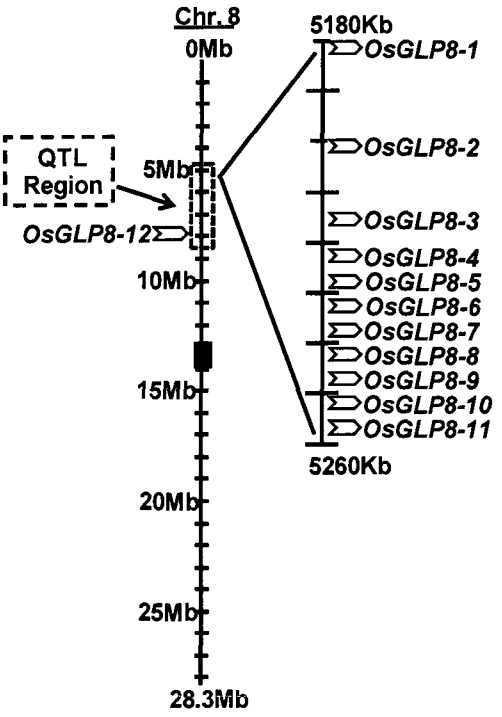

Figure 1: Distribution of *OsGLP* family members within the proposed QTL region on chromosome 8 of *Oryza sativa*. Gene predictions were based on TIGR rice pseudomolecules, Build 4.0. The chromosome and inset are represented as 1 Mb and 10 Kb increments, respectively. The centromeric region is shown as a black rectangle, and relative positions of the 12 *OsGLP* genes are shown as white arrows.

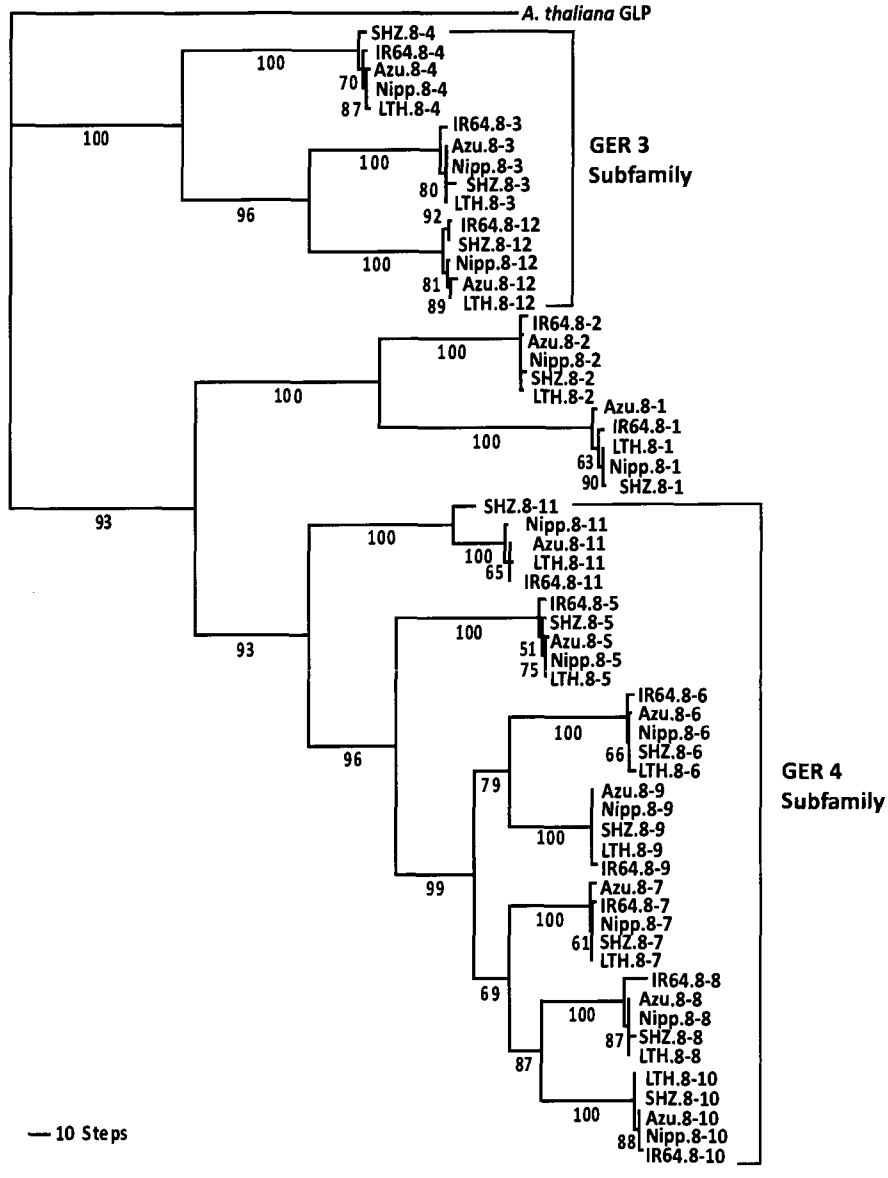

Figure 2. Phylogenetic comparison of allelic versions of *OsGLP* genes. Genomic sequences of *OsGLP* genes from two resistant cultivars, SHZ-2 and IR64, two susceptible cultivars, Azucena (Azu) and LTH, and the reference rice cultivar, Nipponbare (Nipp) were aligned using the ClustalW2 algorithm [28]. An *Arabidopsis thaliana* germin-like protein sequence was chosen as an outgroup. This tree represents a random tree from a heuristic Maximum parsimony search. Bootstrap support values were calculated from 1000 replicates with 1000 trees saved each replicate. *OsGLPs* group into previously described germin subfaimiles, GER3 and GER4. *OsGLP8-1* and *8-2* fall between the subfamilies and are not catagorzied.

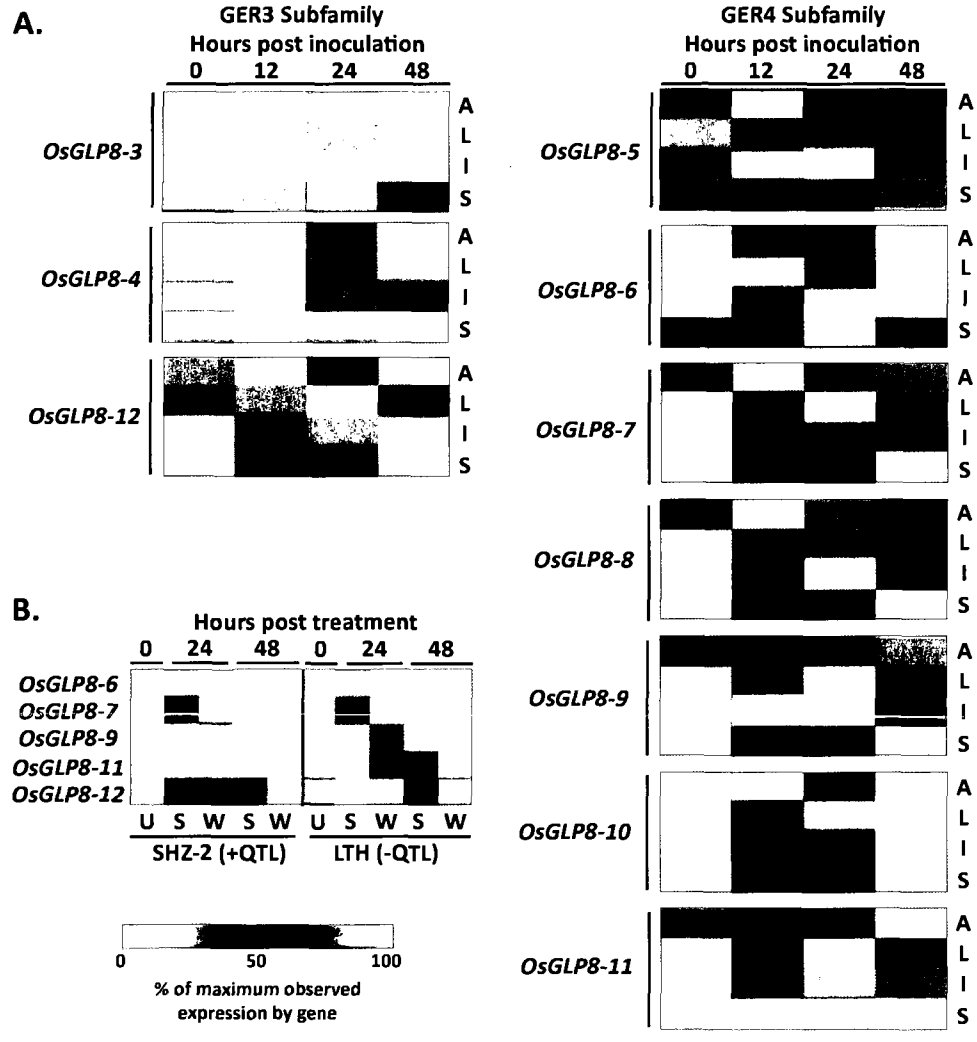

Figure 3. *OsGLP* genes are induced by rice blast infection and mechanical wounding. (A) Gene expression patterns of *OsGLP* genes after challenge with *M. oryzae*. Three week old plants of two susceptible, Azucena (A) and LTH (L), and two resistant, IR64 (I) and SHZ-2 (S), rice cultivars were challenged with virulent *M. oryzae* isolate, P06-6, and sampled at 3 time points post inoculation. *OsGLP* transcript levels were assayed with gene specific primers by RT-PCR, and average band intensities (*n*=3) are shown in the heatmaps. *OsGLP* genes in both GER3 & GER4 subfamilies were induced by *M. oryzae* infection. Differential expression between resistant and susceptible cultivars was observed for *OsGLP8-6* and *8-12*. (B) Induction of selected *OsGLP* genes after pressure spray (S) and mechanical wounding (W) compared to untreated controls (U) in rice cultivars SHZ-2 and LTH. Multiple *OsGLP* genes were induced by both treatments in the two cultivars. Transcripts of *OsGLP8-11* were not observed in the most resistant cultivar, SHZ-2.

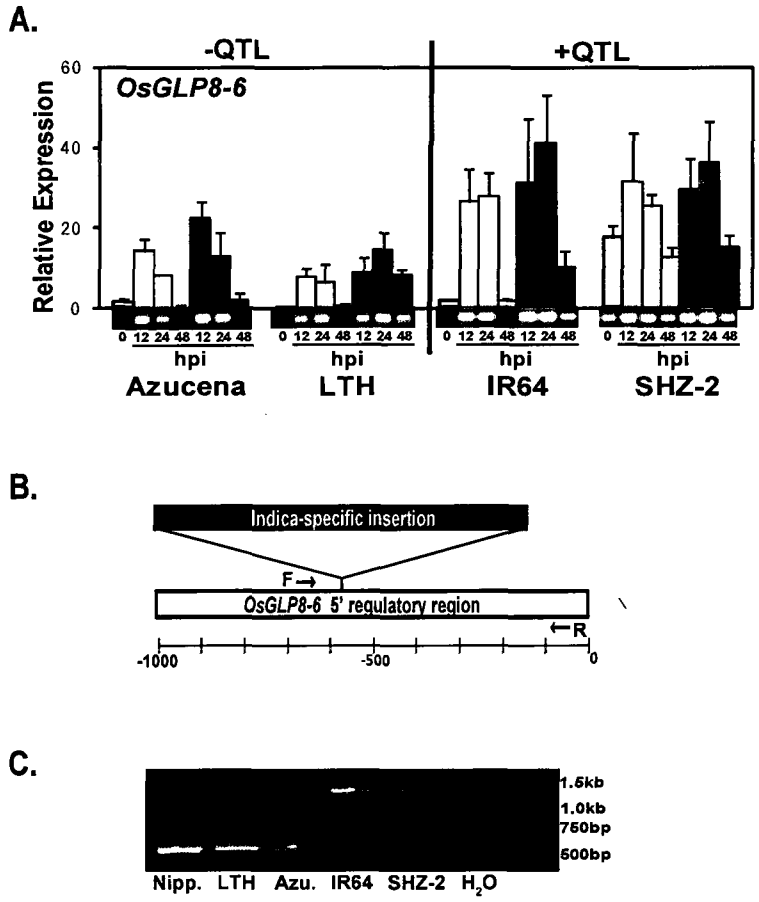

Figure 4: Differential expression of *OsGLP8-6* between resistant and susceptible cultivars. (A) SHZ-2, a highly resistant cultivar, showed higher constitutive expression (0 hours post inoculation, hpi) than the other cultivars. Both resistant (+QTL) cultivars had higher relative induction by mock inoculation (white bars) and *M. oryzae* infection (black bars) compared to susceptible (-QTL). (B) An 858bp insertion was identified in the *OsGLP8-6* 5' regulatory region of the 93-11 (ssp. *indica*) reference genome, and was not found in the Nipponbare (ssp. *japonica*) reference genome. (C) Primers designed 590bp upstream from the initiation codon amplified the insertion. Products of predicted sizes were amplified from the *japonica* cultivars, Nipponbare, LTH and Azucena and larger bands were observed from the *indica* cultivars, IR64 and SHZ-2.

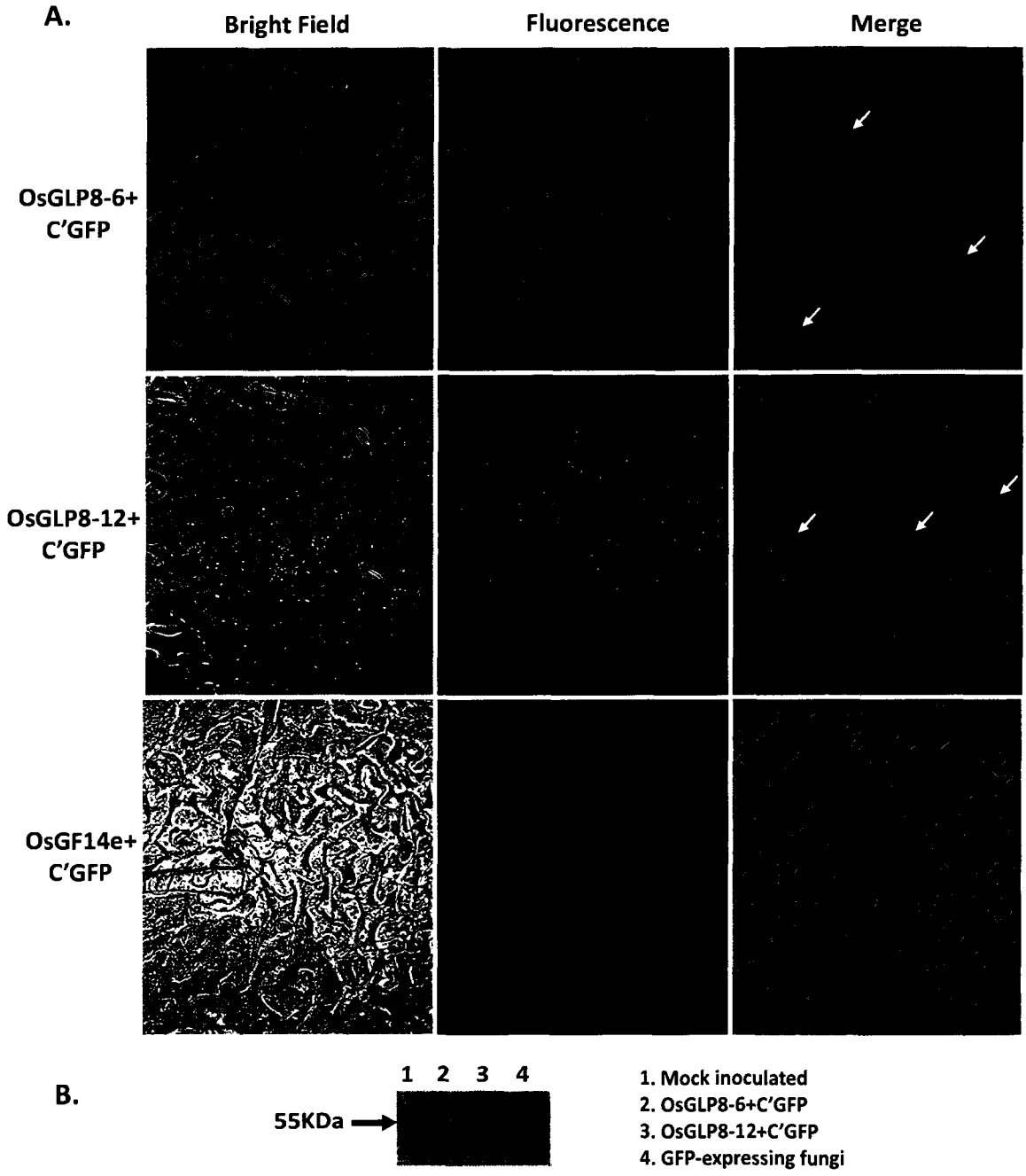

Figure 5. Subcellular localization of OsGLP8-6 and OsGLP8-12. (A) Fusion proteins with C'GFP tags were transiently expressed in *Nicotiana benthimiana* via *Agrobacterium tumafaciens* infiltration, and epidermal peels were examined by fluorescence microscopy 4 days post inoculation at 200X magnification. Both GLP proteins show punctate patterns in fluorescent images compared to diffuse fluroescence for the cytoplasmic protein, OsGF14e. Red structures are autofluorescent chloroplasts. GLP proteins were associated with vesicle structures in the merged images (white arrows). (B) Fusion proteins were detected by immunoblot with anti-GFP antibodies. Both show expected sizes around 56 KDa compared to GFP alone at 26KDa. OsGLP12+C'GFP showed two bands indicating multiple isoforms.

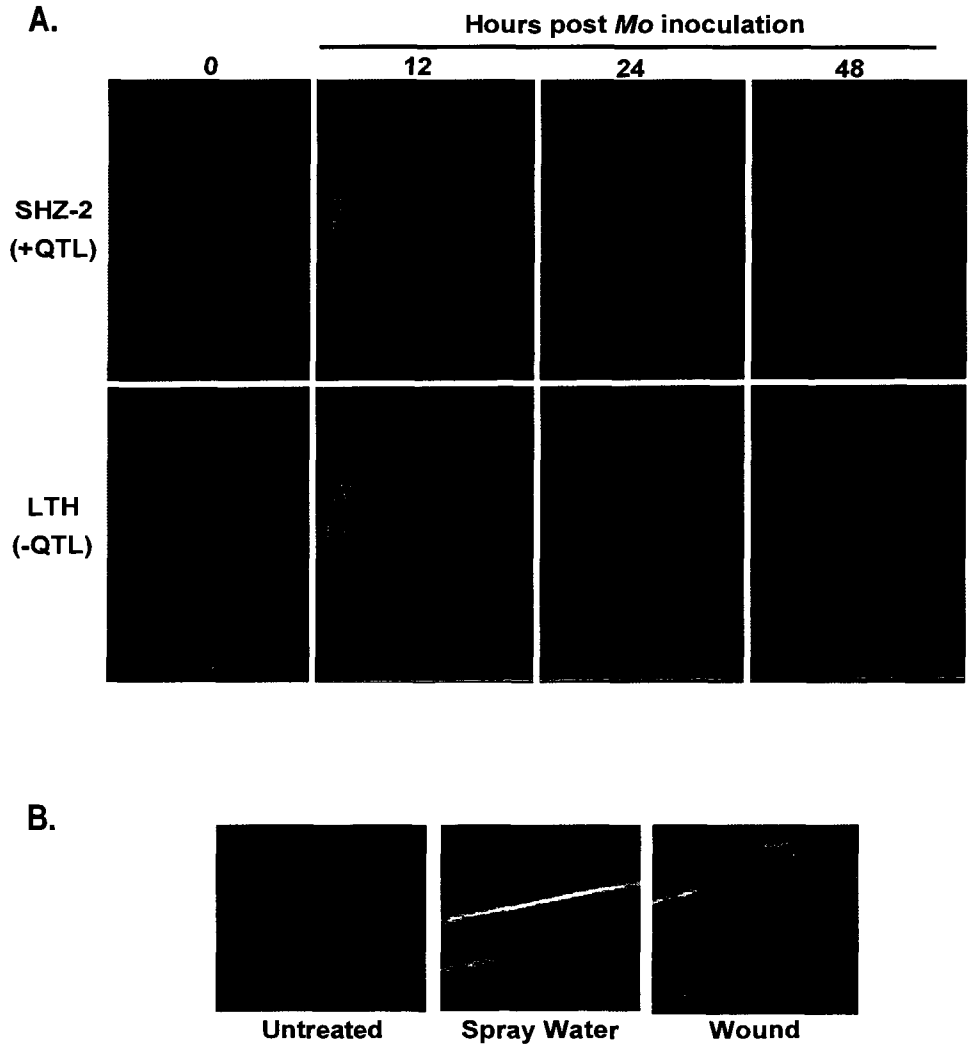

Figure 6. Hydrogen peroxide (H_2O_2) accumulation in leaf tissue of resistant and susceptible cultivars after stress treatments. H_2O_2 was visualized using 3,3'-diaminobenzidine (DAB) [33] and photos were taken with a dissecting microscope. Oxidation of DAB by H_2O_2 results in a dark brown color. (A) Twenty-one day old plants of highly resistant cultivar, SHZ-2, and susceptible cultivar, LTH, were spray inoculated with *M. oryzae* isolate, P06-6, and leaves were collected at three time points post inoculation. Leaves of SHZ-2 showed a higher number of DAB stained spots at 24 and 48hpi compared to LTH. (B) Leaves from SHZ-2 were subjected to a mock spray treatment or mechanical wounding with a needle and were harvested 24h post treatment. H_2O_2 accumulated in scattered spots after the mock spray and in areas directly surrounding the needle wounds.

CHAPTER 4

A QTL-associated oxalate oxidase confers partial resistance to fungal pathogens of rice

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S. Lee generated the silencing construct and transgenic plants. R. Davidson performed molecular characterizations of mutants and disease assays. C. Vera Cruz provided parental and advanced backcross lines, and G. Carillo performed genotyping and field phenotyping of the lines. H. Leung and J. Leach contributed to the conceptual design of the study.

Abstract

Oxalate oxidases (OXO) are considered pathogenesis related (PR) proteins based on gene and protein induction in cereal crops after fungal infection. Previous studies showed that a molecular marker for an *OXO* gene was significantly associated with reductions in rice blast disease symptoms in multiple rice quantitative trait loci (QTL) mapping populations. Four paralogous *OsOXO* genes on chromosome three co-localize with the physical QTL region. Using a gene silencing approach, we validate the involvement of one of four *OsOXOs* (*OsOXO4*) as a partial contributor to broad spectrum fungal disease resistance in rice. The *OsOXO*-RNAi silenced plants show increased susceptibility to two fungal rice pathogens, *Rhizoctonia solani* and *Magnaporthe oryzae*, and to the broad host range pathogen, *Sclerotinia sclerotiorum*. To confirm the role of *OsOXO4* in QTL-based disease resistance, an advanced backcross population was tested for the disease phenotypes observed in the silenced mutants. Lines with the resistant donor *OsOXO4* allele were resistant to *R. solani* and *M. oryzae* while those with the recurrent parent *OsOXO4* allele were susceptible. The partial loss of disease resistance in the silenced mutants and the co-segregation of polymorphic *OsOXO4* alleles with fungal resistance in the advanced backcross population show that *OsOXO4* functions as a component of complex, QTL-based disease resistance in rice. The non-specific resistance provided by *OsOXO4* suggests possible roles in basal defense and/or PAMP triggered immunity.

Introduction

Germins (also called "true germins") were first described as protein markers for germination in mature wheat (*Triticum aestivum*) embryos [1]. Transient induction of the proteins was observed 24 h after imbibing with water and they accounted for 0.1% of total soluble proteins [2, 3]. The same was observed for germins in barley (*Hordeum vulgare*) seedling tissue [4]. An early assumption was that germin proteins were critical for cell expansion during germination because they were found in cell wall, soluble and apoplastic protein fractions of expanding shoots and roots [1, 4-6]. Later, germins were found to be enzymes with oxalate oxidase (OXO) activity, and they were noted for extreme stability to heat, proteolysis and reducing agents such as sodium dodecyl sulfate [7-10]. The denatured monomer subunits are inactive while a large polymer complex is the active form. The crystal structure of the germin-type OXO complex was solved as a homohexamer with cleaved N' signal peptides and one manganese ($Mn^{2/3+}$) ion per monomer subunit [11, 12].

Proteins from barley seedlings with OXO activity had been purified and studied before the connection to germins was realized [13]. They have importance in human medical diagnostics due to their enzyme activity of converting oxalate to carbon dioxide and hydrogen peroxide [6]. Excess oxalate in human diets leads to medical conditions such as bladder and kidney stones, and oxalate levels are reliably detected using barley OXO assays [14, 15]. In plants, oxalate is an inert end product of carbon metabolism formed by the oxidation of ascorbate or other organic acids [6, 16-18]. At saturation, oxalate binds readily to calcium ions and is stored as insoluble calcium oxalate crystals in

the vacuole and/or cell wall [19]. This phenomenon is widely observed in plants that accumulate oxalate such as spinach, beet and rhubarb [20]. Interestingly, cereal crops have relatively low levels of oxalate on a dry weight basis [21].

OXO proteins belong to germin family subgroup within the cupin superfamily of proteins [10]. The germin family includes proteins with the characteristic germin-box motifs including OXOs and germin-like proteins (GLPs) [22]. Unlike GLPs, germin-type OXO proteins and/or gene orthologs are only found in true cereals. Gene families of *OXO*s occur in wheat, barley, ryegrass (*Lolium perenne*), rice (*Oryza sativa*) and maize (*Zea mays)* [3, 23-26]. The apparent lack of germin-type OXOs in dicots and other monocots suggests that they may play unique roles in oxalate metabolism of cereals.

OXOs are not only involved with plant development of cereals, but they are also transiently induced after pathogen infection. Therefore, they were designated as pathogenesis related (PR) proteins based on studies in wheat and barley [27]. In wheat, transcripts of an OXO gene, *gf2.8*, and the corresponding proteins increased within one to three days after infection with the powdery mildew fungus, *Blumeria graminis* f. sp. *tritici* [28]. Higher levels of *gf2.8* transcripts occurred in wheat mesophyll tissues compared to epidermal tissues. Another study found that the enzyme activity of gf2.8 was induced by auxin application, wounding, virus inoculation and heavy metal stress [29]. Similarly, barley OXO (HvOxOa) enzyme activity was transiently induced in leaves infected with *Blumeria graminis* f.sp. *hordei* [4], and gene and protein induction were observed in mesophyll tissue during both compatible and incompatible interactions with *B. graminis* [23]. Wound-dependent induction of multiple ryegrass OXOs was temporally

associated with increased OXO activity and H_2O_2 in leaf tissue [24]. In rice, one *OsOXO* gene was transiently induced in leaves after inoculation with *Magnaporthe oryzae*, the rice blast fungus [25]. These data suggest that OXOs are PR proteins among many different cereal crops.

Another potential substrate of the OXO enzyme is oxalic acid, a pathogenicity factor produced by necrotrophic pathogens such as *Sclerotinia sclerotiorum*. *S. sclerotiorum* has a broad host range and infects mostly herbaceous dicot plants, though a few occurences of disease on cool weather cereal crops have been reported [30]. It is a brute force pathogen that produces and secretes cell wall degrading enzymes and oxalic acid on host tissues. Fungal mutants with defective oxalate biosynthesis pathways caused significantly less disease than wild type isolates [31]. The mode of action of fungal-produced oxalic acid is thought to be suppression of the oxidative burst that occurs in host plants shortly after pathogen inoculation [32, 33]. For protection against diseases caused by *Sclerotinia* spp. and other oxalic acid-producing fungi, cereal OXO alleles have been over-expressed in a number of dicot host plants including sunflower, peanut, soybeans, rapeseed and chestnut [34-38].

In rice, correlative evidence suggests that *OsOXOs* contribute partially to the complex trait of quantitative disease resistance. An OXO candidate defense response (DR) gene marker from barley (*HvOxOa*) was associated with reductions in disease symptoms in multiple rice mapping populations [39, 40]. *HvOxOa* mapped within quantitative trait loci (QTL) intervals for bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae,* as well as rice blast (*M. orzyae*). Interestingly, there are multiple QTL for

partial resistance to sheath blight disease (*R. solani*), in the same region of rice chromosome 3 (Chapter 1). This region contains a tandem cluster of four paralogous *OsOXO* genes [25], (Chapter 1). The history of cereal OXO involvement in fungal defense response and the genetic and physical association of *HvOXOa* with a region of overlapping disease resistance QTL strongly suggest that rice *OsOXO*s are responsible for the partial resistance detected in mapping studies. Additionally, a related gene family of germin-like proteins was shown to contribute collectively to QTL-based fungal disease resistance [41]. Therefore, we investigate the role of the *OsOXO* gene family in broad spectrum fungal disease resistance in rice using an RNA-interference (RNAi) gene silencing strategy. We further test their role in QTL-based disease resistance by associating phenotypes observed in the silencing mutants with phenotypes of advanced breeding materials with known introgressions of resistant donor-*OsOXO* alleles.

METHODS

Fungal growth conditions & inoculation assays

M. oryzae isolates were grown on oatmeal agar at 26°C and stored at -20°C on sterile filter papers [42]. *R. solani* and *S. sclerotiorum* were grown on Difco potato dextrose agar (Becton Dickinson, Franklin Lakes, NJ) at 26°C. *R. solani* was stored at -20°C on sterilized, then infected rye grains. *S. sclerotiorum* were stored as sclerotia at 4°C; sclerotia were surface disinfested with 10% bleach for 1 min before plating.

Rice blast assays were performed on 14 to 21-day-old plants with *M. oryzae* isolate Che06861 as described [42]. Detached leaves were inoculated with 5 µl drops of 5×10^4 spores/ml spore suspension and were photographed seven days post

inoculation. The susceptible checks for the disease assay were rice cultivar Azucena and virulent *M. oryzae* isolate P06-6 [41].

Greenhouse sheath blight screenings were performed on 14-day-old plants using the microchamber assay [43] and *R. solani* isolate RM01401. Plants were incubated in microchambers for 14 days post inoculation and were scored for both disease index (DI; lesion height/plant height*9) and visual index (0-9 scale). Detached leaf sheath blight assays were carried out on 21-day-old rice plants as described [44]. Plugs (1 cm) of *R. solani* were placed on adaxial surface of rice leaves. Leaves were evaluated for lesions and photographed at 72 h post inoculation. Resistant and susceptible checks for both sheath blight assays were rice cultivars, Jasmine 85 and Lemont, respectively [45].

White mold detached leaf assays were performed on 28-day-old rice leaves according to [46] at 22°C and 26°C. Agar plugs (1 cm) of *Sclerotinia sclerotiorum* were placed on the abaxial surfaces of rice leaves (one plug per leaf). Leaves were evaluated for lesions and photographed four days post inoculation. A susceptible bean (*Phaseolus vulgaris*) cultivar was used as a control.

Development of transgenic rice lines

A conserved portion among the four *OsOXO* coding sequence was amplified from the cultivar, Kitaake, with the primers, (Chr3Oxo F, 5' CCGCCGGCGACGAGTTC 3' and Chr3Oxo R, 5' GGTGCCGATGATGCCGACGAGGAG 3'). The gene fragment was purified and ligated into the entry vector, pENTR-dTOPO (Invitrogen, Carlsbad, CA). The fragment was recombined into the pANDA silencing vector [47] using LR Clonase (Invitrogen, Carlsbad, CA). After amplification in *E. coli*, purified plasmids were

transformed into *Agrobacterium tumafaciens* strain EHA105. Stable transgenic rice plants were produced in the japonica cultivar, Kitaake, by described methods, and ex-plants were selected by adding hygromycin to the selection and regeneration media.

Plant materials and growth conditions

Oryza sativa varieties Moroberekan and Vandana, as well as advanced backcross lines from a cross between Moroberekan x Vandana were obtained from the International Rice Research Institute (IRRI, Los Baños, Philippines). Contribution of candidate DR gene markers, including *OsOXO*, to rice blast disease phenotypes in BC_3F_4 lines has been documented [40]. A set of BC_3F_4 lines with partial blast resistance and various combinations of Moroberekan-derived candidate DR genes were intermated in all pairwise combinations, advanced to BC_3F_6 (Carrillo, Thesis). These rice lines were extensively phenotyped for rice blast disease symptoms in India and the Philippines and were genotyped with 172 SSR markers and 10 candidate DR gene markers [25](Carrillo, thesis).

Rice plants were grown in a custom potting mix (1:1:2, peat: sand: Pro-Mix BX soilless potting media). Transgenic and wild-type Kitaake plants used for detached leaf blast, sheath blight and white mold disease assays were grown in a growth chamber at 28°C in 80% humidity with a photoperiod of 14 h light/10 h dark. For other assays, transgenic plants, Kitaake, Lemont, Jasmine, Moroberekan, Vandana and advanced backcross lines were grown in the greenhouse.

RNA isolation, cDNA preparation and real-time quantitative reverse transcriptase PCR

RNA was extracted from non-stressed rice leaf tissue using Trizol (Invitrogen, Carlsbad, CA) and the manufacturer's protocol. RNA samples were treated with 1.5 units of DNAse (Promega, Madison,WI) per 1 ug of RNA. RNA concentrations were determined using a spectrophotometer, and RNA samples were visualized in ethidium bromide stained gels. PCR was performed with RNA template to verify no product amplification due to DNA contamination. Single-strand cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's protocol with 1 ug starting RNA and 50 pmol of oligo dT_{20}. Hygromycin expression was detected by reverse transcription PCR using the primers, HygF and HygR [41], with a 60°C annealing temperature.

OsOXO gene transcript levels were measured by real-time quantitative reverse transcriptase PCR (RT-qPCR) using the primers, OXO_RT_F4 (5'-GCCGGCGAGACGTTCGTCATCCCG-3') and OXO_RT_R2 (5'-ACGAAGACGATGCCAGGGTTCTG-3'). The primers were designed to amplify all four paralogous *OsOXO* genes on rice chr 3. The reference gene, 18s ribosomal RNA, was used to normalize samples [48]. RT-qPCR transcripts were detected using the SYBR green Supermix for iQ (Quanta Biosciences, Inc., Gaithersburg, MD) and the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). PCR reactions (25 ul) contained 12.5 ul Supermix, 6.5 ul diluted template cDNA (1:1 cDNA:water) and 6 ul of 5 uM primer mix. Both primer sets were tested for efficiency on genomic DNA dilutions of (1:5, 1:25, 1:125, 1:625) starting with 25 ng/ul, and melt curves of PCR products were

examined. All RT-qPCR primer/template combinations were performed with three technical replications per plate. An annealing temperature of 60°C was used with OXO_RT and 18s primers and both primer sets were run on the same plate. Average OXO_RT threshold cycle (C_t) values were normalized by subtracting the average C_t of the reference gene (ΔC_t). Transcript amounts for transgenic plants were calculated relative to the average ΔC_t of control plants for a given experiment ($\Delta\Delta C_t$). Fold changes were calculated as $2^{|\Delta\Delta Ct|}$ [49].

Statistical analyses

Linear regressions of sheath blight disease index scores x fold change were performed using SAS 7.1 and proc reg (SAS Institute, Inc., Cary, NC). Chi square calculations revealed 3:1 segregation in the 14-19 T_2 population and were done in SAS using the prob chi function. Analysis of variance for sheath blight disease index scores was conducted with SAS and proc GLM for Moroberekan, Vandana and advanced backcross lines and mean values were compared with an REGWQ multiple testing correction at $p<0.05$.

RESULTS

OXO-RNAi transgenic lines are silenced for one *OsOXO* gene.

The four *OsOXO* genes on rice chr 3 are highly similar with pairwise gene sequence identities in the range of 90-97%. The high similarity made it difficult to obtain gene-specific primers, so a set of RT-qPCR primers that amplify all four genes were used for these studies. The efficiency of the OXO_RT primers in a dilution series of genomic DNA was 96.9% and the correlation coefficient of the standard curve was 0.997. Based

on an alignment of the four *OsOXO* gene sequences from the Nipponbare reference genome, the OXO_RT primers are expected to amplify three unique amplicons of the same size; the products from *OsOXO3* and *OsOXO4* are identical. As expected, melt curves of the PCR products from genomic DNA template contained three different melt peaks corresponding to three unique products. PCR products from cDNA, however, resolved to only one melt peak, presumably the *OsOXO4* gene, which was the only one of four *OsOXO* genes detected in previous transcript profiling studies [25, 50].

Four T_0 explants (3, 5, 14 and 38) and four control plants including two wild-type (Kitaake) and two lines transformed with the empty vector were screened by RT-qPCR. A range of 40 to 100-fold down regulation of *OsOXO4* was observed among the independent transgenic lines compared to the control average (n=4) (Figure 1). All four T_0 plants and empty vector controls showed expression of the hygromycin transgene by RT-PCR, but the wild-type Kitaake plants did not (data not shown). These T_0 lines (3, 5, 14 and 38) were advanced to T_1 populations, and were screened for interactions with *M. oryzae* and *R. solani.* T_2 families were advanced from the parents most susceptible to both pathogens.

Increased silencing in OXO-RNAi T_2 lines correlates with loss of resistance to fungal pathogens

One T_2 family, derived from 14-19 T_1 parent, showed loss of resistance phenotypes to both pathogens and was selected for advanced screening (Figures 2A and 3A). Most (23 out of 30) T_2 plants had sheath blight disease index scores (average DI = 1.3, n=23) similar to wild-type and empty vector controls (average DI = 1.5, n=11).

Disease scores of seven out of 30 plants (average DI = 2.4, n=7) were higher than controls, but lower than the susceptible cultivar, Lemont (average DI = 4, n=7). These results show increased susceptibility (loss of resistance) to *R. solani* in approximately one-fourth (7/30 = 23.3%) of the T_2 population. Twelve of these 14-19 T_2 plants with contrasting sheath blight phenotypes were tested for silencing of *OsOXO* by RT-qPCR (Figure 2C). A range of 10 to 60-fold down regulation of *OsOXO4* was observed among T_2 plants compared to the control average (n=7). There is a strong relationship between increased gene silencing of *OsOXO* and increased sheath blight disease index (Figure 2C; p-value =0.04).

A separate group of ten 14-19 T_2 plants, propagated in the growth chamber, were screened for resistance to *M. oryzae* isolate Che06861 (Figure 3A). The wild-type rice variety Kitaake shows moderate resistance to this isolate that is distinct from single gene type resistance [41]. Four of ten T_2 plants showed mild rice blast disease including necrotic lesions that were more severe than those observed on Kitaake. The symptoms on T_2 plants, however, were not as severe as the susceptible check (rice cultivar Azucena and *M. oryzae* isolate P06-6) which showed lesions and considerable fungal mycelia growth (Figure 3A). The minor blast phenotypes indicate a partial loss of resistance to *M. oryzae* in the T_2 plants. Seven of ten plants were screened for expression of *OsOXO* by RT-qPCR (Figure 3B). Four T_2 plants showed a range of 3.5 to 21-fold down regulation of *OsOXO4* compared to the control average (n=4, Figure 3B). Although the increased symptoms were relatively minor, increased silencing of *OsOXO4* corresponded with increased rice blast disease.

The genotype of the 14-19 T_1 parent was tested in two ways. First, a total of 19 T_2 plants were screened for expression of the hygromycin transgene by 40 cycles of RT-PCR. Twelve of 19 plants showed expression of hygromycin and seven did not. A chi square test shows that these proportions are not significantly different from a three to one segregation pattern ($\chi 2 = 1.42$, $p=0.23$). In the T_2 sheath blight greenhouse population of 30, approximately one-fourth showed an increased disease phenotype compared to controls also consistent with a 3:1 segregation pattern ($\chi 2 = 0.04$, $p=0.83$).

Gene silencing and loss of disease resistance persists in the OXO-RNAi T_3 generation

T_3 families were advanced from two T_2 plants (14-19-30 and 14-19-35) with the highest levels of silencing. Fourteen plants per family were screened for sheath blight resistance using a detached leaf assay that shows more exaggerated symptoms compared to the microchamber assay (Figure 4A), [44]. The 14-19-30 family was segregating, with half (7/14) of the plants showing more severe lesions at inoculation sites than control plants. The 14-19-35 family showed a range of phenotypes from mild to severe, but all 14 had larger lesions at inoculation sites compared to controls. Presence of the hygromycin transgene in genomic DNA was tested by PCR for the two T_3 families. Twelve of 14 plants in the 14-19-30 family had the transgene as did all 14 plants in the 14-19-35 family (Figure 4A). The two segregants of the 14-19-30 did not show sheath blight symptoms. This demonstrates that the 14-19 T_2 population was indeed segregating for the hygromycin transgene since wild-type segregants were detected among their progeny.

The 14 presumed homozygous plants of the 14-19-35 family were also screened for resistance to *M. oryzae* using a detached leaf assay (Figure 4B). As with the T_2 plants, increased disease, although mild, was observed in most of the T_3 plants. Six of the lines were screened for silencing by RT-qPCR and a range of 1.5 to 12-fold down regulation of *OsOXO* was measured compared to wild-type average (n=2; Figure 4C). The T_3 plant, 14-19-35-10, had the least amount of silencing and showed the least sheath blight disease among the sibling progeny (Figure 4A).

A silenced family of OXO-RNAi T_3 plants are susceptible to a broad-host range fungal pathogen

Five T_3 plants derived from the silenced 14-19-35 family were screened for resistance to the broad-host range pathogen *S. sclerotiorum,* a producer of oxalic acid (Figure 5), [31]. Normally, S. *sclerotiorum* does not occur on rice, likely because they differ in their optimal growth environments. Rice grows best at temperatures ranging between 26-28°C, and *S. sclerotiorum* infects its legume hosts most effectively at 21-22°C [46]. Detached leaf assays were performed for five T_3 and five wild-type rice plants at both temperatures. At optimal temperatures for rice (26-28°C), there was no disease on all leaves after seven days of incubation. However, at optimal temperatures for *S. sclerotiorum* (22°C), transgenic plants show more disease symptoms than wild-type at four days post inoculation (Figure 5). Leaves of bean (*P. vulgaris*), a natural host of *S. sclerotiorum,* showed lesions at two days post inoculation (data not shown). Though the wild-type rice plants showed lesions at seven days post inoculation, symptom development was clearly delayed compared to the *OsOXO* silenced plants.

Presence of a resistant donor *OsOXO* allele correlates with fungal disease resistance in advanced breeding materials

Moroberekan and Vandana are inbred parental cultivars of advanced breeding populations with contrasting disease phenotypes that have been well studied as sources of complex disease resistance [25, 40]. An advanced backcross population (BC_3F_4) derived from a cross between the two parents was made with the rice blast susceptible variety Vandana as the recurrent parent [40]. Molecular mapping showed that multiple DR gene markers, including a polymorphic *OXO* marker, were significantly associated with rice blast disease scores among the BC_3F_4 population (OXO; $R^2 = 0.25$), [40], and that Moroberekan was the donor of the resistant alleles. Subsequent genotypic data confirms that intermated BC_3F_6 lines, 19-6-7, 19-6-56, 19-6-99 contain the resistant donor *OsOXO* allele from Moroberekan and are highly resistant to *M. oryzae* across multiple environments [25]. Line 19-3-196 has the susceptible *OsOXO* allele from Vandana and is highly susceptible to *M. oryzae* [25].

Because a susceptible sheath blight phenotype was observed among the *OsOXO*-RNAi lines, a logical hypothesis was that Moroberekan, a donor of partial resistance through *OsOXO*, is more resistant to *R. solani* than Vandana, a susceptible variety. Sheath blight phenotypes were tested in microchamber assays for Moroberekan, Vandana and resistant and susceptible checks, Jasmine 85 and Lemont (n=12; Figure 6). As expected, Moroberekan was more resistant than Vandana to *R.solani* similar to the resistant and susceptible checks, Jasmine 85 and Lemont, respectively.

Sheath blight disease phenotypes were assayed on the two parents and selected BC_3F_6 lines, 19-6-7, 19-6-56, 19-6-99 and 19-3-196 (n=5 to 9) to look for agreement with previously described rice blast phenotypes (Figure 7), [25]. Average disease indexes for the three backcross lines with the Moroberekan *OsOXO* allele were not significantly different from Moroberekan, and were much lower than Vandana. One backcross line, 19-3-196, with the Vandana *OsOXO* allele showed more disease than all the other lines including Vandana. Average sheath blight disease indexes correlated with corresponding rice blast results from two independent field locations in Almora, India and IRRI, Philippines [25]; Figure 7B). This data strongly suggests that presence of the resistant donor *OsOXO* allele contributes to broad-spectrum fungal resistance in the advanced backcross population.

DISCUSSION

We demonstrate that reduction of *OsOXO4* gene transcripts results in loss of resistance to two economically important fungal pathogens of rice, *R. solani* and *M. oryzae*. In multiple generations of transgenic plants, increased gene silencing corresponded with increased disease symptoms (Figures 2, 3 and 4). Silenced *OsOXO*-RNAi lines also showed increased susceptibilty to the broad host range pathogen, *S. sclerotiorum* (Figure 5). These data are consistent with multiple functions for *OsOXO4,* such as providing a basal level of resistance for rice plants and degrading pathogen-produced oxalic acid. We further show that the broad-spectrum, partial resistance provided by *OsOXO4* is a component of multi-genic QTL-based disease resistance. Advanced backcross lines containing the donor resistant *OsOXO4* allele were resistant to

rice blast and sheath blight while a line with the recurrent parent *OsOXO4* allele was susceptible to both diseases (Figure 7). Understanding the genes underlying complex resistance is critical for accelerating breeding progress and is especially important for sheath blight control for which there are no major gene resistances among breeding germplasm [51].

There are only four *OsOXO* genes annotated in the rice genome, and they are arranged as a tandem cluster at the end of chromosome 3 (Chapter 1); [25]. In the wild type variety Kitaake only one of four gene transcripts was detected in RT-qPCR experiments. This is corroborated by evidence in genome wide expression libraries of rice. Among eighty transcript libraries from five varieties in the Massively Parallel Signature Sequencing (MPSS) Rice Expression Atlas (University of Delaware) [50], only transcript signatures of *OsOXO4* were detected (Chapter 1). The Rice Genome Annotation Digital Northern Database (Michigan State University) contains expressed sequence tag (EST) evidence for three of the four *OsOXO* genes [52]. Transcripts of *OsOXO1* were found only in floral tissues, *OsOXO3* in leaves and roots and *OsOXO4* in leaves, roots and seeds. *OsOXO4* was induced by pathogen stress [25]; (Chapter1, Seweon Lee, unpublished) and is so far the only *OsOXO* gene in the cluster associated with rice defense response.

The correlation of gene silencing and disease confirms a role for *OsOXO4* in general rice defense, but does not explain the mechanism of partial resistance to fungi with different infection strategies. *R. solani*, the sheath blight pathogen, is a

necrotrophic fungus that uses an infection cushion and possibly host-specific toxins to invade plant sheaths [53, 54]. *M. oryzae* is a hemi-biotrophic fungus that attaches to the leaf surface with an appresorium, and forms a penetration peg beneath the appressorium to breach cell walls. Both require crossing of the plant cell wall. The predicted role of OXO in plant development and defense is related to its production of H_2O_2 and its sub-cellular localization in the plant cell wall. The proteins were originally detected in tissues undergoing cell expansion, and they became increasingly insoluble after fungal infection [1, 28]. The location and activities of OXOs lead to the prediction that they participate in cell-wall remodeling processes that require H_2O_2 for oxidation of cell wall polymers and for subsequent polymer cross-linking [6][33]. A reasonable hypothesis for the function of OXO proteins is that they protect plant tissues by re-enforcing cell walls surrounding fungal entry sites [28].

In the context of plant innate immunity, cereal germin-type OXOs including rice *OsOXO4* likely contribute to constitutive basal defenses and to inducible immunity triggered by pathogen associated molecular patterns (PAMPs) [55][56]. The putative role in constitutive defenses is based on the presence of *OXO* transcripts in non-stressed leaf tissue (Chapter 1, this study) and the constitutive OXO gene and protein expression in wheat and barley leaf, seed and root tissues [57], (Chapter 1). OXO proteins may play roles in developmental processes [4] in the plant cell wall. Thus, their constitutive presence and activity could ensure the invading pathogen encounters a structural barrier as well as the continual presence of the active oxygen species H_2O_2. Another basal defense provided by OXO is its ability to degrade a pathogenicity factor produced

by some fungi, oxalic acid. This was demonstrated by accelerated formation of lesions in the silenced mutants after inoculation with the necrotrophic, broad host range pathogen, *S. sclerotinia* (Figure 5). The fact that germin-type OXOs are found only in cereal crops may explain the limited reports of *Sclerotinia*-caused disease on these plant species. The notion is further supported by successful control of *Sclerotinia* diseases in dicots by overexpression of cereal OXO alleles [34-37].

OXOs are constitutively expressed, but they are also inducible PR-proteins. *OsOXO4* was induced to higher levels by a virulent strain of *M. oryzae* in several rice varieties (Seweon Lee, unpublished), and was induced in resistant and susceptible interactions with both bacterial (*Xanthomonas oryzae* pv. *oryzae*) and fungal (*M. oryzae*) pathogens [25, 50]; (Chapter 1). Orthologous wheat and barley OXOs are also induced by fungi and viruses [4, 23, 28, 57]. Barley OXO transcripts (HvGER1) increased after salt stress, wounding and H_2O_2 application [57]. The non-specific induction of OXO genes by various pathogens suggests that they are induced by a variety of PAMP elicitor molecules. In addition, they may be induced by their own enzyme product, H_2O_2, in a feedback loop-type mechanism.

The disease phenotypes in the OXO-RNAi silenced mutants were mild compared to susceptible checks. The minor phenotypes are consistent with *OsOXO4* contributing only partial resistance to a complex, multi-genic trait. The amount of resistance contributed by *OsOXO4* is largely dependent on the genetic background and allelic states of other inherent components of quantitative disease resistance. In the advanced

backcross population derived from a cross between Moroberekan and Vandana, OXO is an important contributor as it showed the strongest correlation with disease phenotypes among all candidate defense gene markers tested [40](Carrillo, thesis). The correlation of resistant rice blast and sheath blight phenotypes in the intermated BC_3F_6 lines with presence of the resistant donor *OsOXO4* allele from Moroberekan (Figure 7B) supports their role in broad spectrum QTL-based disease resistance.

Discovering genes contributing to broad spectrum, quantitative resistance is a much-needed and promising advance toward achieving durable, long-lasting resistance. The discovery that a gene encoding a PR protein, *OsOXO4*, is a contributor to QTL is consistent with similar findings for a related group of genes, the *OsGLP*s. Genes like *OsOXO* and *OsGLP* are desirable sources of resistance because they have dual functions in plant development and defense. Hypothetically, because these genes do not target single pathogen components for their effect, the protection they provided will be difficult for pathogens to overcome relative to single-major gene resistances. Future challenges for rice breeders include identifying natural variation at these loci and incorporating them into adapted varieties.

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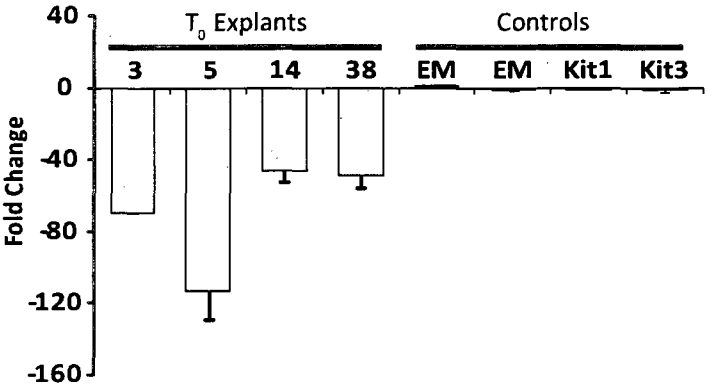


Figure 1. Transgenic OXO-RNAi T_0 plants are silenced for *OsOXO*. Stable transgenic rice plants with a conserved silencing trigger identical to all four *OsOXO* genes were generated by plant transformation. Expression of *OsOXO* genes was assessed by qRT-PCR using primers designed to amplify all *OsOXO* genes. Multiple independent T_0 plants showed gene silencing expressed as fold change relative to average of the controls (n=4), empty vector (EM) plants and wild type Kitaake (Kit).

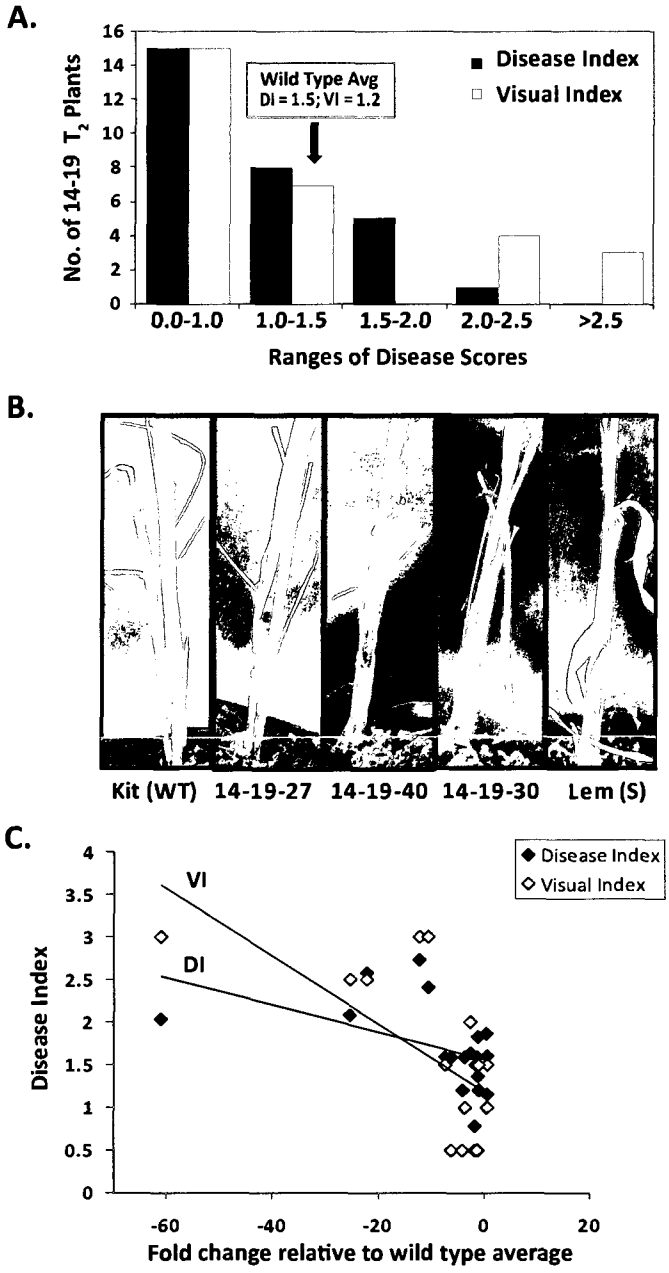


Figure 2. OXO-RNAi plants are susceptible to sheath blight disease. (A) Thirty plants from the OXO-RNAi 14-19 T_2 family were screened for resistance to *R.solani* using the microchamber method [43]. The histogram shows distributions of two disease measures, disease index (DI) and visual index (VI) compared to control plants (0 = no disease and 2.5 = moderately susceptible). (B) The range of sheath blight symptoms observed at 14 days post inoculation among transgenic plants compared to the wild type (Kit) and the susceptible check, Lemont (Lem). (C) Silencing of *OsOXO* correlates with increased sheath blight disease. Expression of *OsOXO* genes were assessed by qRT-PCR which is expressed as fold change relative to wild type average (n=6). Both measures of disease were plotted against fold change.

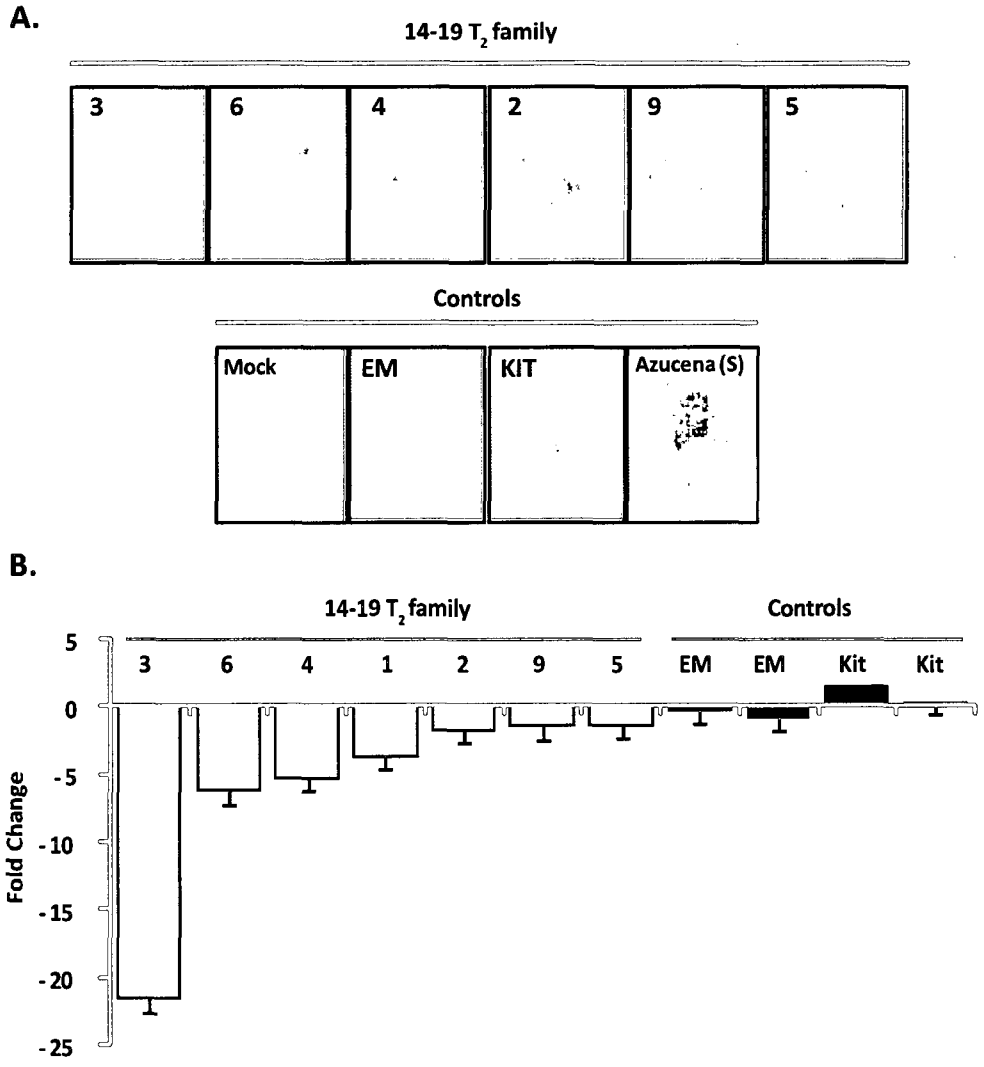


Figure 3. OsOXO-RNAi plants show mild rice blast phenotypes. (A) Plants from the 14-19 T_2 family were inoculated with *M. oryzae* isolate, Che06861. Photos were taken under the dissecting microscope at 7 days post inoculation. A range of rice blast symptoms were observed compared to controls, empty vector (EM) and wild type (KIT) and the susceptible check, Azucena. (B) Expression of *OsOXO* was assessed by qRT-PCR and is shown as fold change relative to control average (n=4). Gene silencing corresponded to the mild rice blast phenotypes.

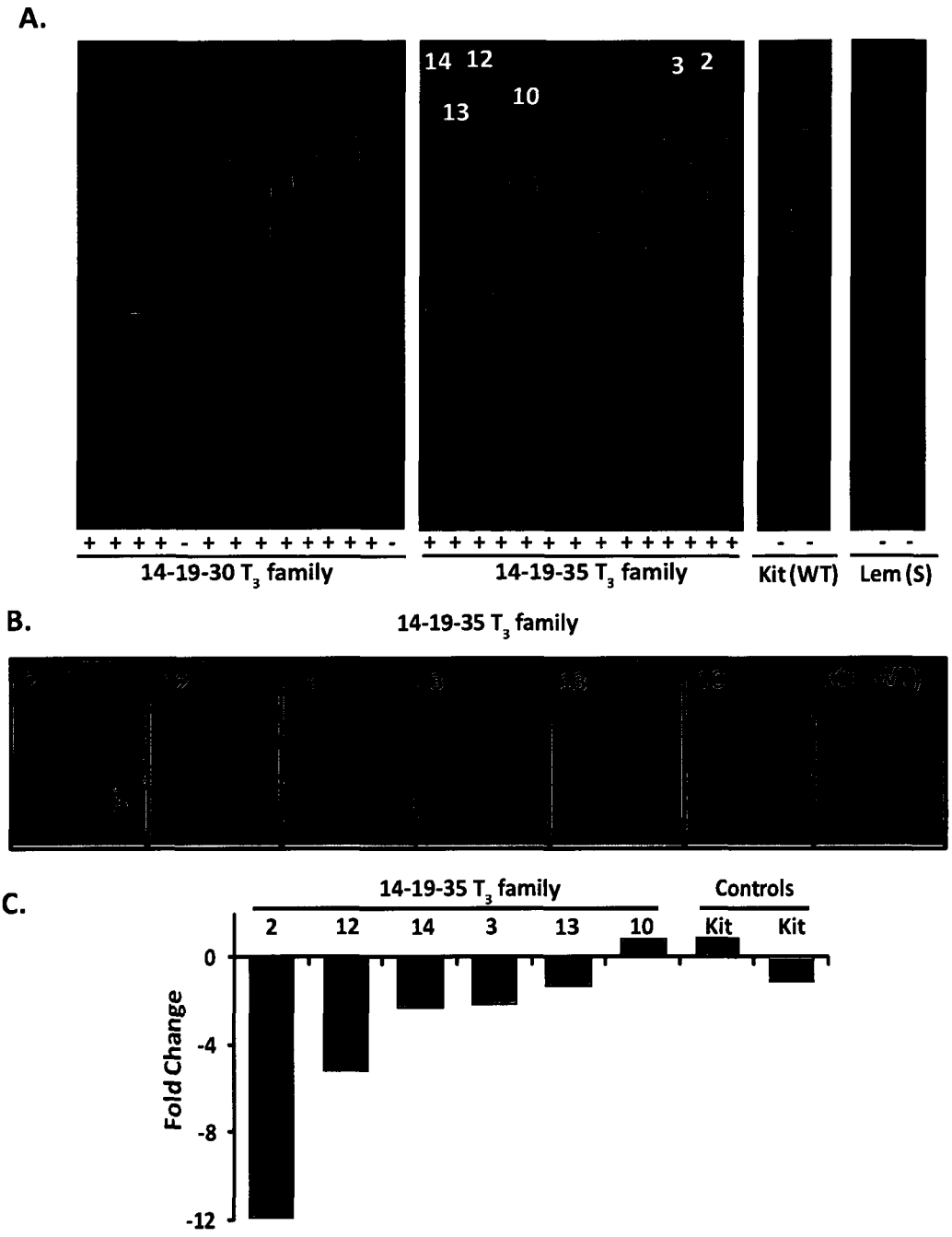
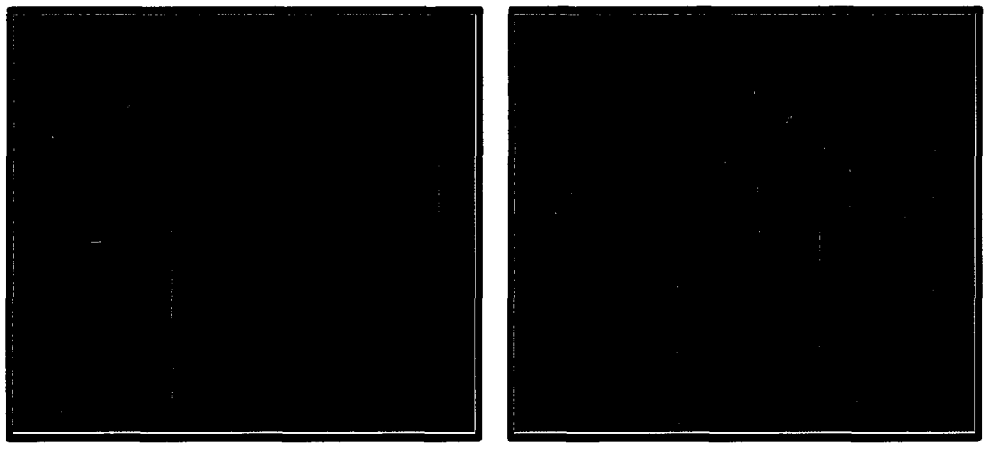


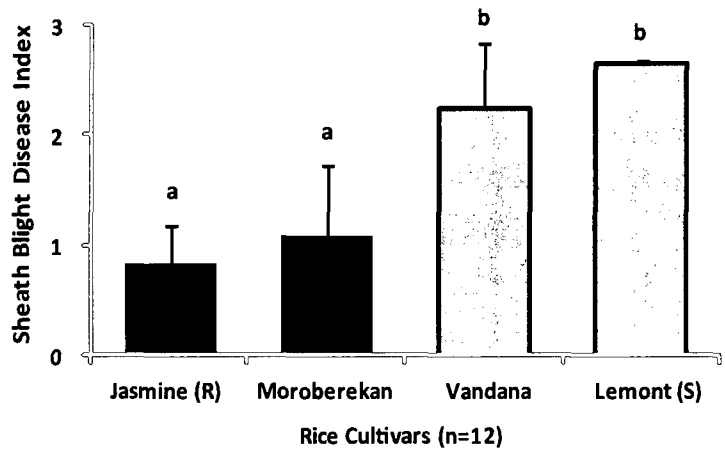
Figure 4. Disease phenotypes and silencing for OXO-RNAi T_3 generation. (A) Progeny from two susceptible T_2 plants were advanced and screened for sheath blight resistance using a detached leaf assay compared to wildtype (Kit) and susceptible check (Lem). Plants were also genotyped for the presence (+) or absence (-) of the hygromycin transgene in genomic DNA. (B) Selected plants of the 14-19-35 family were screened for resistance to *M. oryzae* using a detached leaf assay [42]. (C) *OsOXO* expression was screened in the same plants in (B), and the corresponding plants from (A) are indicated with white letters. Gene silencing and disease phenotypes persisted in T_3 generation.



Kitaake (WT) **14-19-35 T_3 Plants**

Figure 5. Silenced OXO-RNAi T_3 plants show increased susceptibility to the broad host range pathogen, *Sclerotinia sclerotiorum*, causal agent of white mold of bean. Five plants from the T_3 family described in Figure 4 and Kitaake wild-type (WT) were inoculated with *S. sclerotiorum* using a detached leaf assay [46] at 21-22°C. Photos were taken 4 days post inoculation (dpi). The assay was performed at 26-28°C and there were no symptoms at 7 dpi (not shown).

A.



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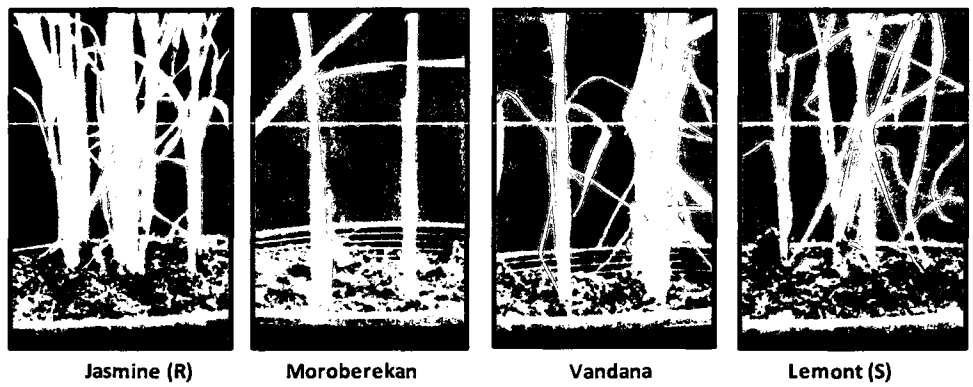

Figure 6. Sheath blight phenotypes of Moroberekan and Vandana compared to resistant and susceptible checks. Moroberekan and Vandana are parents of an advanced backcross population that has been extensively genotyped and evaluated for rice blast resistance [25, 40]. (A) They were evaluated for sheath blight resistance using the microchamber method [43] and scored by disease index. ANOVA using SAS and proc GLM was used to compare means using an REGWQ multiple testing correction at p=0.05. Moroberekan, the rice blast resistant parent, shows comparable resistance to *R. solani* as the resistant check, Jasmine. Vandana, the susceptible, recurrent parent, showed higher disease index than Moroberekan, and was not different from the susceptible check, Lemont. (B) Representative sheath blight symptoms on four rice cultivars.

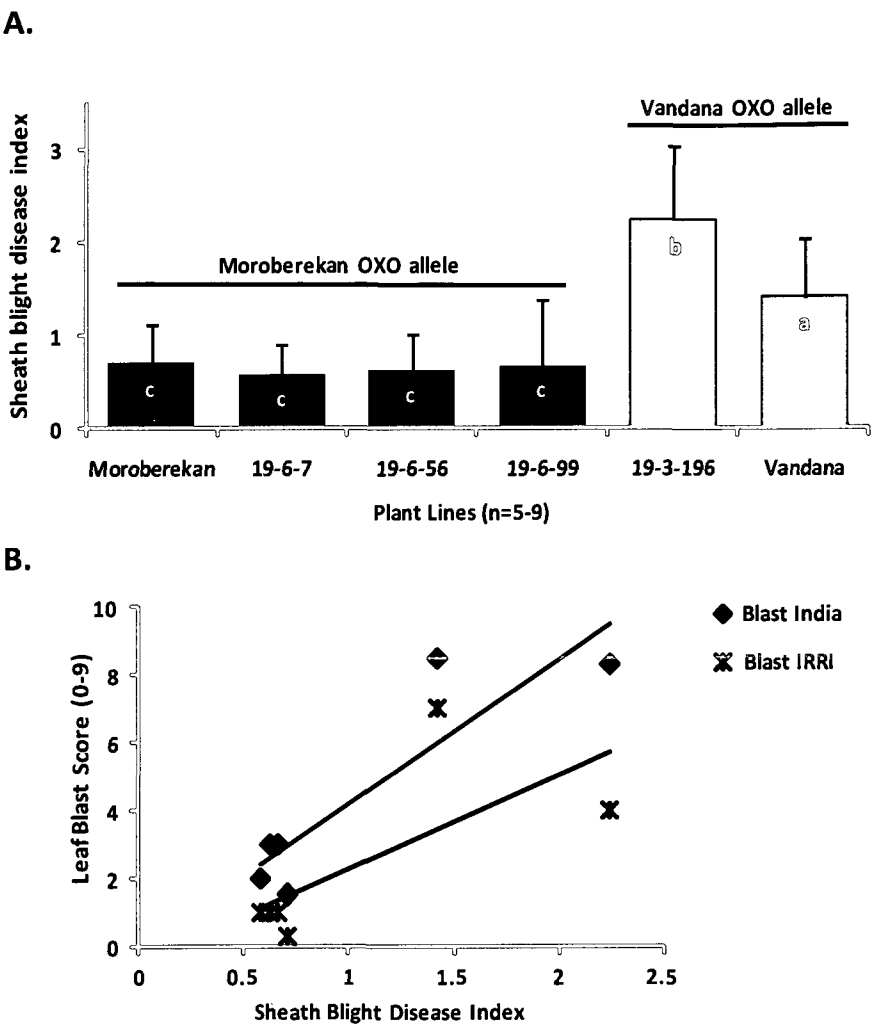

Figure 7. Sheath blight and rice blast resistance correlate with presence of resistant-OsOXO alleles among advanced backcross (BC) lines. (A) Moroberekan, Vandana and four advanced BC lines were inoculated with *R. solani* using the microchamber method [43]. Average disease index scores were compared with ANOVA (SAS, proc GLM) and an REGWQ multiple testing correction at p=0.05. The lines with Moroberekan *OsOXO* alleles show the same phenotypes as that parent. The same was observed for one BC line with the Vandana *OsOXO* allele and Vandana. (B) Average leaf blast scores for the parents and BC lines taken from [25] were plotted against corresponding sheath blight disease indexes. A positive correlation was observed between the two different disease scores including the blast scores obtained from Almora, India and IRRI, Philippines.

CHAPTER 5

CONCLUSIONS

The body of work presented in this dissertation represents a comprehensive use of bioinformatic and molecular biology tools in combination with traditional plant pathology methods to address hypotheses regarding functional relationships of genes governing partial disease resistance phenotypes. Rice is the first crop species to have a high quality and well-annotated genome. In recent years, genome-wide transcriptome and single nucleotide polymorphism (SNP) data have become available (Appendix I). In addition, there are excellent genetic stocks such as quantitative trait loci (QTL) mapping populations that have been well characterized both genotypically and phenotypically. Given the abundance of information and plant materials, it is an ideal opportunity to apply computational tools to applied goals in rice breeding and cultivar improvement.

Genes and proteins in the germin family have been studied across plant taxa for over 30 years, though few of the efforts have been focused on rice. In the rice genome, there are 40 genes with the characteristic germin-box motif, and as many as six may be non-functional based on lack of expression evidence. The meta-analysis of phylogenetic relationships and gene expression evidence of germin family proteins in multiple plant species shows that GLPs are present in bryophytes and as gene families in monocots and dicots. In contrast, OXO gene families have been found only in true cereals suggesting that they participate in unique biochemical pathways related to oxalate metabolism and defense response in those plants. The

subfamily groupings presented in Chapter 2 provide a concise summarization of germin family sequences, information and literature and help clarify the ambiguous nomenclature that has evolved over the years. The review article (Chapter 2) not only clears up the nomenclature, but it also allows predictions of enzyme activity and stress responsiveness of germin family proteins based on phylogenetic relationships of amino acid sequences. Our data shows that germin proteins with superoxide dismutase (SOD) or oxalate oxidase (OXO) enzyme activities are most likely to be involved with disease resistance. This will likely be amended as currently unknown functions of other germin proteins are elucidated.

Germin-like proteins (GLPs) and oxalate oxidases (OXOs) were previously classified as pathogenesis-related (PR) genes based on studies in wheat and barley. Now, we conclude that these genes behave similarly in rice. PR genes or proteins are those that are observed to accumulate in downstream defense responses. In general, *OsGLP*s are induced during early stress responses (12 hpi; Chapter 3), and *OsOXO4* is induced at later time points (24-48 h; Chapter 2) which is consistent with observations in barley. Intriguingly, both *OsGLP*s and *OsOXO4* are induced by multiple stimuli demonstrating their flexibility in responding to attack. Both are induced by fungi, bacteria, and mock inoculations, and *OsGLP*s are also induced by mechanical wounding. In rice-bacterial systems, gene induction occurs after both compatible and incompatible interactions (Chapter 2). These types of stimuli are known to upregulate diverse plant hormone pathways suggesting that germin family genes may be turned on during multiple, overlapping defense response pathways. Predictions of small RNAs putatively targeting *OsGLP*s after rice blast infection and abscisic acid application (Chapter 2) and the identification of overrepresented cis-elements in 5' regulatory regions of *OsGLPs* (Chapter 3) provide well-founded hypotheses for future experiments regarding germin family gene regulation.

Gene silencing studies confirm roles for *OsGLP*s (Appendix II) and *OsOXO4* (Chapter 4) in broad-spectrum disease resistance in rice. Additionally, germin family proteins are also associated with developmental processes across various tissue types (Chapters 2, 3 and 4). These results raise questions of how these developmental genes/proteins confer partial resistance to diverse pathogens with entirely different infection strategies. Our data shows that *OsGLP*s are probably localized in the plant cell wall, and the same is likely true for *OsOXO4* based on a putative N' signal sequence. The predicted enzyme activities, SOD for OsGLPs and OXO for OsOXO4, have not been validated in rice, and these are the logical next experiments toward understanding their mechanisms in development and resistance. Both types of enzyme activities produce hydrogen peroxide (H_2O_2) though it is derived from entirely different substrates. The putative SODs likely serve as ROS scavengers that protect plants from oxidative stress induced by highly unstable superoxide radicals. However, it is also possible that *OsGLP*s participate in coordinated regulation of superoxide to produce H_2O_2 for growth and development of the cell wall. For those with OXO activity, the oxalate substrate is present in the plant, but may also be produced by the pathogen. It is not unreasonable to think that OsOXO4 metabolizes substrates from both sources given its constitutive presence in developing plant tissues and its ability to protect rice from the oxalic acid-producing fungus, *Sclerotinia sclerotiorum*. It would be interesting to compare endogenous oxalate contents across rice cultivars and relate them to disease phenotypes. Additionally, it is not known whether *Magnaporthe oryzae* or *Rhizoctonia solani* produces oxalic acid.

Finally, this work addresses questions about what types of genes confer resistance governed by QTL. Multiple hypotheses have been put forth including genes involved in morphology/development, basal defense or PTI, chemical warfare or signal transduction, weak forms of R-genes or ambiguous genes with no known function. In the case of the major effect

QTL on chromosome (chr) 8, an entire family of *OsGLP*s, especially those in the GER4 germin subfamily, contribute collectively to disease resistance (Chapter 3, Appendix II). This conforms to the notion of QTL consisting of multiple genes contributing additive effects. Allelic differences in gene expression, and not detrimental changes in coding sequences, were most consistent with resistant versus susceptible disease phenotypes. The correlation of timing of gene expression and H_2O_2 production after rice blast infection further supports the idea that timing of response is a key tactic for resistant cultivars. Only one germin gene, *OsOXO4*, corresponding with the chr 3 QTL contributes to partial disease resistance (Chapter 4). The main difference between disease phenotypes of the OsGLP-RNAi mutants and the OsOXO-RNAi was severity of infection. This reflects the differences in contribution of the genes to the overall phenotype in the cultivar, Kitaake, with multiple *OsGLP* genes contributing more than *OsOXO4*. Our results support two hypotheses regarding genes functioning in QTL; genes involved in development and downstream defense responses.

Collectively, the bioinformatic and functional work summarized here adds knowledge about sources of broad-spectrum disease resistance in rice that can be applied to other plant species. To continue this work, more candidate genes must be functionally validated in the lab and orthologues identified from other species. Ideally, the functional knowledge is, then, transferred to breeding programs for use in marker assisted selection. Success in utilizing QTL-associated candidate defense response genes will be achieved by screening diverse rice germplasm at the desired loci, incorporating 'good' alleles into agronomically favored cultivars, and testing them across diverse environments.

APPENDIX I.

Genome-wide SNP variation reveals relationships among landraces and modern varieties of rice

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R. Davidson validated large effect SNPs, calculated allele frequencies, analyzed relationships between genomic introgressions and quantitative trait loci, and contributed to the OryzaSNP database search pages. R. Davidson contributed to the manuscript and created Figures 1 and 4.

Title: Genome-wide SNP variation reveals relationships among landraces and modern varieties of rice

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Abstract

Rice, the primary source of dietary calories for half of humanity, is the first crop plant for which a high-quality reference genome sequence from a single variety was produced. We used resequencing microarrays to interrogate 100 Mb of the unique fraction of the reference genome for 20 diverse varieties and landraces that capture the impressive genotypic and phenotypic diversity of domesticated rice. Here we report the distribution of 160,000 non-redundant single nucleotide polymorphisms (SNPs). Introgression patterns of shared SNPs revealed the breeding history and relationships among the 20 varieties; some introgressed regions are associated with agronomic traits that mark major milestones in rice improvement. This comprehensive SNP data provides a foundation for deep exploration of rice diversity and gene-trait relationships, and their use for future rice improvement.

Introduction

The genomes of domesticated rice, *Oryza sativa,* contain a wealth of information that can explain the large morphological, physiological, and ecological variation observed in the many varieties cultivated for food. To meet population demands by 2025, rice production must increase by 24% (1). The innovative use of genetic diversity will play a key role in reaching this ambitious goal.

The availability of complete genome sequences provides a starting point to understand the tremendous diversity of the rice gene pool at a fine scale. Among the organisms with a high-quality genome sequence from at least one individual or strain, such as human, mouse, and *Arabidopsis*, genome-wide surveys of single nucleotide polymorphism (SNP) variation in small or moderately sized samples have captured significant portions of within-species variation. In human and mouse, for example, a sampling of 71 and 15 individuals captured 80% and 43% of the genotypic variation, respectively (2, 3). In the model plant, *Arabidopsis*, 20 diverse varieties captured over 90% of the common genotypic variation in the species (4).

We initiated the OryzaSNP project (www.oryzaSNP.org) to discover genetic variation within 20 rice varieties and landraces. These varieties, the OryzaSNPset collection (SI Table 1), are genetically diverse and actively used in international breeding programs because of their wide range of agronomic attributes (5). Most varieties belong to the two main groups, indica and japonica, including tropical and temperate japonica, while others represent the aus, deep water and aromatic rice groups. Adapting a hybridization approach previously used for human, mouse, and *Arabidopsis* (3, 6, 7), we determined SNP variation in 100 Mb of the rice genome, representing ~80% of the non-repetitive portion of the 390 Mb Nipponbare

reference genome (8). Here, we describe the discovery of 159,478 high-quality, non-redundant single nucleotide polymorphisms (SNPs) distributed across the entire genomes of the OryzaSNPset. Relative to the model dicotyledenous plant *Arabidopsis* (4), typical haplotype blocks in indica rice varieties are longer (approximately 200 kb). Observed patterns of shared SNPs among groups indicate introgression due to recent breeding or historical out-crossing events.

Results and Discussion

SNP Prediction and Coverage. The non-repetitive sequence (100.1 Mb) used to design six ultra-high density tiling arrays was selected from the high quality reference genome of *O. sativa* variety Nipponbare (temperate japonica) (8). The arrays interrogated 26.2% of the genome with low repeat and therefore high genic content for SNP discovery, and targeted full or partial sequences corresponding to 57% of the 41,042 non-TE-related gene models in the TIGR r5 database.

We randomly selected regions represented on the arrays for dideoxy sequencing to generate 3.6 Mb of double-stranded sequence across all 20 varieties, corresponding to 1.8 Mb of non-redundant sequence in the reference genome. From these sequences, a gold standard set of curated polymorphisms from unambiguous alignments was compiled for quality assessment and training of our SNP predictors.

Two different computational methods were used for SNP discovery. A model-based (MB) approach, which considered the hybridization signature of a feature (corresponding to a position in the reference genome) and its tiling neighbors (corresponding to sequence bases in the immediate vicinity of that position) (2, 3, 6, 7), identified 242,196 non-redundant SNPs at non-repetitive sites (Table 1, SI Table 3). We also applied a support vector machine (SVM), machine learning

(ML) approach that had been used previously for *Arabidopsis* array data (7). Training sets for the modified ML method included the SNPs in the gold standard data set, the experimental hybridization data, information about repetitive oligonucleotides tiled on the arrays, and known polymorphisms in the indica genome. The latter were identified by comparing the Nipponbare genome with a second reference genome from the indica cultivar 93-11 (9), and their use improved the performance of the ML method for SNP detection in indica varieties.

A set of 316,373 SNPs at non-repetitive sites were predicted by the ML method (Table 1; SI Table 3). Assessed on the gold standard SNP data set, a false discovery rate (FDR) of 8.3% and a recall of 20.9% were observed for ML at non-repetitive sites across all varieties, compared to 9.1% and 14.4%, respectively, for the MB-detected SNPs. Several SNPs or insertion/deletion polymorphisms in close proximity can suppress hybridization and reduce SNP detection, and could account for low recall rates (7, 10). Together, the two datasets (MBML-union dataset) included 397,348 SNPs. Of these, 159,879 SNPs were predicted by both methods (MBML-intersect dataset, SI Table 3), and constitute a high quality subset (FDR 2.9%; recall 11.0%) used in subsequent analyses. About one fourth of the high quality MBML SNPs were validated at 97% accuracy (S.R. McCouch, personal communication). The genome-wide average of SNPs/kb using the MBML-intersect data was 1.6, and the transition/transversion rate (2.1) is similar to other species (11).

Allele frequencies for two-thirds of all sites were ≥ 0.15, and approximately one third of SNPs were present in seven to twelve varieties (Fig. 1b). The frequency distribution in rice differs markedly from that observed in *Arabidopsis*, where 50% of non-synonymous and 40% of synonymous SNPs occurred in only

one accession (12, 13). This is likely because the *A. thaliana* set included strains chosen for maximal genetic diversity (14), and thus had less population structure than the OryzaSNPset.

Most OrzyaSNPset varieties are donors of agronomic traits, with mapping populations available, enabling rapid application of the discovered SNPs in mapping experiments. As expected, many more coding region SNPs occurred in indica/aus (86.4%) than in japonica varieties, which include the Nipponbare reference from which the array was designed (13.6%). The highest number of SNPs occurred in the aus varieties (http://www.oryzasnp.org). Overall, between 26,700 and 57,700 SNP were detected in japonica x indica pairs in the MBML-intersect dataset. Over 33,000 SNPs distinguish IR64 and Azucena, the parents of widely used doubled haploids and recombinant inbred lines (15, 16) and references therein). Within the indica group, over 17,000 SNPs are predicted between Zhenshan 97B and Minghui 63, parents of Shanyou 63, the most popular hybrid rice in China from 1985 to 2000.

SNP Annotations and Large Effect SNPs. Most high quality MBML intersect SNPs (91,150/159,879) were located within gene models (Table 2). The proportions of genic SNPs identified as coding, intronic or UTR (43.5%, 41.6% and 15.7%, respectively) were different from the proportions identified in *Arabidopsis* [64.1%, 26.8% and 9.1%, respectively; (7)]. The larger rice introns (397 bp) contained more SNPs than *Arabidopsis* introns [168 bp; (17)].

Although the ratio of non-synonymous to synonymous SNPs in the array data was 1.2 across all gene models, the ratio dropped to 1.0 for sites in Pfam domains, regions expected to have fewer amino acid substitutions due to domain conservation (Fig. 1a, http://www.oryzasnp.org). Genes coding for cellulose

synthase, mitochondrial carrier, and amino acid transporter domains, all of which have transmembrane domains, had the lowest non-synonymous to synonymous substitution ratios. In contrast, sequences encoding leucine rich repeat and NB-ARC domains had a significantly higher ratio of non-synonymous to synonymous SNPs than average (Fig. 1a). As these domains are common in plant disease resistance proteins (26), this finding is consistent with these proteins being particularly diverse due to pathogen pressure (7, 18-20).

About 2.7% of the rice genes contained large-effect SNPs that are expected to affect the integrity of encoded proteins. These include changes predicted to disrupt intron splicing (73 donor site SNPs, 66 acceptor site SNPs), to introduce premature termination codons (388 SNPs), to eliminate translation initiation sites (41 SNPs) and to replace nonsense with sense codons (71 SNPs). Dideoxy sequencing was used to validate a subset of 209 large-effect SNPs across the 20 varieties (SI Table 4). The 16.3% FDR in this set was higher than the MBML average of 2.9% (SI Table 3), in agreement with what was observed in *Arabidopsis* (21). The FDR was evenly distributed across rice varieties and allele frequencies and, thus, was not biased against particular SNP calls.

Fifty percent of SNPs resulting in premature stops were rare, occurring in less than three varieties (Fig. 1b). Although this low frequency skew suggests that premature stops are detrimental, such loss of function changes are known to underlie many traits selected during domestication (22). The frequency distributions of SNPs changing intron splice sites and start/stop codons may reflect more neutral selection as compared to SNPs within coding regions. Alternatively, these gene structure features may not be shared with Nipponbare (Fig. 1b).

Phylogenetic Relationships, Population Structure, and Decay of Linkage Disequilibrium. The phylogenetic tree produced using the MBML-intersect dataset (Fig. 2a) revealed three distinct groups, with temperate and tropical japonicas closely allied in one group and the other groups correlating with aus and indica types, consistent with other analyses (12, 23). Dom-sufid (aromatic) grouped among the temperate japonica, a discrepancy from the ancestral placement relative to tropical japonica (23). This discrepancy was not observed when using the MB data only, and may result from the low prediction rate of SNPs for Dom-sufid by ML. Analysis of population structure by the Bayesian clustering program InStruct (24) also revealed the three groups (Fig. 2b).

The extent of linkage disequilibrium (LD) impacts both the genotyping effort required for whole genome association scans, as well as the resolution with which causal regions can be localized. LD reflects the strong population structure of the OryzaSNPset (Fig. 2c). For the MBML intersect dataset, LD extends to around 200 kb for the indica group, a higher estimate than reported previously (12-14). The limited number of SNPs among the japonica varieties renders the LD estimation for that group unreliable. When we focused on the regions used by Mather et al (14), LD levels similar to our genome-wide LD decay pattern were observed.

Extended Haplotype Sharing Identifies Large Scale Introgressions. Modern rice germplasm is to a large extent shaped by directed breeding, and the OryzaSNP dataset affords an opportunity to assess the degree of introgression and relationships among varietal groups. Most OryzaSNPset varieties are widely used in breeding programs and are under strong selection; thus, important aspects of breeding history can be inferred from introgression patterns among the three groups, indica, japonica and aus. Using a haplotype sharing ratio method in a

comparison of all group-pairs, we identified patterns of introgression among the three varietal groups along the 12 chromosomes (Fig. 3 and SI Fig. 1).

Large introgressions revealed by the SNP data reflect the breeding history of some rice varieties. For example, the japonica varieties Cypress and M202 show large regions on chr 1 introgressed from indica or aus (Fig. 3). These modern US semi-dwarf varieties were previously known to harbor introgressions from the indica variety IR8, the donor of the semi-dwarf gene *Sd1* important in the Green Revolution. The *Sd1* locus (25) is located at approximately 38.7 Mb on chr 1, corresponding to the overlapping introgression regions we observed in Cypress and M202.

The OryzaSNP data also confirmed introgressions from the aus group, a pool of traditional varieties commonly used as donors for abiotic stress tolerance traits into cultivated varieties. On chr 1, the indica variety Pokkali contains aus introgression regions that correspond to flanking markers and candidate genes underlying a salt tolerance QTL (*Saltol*) between 10.7 and 12.3 Mb (Fig. 3) (26). Moroberekan, a temperate japonica traditional variety from Africa and a popular donor for disease resistance and drought tolerance (27), contains several regions on chr 6 introgressed from indica or aus (Fig. 3), one of which co-localizes to a large cluster of NB-ARC type resistance genes between 9.2 and 11.1 Mb (28). These intriguing introgression patterns suggest that Pokkali and Moroberekan, "landraces" indigenous to India and Africa, respectively, were involved in crossbreeding with exotic germplasm by early rice farmers.

Two indica varieties from China, Minghui 63 and Zhenshan 97B, parents of a popular hybrid rice, Shanyou 63, are well characterized by QTL mapping for various traits and for gene action controlling heterosis (29, 30, 31). Minghui 63

shows an introgression on chr 6 (0-4 Mb) from japonica that colocalizes with QTL reported in several studies for traits including leaf area, vascular bundle number, root features, plant height, cooking quality, and amylose content (Fig. 4a, SI Table 5) (29, 30, 31). The introgression also contains heterotic loci associated with high yield performance in heterozygous vs. homozygous individuals (Fig. 4b, SI Table 5) (32). Of particular interest in this region are SNPs in the *waxy* locus, a starch synthase gene known to affect amylose content in rice grain (14). We detected large effect SNPs in four genes within the region. While the contribution of these genes to heterosis in Shanyou 63 is unknown, their identification demonstrates the power of the OryzaSNP data for identifying candidates at a high resolution (1 SNP per 2.6 kb in this 4 Mb region).

Common patterns of introgression could indicate other selection events. Across the 20 genomes, 295, 66, 12 and 1 positions were found in 2, 3, 4, to 5 varieties, respectively, that carried introgressions of the same type (see plots at top of chromosomes in Fig.3 and SI Fig. 1). These patterns of co-incidence were significantly different from random by a permutation test using 1,000 resamples ($p < 0.025$). A majority (70%) of the co-incident introgressions occur within 300 kb of one another. For example, in the region from 18.9-19.7 on chr 5, there are three positions where introgressions occur in 5, 4, and 3 of the same or other varieties. Using QTL data extracted from the Gramene database (http://gramene.org), this region was found to be associated with grain quality, carbohydrate content, and panicle traits based on an enrichment test ($p < 7.24$ E-42 by Fisher's exact test) (33). These blocks of co-localized, shared introgressions cover about 9% of the genome. The non-random distribution and co-incidence of introgressions may

indicate regions of intense selection, such as those related to desirable traits under domestication.

Haplotype sharing between pairs of accessions identified extended blocks where 90% or more of the SNPs are in common (SI Fig. 2). Pair-wise sharing revealed even more potential introgressions than observed by haplotype sharing across group-pairs. Large regions of aus introgressions in Pokkali occur on chr 5 from 8.3-13 Mb (Fig. 3 and SI Fig. 1) and are part of an area that appears highly conserved across all other indica and japonica.

To further examine the history of varietal selection, we tabulated the number and length of introgressions occurring from other varietal groups (SI Table 6). On average, the modern indica and japonica varieties have 8.4 Mb of introgressions from other groups whereas the aus type has approximately twice the length of introgressions (17.5 Mb). The average length of introgressions, however, appears to be similar across varietal groups (average 0.15 ± 0.04 Mb per introgression). One possibility is that modern varieties have been under strong selection for specific production environments, thus constraining the introgression of chromosomal fragments. On the other hand, the aus types, representing traditional varieties, could be subjected to a lesser degree of selection. Although a larger sample size will be needed to test this hypothesis, this analysis demonstrates the potential of genome-wide SNP datasets for probing the history of varietal selection in rice.

Conclusions

Our study provides comprehensive SNP data from a set of rice varieties that captures the impressive genotypic and phenotypic diversity of this important crop plant. An immediate outcome of our work is the detection of chromosomal

segments introgressed from one varietal group into another shedding light on the breeding history of rice. Some introgressions correlate with known genomic regions responsible for traits transferred between varietal groups, while others represent candidates for additional events of potential significance for breeding. Furthermore, the much-improved knowledge of shared breeding history and genetic relationships enhances traditional methods (*e.g.*, coefficient of parentage, CoP, SI Table 7) for the selection of parents for crossing programs.

The SNP coverage of the rice genome available from our study is more than sufficient to obtain genome-wide tag SNPs, especially for regions highly conserved across varietal types, despite the lower estimates of LD (75-150 kb) in previous studies (12-14). Sequencing of additional rice types including *Oryza rufipogon,* the progenitor of domesticated rice, is an obvious next step to provide more SNPs across all groups.

Lastly, and perhaps most importantly, the OryzaSNP resource provides the foundation for high-resolution genotyping of hundreds to thousands of additional varieties. Compared to studies of other model plants such as *Arabidopsis*, a major advantage of rice is the much more extensive information available for a diverse set of known agronomically important traits from thousands of varieties across many different environments. Detailed knowledge of phenotypes, coupled with a deep genotype database, will create a powerful platform for association genetics and discovery of alleles that can be combined to achieve the much-needed increase in rice yield in the coming years.

Materials and Methods

Plant varieties, reference genome masking and target selection. Each rice variety (SI Table 1) was purified by one round of single seed descent. Rice genome

sequence (Build 4, 8) was masked for repeats (8, 34, 35). Those sequences with no or a single hit (91.6 Mb) and with two to ten hits (77.6 Mb) were chosen for long-range PCR (LR-PCR) primer design (Supporting Information).

Array design, sample preparation, and hybridization. The 13,586 selected LR-PCR amplicons span 11,343 non-overlapping fragments and cover 117.8 Mb of unmasked genomic sequence. This genomic fraction was used to design six high-density oligonucleotide (25-mer) resequencing arrays that queried 100.1 Mb of the Nipponbare genome using a tiling strategy (3, 6, 7). The LR-PCR products for each of the 20 rice strains were combined (at ~8 Mb complexity), fragmented, and labelled (3). Each array, synthesized by Affymetrix, contained ~20 Mb of tiled sequence, and was segmented into three chambers. Each chamber was hybridized with a different DNA/hybridization mixture containing labeled target DNAs of two strains. Hybridized targets were detected using confocal scanners.

Base-calling, SNP detection and normalization. We used the pattern recognition (model-based or MB) algorithms for analysis as described previously (3, 6) using criteria and quality scoring algorithms specified in the Supporting Information (3). To correct for between-array variation and to obtain comparability of the data generated by multiple array experiments, hybridization data were quantile-normalized on the level of amplicon pools across all varieties (36).

Repetitive probe annotation and quality assessment SNPs. Repetitive probes in the reference genome were annotated by identifying oligomers that match at least one other 25-mer in the target DNA, allowing for some degree of degeneracy. The mismatch criteria distinguished between the three match types *exact*, *inexact* and *short 25-mer matches,* and *bulged 25-mer matches* that were restricted to a one-base bulge located only on one strand (7) and Supporting Information, p. S4/5).

We used dideoxy sequencing of randomly selected fragments from a subset of the tiled regions to compile a set of true (curated) SNP and non-SNP positions for quality assessment (Supporting Information). A two-layered approach based on Support Vector Machines (SVMs) was applied to predict SNPs from the hybridization data (7).

SNP annotation. All SNP locations and tiled regions were mapped relative to the IRGSP (8) and TIGR (37) pseudomolecules using the program Vmatch (http://www.vmatch.de/) and SNPs were annotated relative to the IRGSP and TIGR pseudomolecules and to the RAP and TIGR gene models (Supporting Information). For the MBML-intersect SNP set, a total of 158,928 out of 159,879 IRGSP localized SNPs were mapped to the TIGR pseudomolecules. SNP sites annotated as non-synonymous, synonymous or as large-effect changes were extracted from the MBML-intersect data set, and only sites with high confidence base calls for at least 15/20 cultivars were included in calculations of allele frequencies. For each SNP site, the number of varieties with bases different from the reference were plotted by frequency and annotation category. SNP distribution and annotation processes (http://www.oryzasnp.org) are detailed in the Supplemental Information. Sixty loci containing large effect SNPs were randomly selected for validation by PCR amplification and dideoxy sequencing at 2x coverage for all amplicons (SI Table 4 and http://www.oryzasnp.org).

Dendrogram construction and population structure. A pairwise distance matrix using the simple matching coefficient for SNPs at non-repetitive sites was calculated, and an unweighted Neighbor Joining tree was constructed using DARwin 5 (38) (Fig. 2a). Population structure was determined by model-based

inference using InStruct (24) on a random subset of 5,000 MBML-intersect SNPs (Fig. 2b).

Linkage disequilibrium and introgression analyses. Only biallelic non-singleton SNPs in the MBML-intersect dataset were used to calculate LD as the correlation coefficient r^2 between SNP pairs. The mean r^2 value was calculated for 10 kb bins based on all pairs of non-singleton SNPs. Due to the extensive population structure in the sample of 20 varieties, we examined LD decay in each subpopulation separately. Because of the small sample size in the aus group (four varieties), only indica and japonica groups, with eight varieties each, were analyzed. Only SNP pairs with no missing data at both loci in at least six chromosomes of the eight varieties were included in the calculations (Fig. 2c).

To study the ancestral contribution of groups to the genome of each variety, we applied a likelihood ratio test method. All putative introgressions between pairs of groups (indica, aus, and japonica) were examined. For every window of 100 Kb with at least 10 SNPs, the ratio of the average sharing of each variety to its own and another group was calculated when at least three pairs of comparison occurred in each group. Regions with an average sharing ratio of < 0.5 were defined as introgressions (Fig. 3, SI Fig. 1). Frequencies of introgressions shared across varietes were plotted on these Figures. The length, number of introgressions in each variety, and shared introgressions across varieties were tabulated (SI Table 6). Regions of extensive haplotype sharing, with 90% or more shared SNPs, were determined for each pair of varieties (SI Fig. 2).

QTL and genetic data for Pokkali, Moroberekan, Minghui 63 and Zhenshan 97B were from published studies. Physical locations for flanking markers were acquired from Gramene (http://gramene.org) and MSU Rice Genome Annotation

databases or were inferred by blastn searches of marker-associated sequences and/or marker primers against the reference genome (Figs. 3-4, SI Table 5).

Data release. Processed resequencing data are at http://www.ncbi.nlm.nih.gov/Traces/ and SNP annotations and descriptive information on basic queries are at http://www.oryzasnp.org. The dideoxy sequence data generated for data quality and training purposes and large-effect validation are in GenBank (FI321710-FI329971 and FI494729-FI495095, respectively).

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Fig. 1. Annotation and distribution of SNPs. (a) Non-synonymous (12,788) and synonymous (13,698) SNPs predicted from the MBML intersect found in select Pfam domains of rice genes with 30 or more SNPs. Chi-square significance of the observed non-synonymous and synonymous SNP distributions for each Pfam group is shown using; * = $p<0.05$, ** = $p<0.001$. **(b)** Allele frequencies at non-repetitive MBML-intersect sites for SNPs in all annotation groups (top panel), genic SNPs (middle panel), and large effect SNPs (bottom panel). Only SNP sites from the MBML-intersect with complete data for >15 varieties were considered. The number(s) of varieties with alleles (SNPs) different from the reference variety, Nipponbare, are indicated on the x-axis.

Fig. 2. Phylogenetic relationships, population structure and decay of linkage disequilibrium in the OryzaSNPset. (a) Unweighted Neighbor Joining dendrogram for non-repetitive SNPs in the MBML-intersect data (159,879 sites). Horizontal bar indicates distance by simple matching coefficient. **(b)** Population structure as determined by model-based inference using InStruct (24). The three groups correspond to indica (red), aus (brown) and japonica (blue). **(c)** Decay of LD, expressed as r^2 as a function of inter-SNP distance for filtered MBML-intersect SNPs, in the indica and japonica varieties, for each chromosome (light) and over all (bold). Limited numbers of japonica SNPs bias LD estimates.

Fig. 3. Introgressed regions detected in rice chr 1, 5 and 6. The origin of an introgression is indicated by the color of the varietal group (red from indica, blue from japonica and brown from aus). Each vertical line corresponds to a window of 100 kb. If the source of an introgression is ambiguous, each potential donor is

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Fig. 4. Relationships among SNP genotypes of parental varieties, Zhenshan 97B (ZSh) and Minghui 63 (M63), QTL regions, and F_1 heterotic loci on chr 6. **(a)** Molecular marker data from five QTL studies (29-31, 39, 40) were assembled and flanking markers were physically mapped to the rice genome (green bars). Parental genotypes at 17,317 SNP sites are shown as red (A allele), blue (B allele) or gray (missing data) lines and introgressions are shown as black bars. QTL for traits including leaf area, vascular bundle number, plant height, root number, cooking quality, amylose content, gel consistency and shoot weight co-localize with a large introgression in M63. **(b)** F_1 hybrid genotypes at 1,564 SNP sites in the overlapping QTL region at 0-4 Mb. Heterozygous SNP sites are black lines; small = intergenic, medium = UTR/intron/synonymous and large = non-synonymous. Blue bars show heterotic loci associated with high yield performance in heterozygous vs. homozygous individuals (32).

Table 1. SNP predictions at non-repetitive sites for varieties by variety group and prediction method.

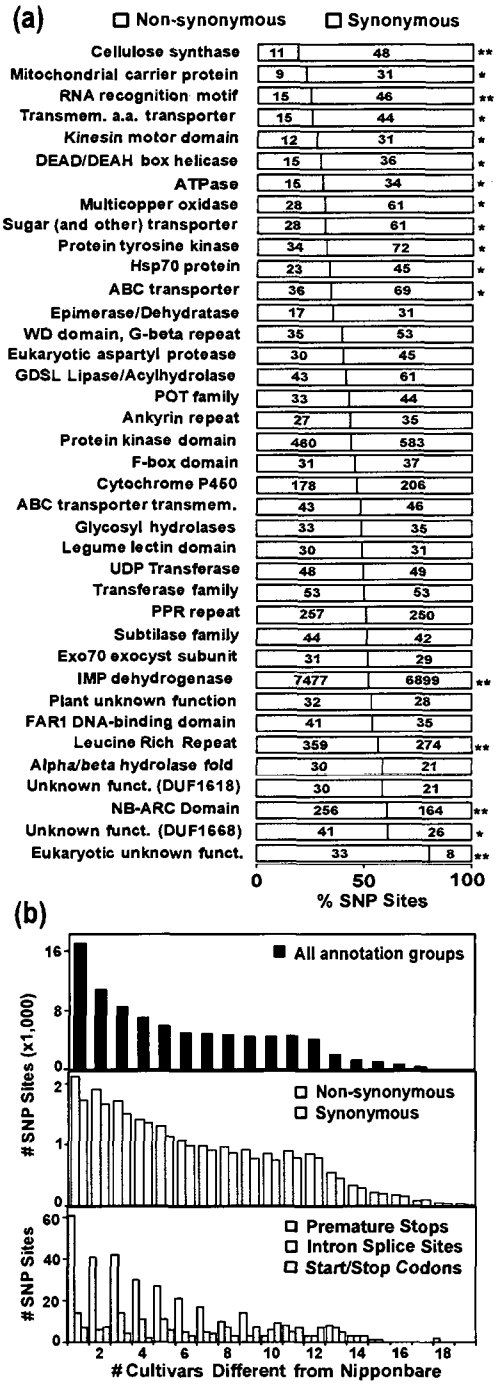
	Mean SNPs per variety [Recall (%):FDR (%)][a]							
Group (*n*)	**MBML-union**		**MBML-intersect**		**MB only**		**ML only**	
Temp. japonica (4)	14,882	[NR:NR]	2,028	[NR:NR]	11,044	[NR:NR]	1,810	[NR:NR]
Trop. japonica (3)	50,221	[18.4:14.6]	20,012	[7.3:8.5]	12,543	[2.1:34.4]	17,666	[9.0:16.0]
Aromatic (1)	51,817	[NR:NR]	2,022	[NR:NR]	48,747	[NR:NR]	1,048	[NR:NR]
Aus (4)	137,114	[24.9:11.6]	63,054	[12.4:2.1]	28,195	[2.7:25.4]	45,865	[9.8:17.3]
Indica (8)	126,702	[25.3:10.4]	54,903	[11.0:2.5]	29,684	[3.8:25.5]	42,115	[10.5:11.7]
All varieties (20)[b]	91,203	[27.8:12.3]	38,080	[9.7:3.2]	24,040	[10.2:24.7]	29,083	[7.9:14.0]

[a] Recall and FDR not reported (NR) where fewer than 50 SNPs were available for evaluation, because of very low statistical power.

[b] Numbers are means over all varieties.

Table 2. Annotations of non-repetitive SNPs relative to the TIGR rice gene models. SNPs on the TIGR rice pseudomolecules were classified as Genic or Intergenic, and locations within gene models annotated. The sums of coding, intronic, 5' UTR and 3' UTR SNPs within a column are greater than total genic sums since they are given for all overlapping gene models.

	MB	ML	MBML-union	MBML-intersect
Genic	135,119	171,750	215,032	91,150
Coding	57,935	74,007	91,992	39,652
Intronic	56,693	71,779	90,301	37,883
5' UTR	7,374	10,116	12,660	4,794
3' UTR	14,195	17,179	21,751	9,551
Intergenic	105,306	142,764	179,646	67,778
Total SNPs	240,425	314,514	394,678	158,928



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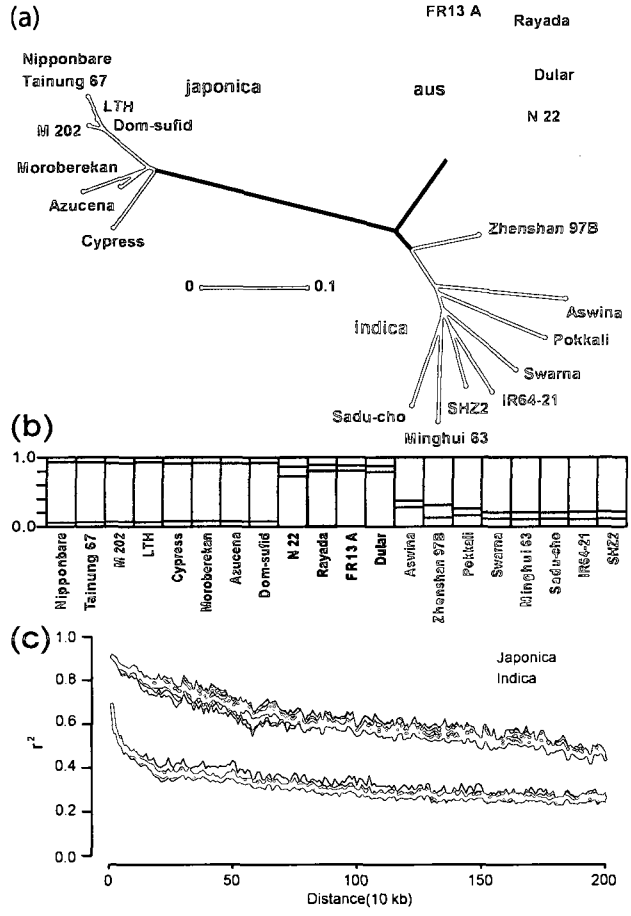

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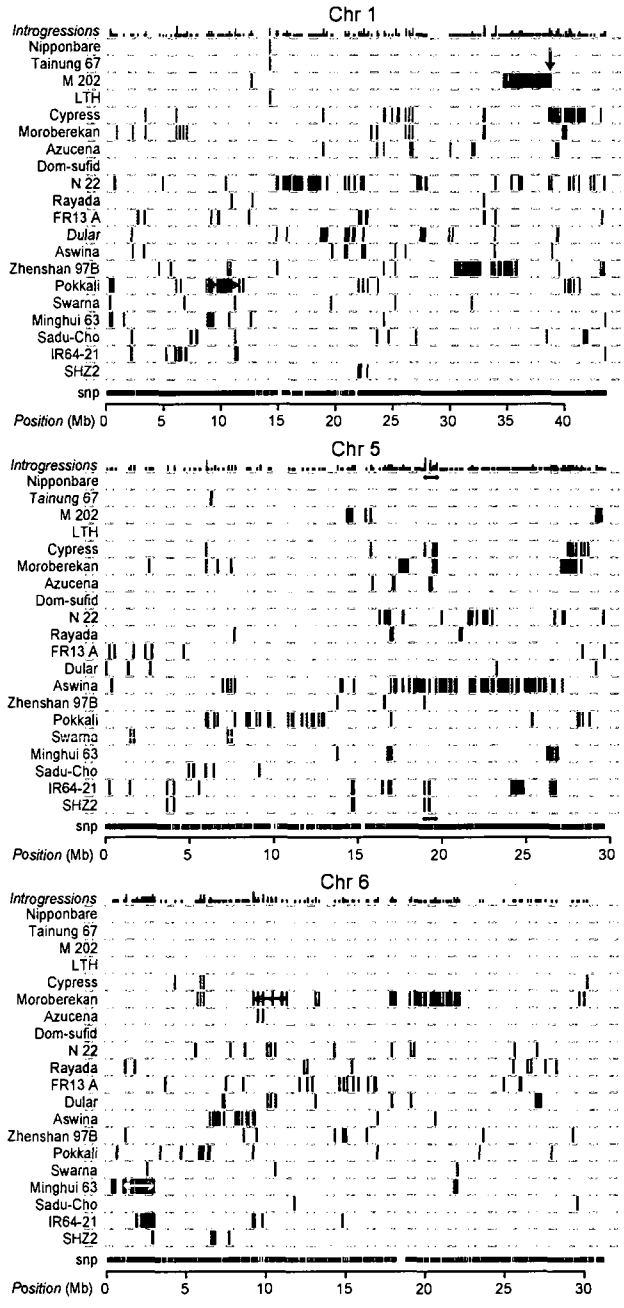

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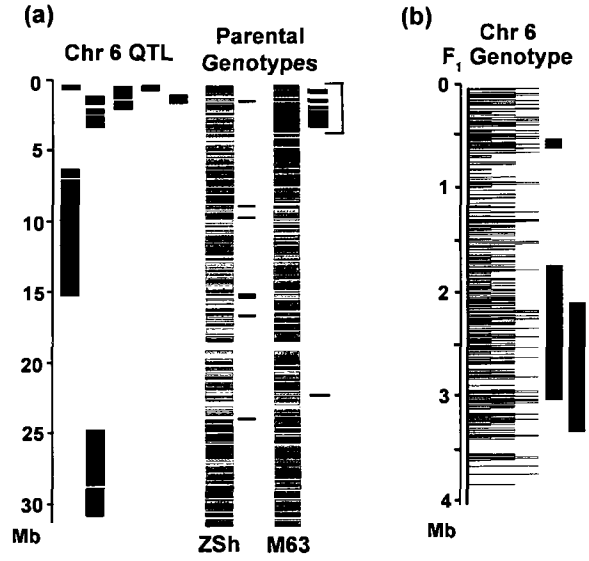

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APPENDIX II.

A germin-like protein gene family functions as a complex QTL conferring broad-spectrum disease resistance in rice

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R. Davidson screened *OsGLP*-silenced mutants for resistance to sheath blight, performed expression profiling of *OsGLP* genes after rice blast infection and computed all statistical analyses in the manuscript. R. Davidson contributed to the manuscript and constructed Figures 3, 5 and 6.

Title:

A Germin-Like Protein Gene Family Functions as a Complex QTL Conferring Broad-Spectrum Disease Resistance in Rice

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[W]The online version of this article contains Web-only data.

ABSTRACT

Plant disease resistance governed by quantitative trait loci (QTL) is predicted to be effective against a broad spectrum of pathogens and long lasting. Use of these QTL to improve crop species, however, is hindered because the genes contributing to the trait are not known. Five disease resistance QTL that co-localized with defense response genes were accumulated by marker aided selection to develop blast-resistant varieties. One advanced backcross line carrying the major effect QTL on chromosome (chr) 8, which included a cluster of 12 germin-like protein (*OsGLP*) gene members, exhibited resistance to rice blast disease over 14 cropping seasons. To determine if *OsGLP* members contribute to resistance, and if the resistance was broad-spectrum, a highly conserved portion of the *OsGLP* coding region was used as an RNA interference (RNAi) trigger to silence a few to all expressed chr 8 *OsGLP* family members. Challenge with two different fungal pathogens (causal agents of rice blast and sheath blight diseases) revealed that as more chr 8 *OsGLP* genes were suppressed, disease susceptibility of the plants increased. Of the 12 chr 8 *OsGLP*, one clustered subfamily (*OsGER4*) contributed most to resistance. The similarities of sequence, gene organization, and roles in disease resistance of *GLP* family members in rice and other cereals, including barley and wheat, suggest that resistance contributed by the chr 8 *OsGLP* is a broad-spectrum, basal mechanism conserved among gramineae. Natural selection may have preserved a whole gene family to provide a stepwise, flexible defense response to pathogen invasion.

INTRODUCTION

Protection of agronomic crops from losses due to disease has largely relied on the use of genetic resistances in plant breeding programs. In major food crops such as rice, single gene-based (*R*-gene mediated) resistance is effective for some diseases. However, highly variable pathogens, such as *Magnaporthe oryzae*, can adapt rapidly to overcome *R*-gene mediated resistances (Bonman et al., 1992). A viable solution to the vulnerability of single gene resistance is to build a basal level of quantitative resistance, which, because of its multigenic nature, is predicted to delay the evolution of pathogens to virulence (Johnson, 1984). Quantitative resistance is particularly essential for important diseases like sheath blight, caused by *Rhizoctonia solani*, where no single gene resistances are available (Lee and Rush, 1983; Rush and Lindberg, 1984; Li et al., 1995; Pinson et al., 2005; Liu et al., 2008). Unlike most *R* gene resistances, quantitative resistance may also be broad-spectrum and effective against multiple pathogens, although direct evidence of this is limited. Incorporating quantitative trait loci (QTL) into germplasm, however, is hindered by the lack of knowledge of what genes are contributing to the QTL. As no disease resistance QTL have been cloned from rice to date, plant breeders cannot develop the precise molecular markers needed to track and select for the functional genes in crop improvement programs.

To understand the molecular basis for QTL-governed disease resistance in plants and determine its utility to control diseases in cropping systems, we and others have accumulated substantial correlative evidence that defense response (DR) genes contribute to quantitative resistance. DR genes are predicted to function in plant disease resistance and their mRNAs and/or enzymatic activity often are induced after pathogen challenge (Dixon and Harrison, 1990). Using various mapping populations derived from rice cultivars with demonstrated variation in multigenic resistance, chromosomal regions conferring quantitative resistance to several important rice diseases such as bacterial blight, sheath blight, and rice blast were identified, and these disease resistance QTL were shown to co-localize with candidate DR genes (Ramalingam et al., 2003; Liu et al., 2004; Wu et al., 2004). We used five DR genes as markers to demonstrate that the more QTL accumulated into lines, the more rice blast resistance we observed in multi-location trials (Liu et al., 2004). However, causal effects of the DR genes were difficult to demonstrate due to the relatively small effects of individual genes, and the presence of multiple gene family members that may play different roles in defense. Consequently, plant breeders still lacked sufficient confidence to apply the DR genes as selection markers in crop improvement programs.

To establish a causal effect between DR gene function and QTL, we have focused on a major-effect rice blast resistance QTL on rice chr 8 (LOD 7.1-10; contributing over 30% of the phenotypic effect) that co-localized with a barley oxalate oxidase-like gene marker (*HvOXOLP*) in several rice mapping populations (Ramalingam et al., 2003; Liu et al., 2004). Minor QTL for sheath blight resistance have also been identified in this chromosomal region (Pinson et al., 2005). Oxalate oxidase-like genes, now referred to as germin-like protein (*GLP*) genes, belong to the functionally diverse cupin superfamily, and which have been identified in Arabidopsis, grapevine, and many gramineae (Membre et al., 2000; Lane, 2002; Godfrey et al., 2007; Dunwell et al., 2008). Several lines of evidence suggest that GLPs are involved in general plant defense responses (Lane, 2002), including the observation that expression of certain *GLP*s is enhanced after infection with pathogens, feeding of insects, or application of chemicals such as salicylic acid, hydrogen peroxide (H_2O_2), or ethylene (Dumas et al., 1995; Zhang et al., 1995; Wei et al., 1998; Zhou et al., 1998; Federico et al., 2006; Lou and Baldwin, 2006; Zimmermann et al., 2006; Godfrey et al., 2007). Transient overexpression of certain barley *GLP* subfamilies resulted in enhanced resistance to the powdery mildew fungus, and, for some subfamilies, silencing resulted in enhanced susceptibility to the pathogen (Zimmermann et al., 2006). Silencing of a *Nicotiana GLP* increased performance of an herbivore (Lou and Baldwin, 2006).

The mechanism by which GLPs influence plant defense is likely related to their generation of active oxygen species. They are targeted to the cell wall and apoplast, and, while their functions are largely unknown, some members related to the barley HvGER4 subfamily exhibit superoxide dismutase activity (Christensen et al., 2004; Zimmermann et al., 2006; Godfrey et al., 2007). Superoxide produced by NADPH oxidase or peroxidases in response to pathogen attack is predicted to be dismutated to H_2O_2 by the GLP, accounting for the accumulation of H_2O_2 (Bolwell and Wojtaszek, 1997). H_2O_2 is an important component of plant defenses responses, with possible roles in basal defense responses such as the oxidative cross-linking of cell wall proteins and lignin precursors as well as in papillae formation (Olson and Varner, 1993; Wei et al., 1998). H_2O_2 also is involved in hypersensitive cell death, signaling in systemic acquired resistance, and induction of DR gene expression (Chen et al., 1993; Lamb and Dixon, 1997; Alvarez et al., 1998).

Underlying the chr 8 QTL, we predicted 12 putative rice GLPs (*OsGLP*) clustered within 2.8 Mb. Expression profiling studies and gene and promoter sequence analyses suggest that a combination of these *OsGLP* family members contribute to defense responses in rice (R. Davidson, unpublished data). In this study, we use RNAi silencing of the chr 8 *OsGLP* gene family members to confirm their contribution to resistance against two different diseases, rice

blast and sheath blight. Our data show that as more *OsGLP* gene family members, particularly those in the *OsGER4* subfamily, are suppressed, susceptibility of the transgenic plants to the pathogens causing rice blast (*M. oryzae*) and sheath blight (*R. solani*) increases. A rice line carrying the effective chr 8 QTL was planted in the field for over 14 cropping seasons and still exhibits excellent blast resistance. This unique combination of QTL mapping, gene function analysis, and field evaluations provides confidence for selecting the *OsGLP* gene family as a complex QTL in breeding programs.

RESULTS

The Chr 8 QTL Contributes to Disease Resistance

Five QTL from cultivar Sanhuangzhan 2 (SHZ-2), including the major effect chr 8 QTL that is associated with *OsGLP* genes, were introgressed into the susceptible commercial cultivar Texianzhan 13 (TXZ-13) using marker-assisted selection, resulting in backcross (BC) line BC116 (Liu et al., 2004). The presence of the chr 8 QTL in line BC116 was confirmed using three marker-based mapping techniques, (Supplemental Fig. S1). Compared to the recurrent parent TXZ-13, line BC116 exhibited superior resistance to blast over 14 cropping seasons at two locations (Yangjiang, Fig. 1, Supplemental Table S1, and Conghua, data not shown) in Guandong Province, China. Although there are other contributing QTL regions in this line, these results are consistent with the hypothesis that the chr 8 QTL contributes to effective and stable disease resistance in the field.

RNAi Silencing of Rice Chr 8 *OsGLP* Genes

A cluster of 12 highly conserved germin-like protein (*GLP*) gene members were predicted within the rice chr 8 disease resistance QTL region (data not shown). GLP is a general term used to indicate proteins that are not true germins or oxalate oxidase, but they contain a germin motif and their enzyme activities may not be known (Carter et al., 1998). We designated the rice genes as *OsGLP8-1* to *OsGLP8-12,* for *Oryza sativa* Germin Like-Proteins, in their 5' to 3' order on chr 8. Our approach to determine the contribution of individual and collective rice *OsGLP* to the resistance contributed by the chr 8 QTL was by gene silencing. However, our first challenge was to identify sequences unique to chr 8 *OsGLPs.* We predicted 41 gene members of these families on rice chr 1, 2, 3, 4, 5, 8, 9, 11, and 12 using the barley cDNA sequences *HvOxOLP* and *HvOxOa* to scan the rice genome (Fig. 2). All predicted proteins contained exact or slight variations of the characteristic germin box sequence, PHIHPRATEI (data not shown) (Lane,

2002). Most predicted OsGLPs were classified based on amino acid similarities using the nomenclature previously established for barley germins and GLPs (*Hordeum vulgare HvGER;* (Zimmermann et al., 2006) (Fig. 2). In keeping with the barley nomenclature, the rice subfamilies were named *OsGER1* to *OsGER6* (Fig. 2, Supplemental Table S2) (Druka et al., 2002; Zimmermann et al., 2006). The majority of the 12 chr 8 *OsGLP* genes were classified in the rice *OsGER4* and *OsGER3* subfamilies (Fig. 2, Supplemental Table S2). The *OsGER4* subfamily contains seven *OsGLP* members (*OsGLP8-5,6,7,8,9,10,11*) and is most closely related to the barley subfamily (*HvGER4*) that is associated with defense responses (Christensen et al., 2004; Zimmermann et al., 2006).

For selective RNAi-mediated silencing of *OsGLP* genes on chr 8, we used a 500 bp region of *OsGLP8-3* (Supplemental Fig. S2). We predicted this region had sufficient identity to co-silence all chr 8 *OsGLP,* but not more distantly related *OsGLPs.* Silencing experiments were performed in the japonica cultivar Kitaake, which has no *R*-gene mediated resistance against *M. oryzae* isolate Che86061. Ideally, silencing would have been performed SHZ-2, the chr 8 QTL donor, which is predicted to contain a highly effective combination of *OsGLP* genes. However, as SHZ-2 is an indica cultivar and is recalcitrant to transformation, we used the more easily transformed japonica cultivar Kitaake. T_0 and T_1 *OsGLP*-suppressed transgenic plants were phenotypically indistinguishable from untransformed Kitaake plants, but some failed to produce seeds. The genome insertion of the transgene was confirmed by PCR using primers to the vector and transgene (data not shown).

The single RNAi construct suppressed all chr 8 *OsGLP* genes transcribed by Kitaake with different efficiencies among the T_0 transgenic plants, as demonstrated by semi-quantitative reverse transcription (RT)-PCR analysis (Fig. 3A, Supplemental Fig. S3A). Two T_0 plants, 10 and 24, in which all expressed chr 8 *OsGLP* genes were suppressed, were advanced to the T_1 generation. Most genes silenced in T_0 parental plants were also differentially silenced among the T_1 progeny (Fig. 3C, Supplemental Fig. S3B). Fewer genes were suppressed in T_1 plants than in T_0 parents, suggesting reduced silencing in the T_1 generation. A further reduction of silencing was observed in the T_2 generation (data not shown). Transcription of all 12 genes was assessed, but expression of *OsGLP8-4* and *8-10* were not observed in Kitaake under our experimental conditions. Transcripts of *OsGLP8-8* were not observed in T_0 and transcripts of *OsGLP8-1* and *8-3* were not observed in T_1 plants, possibly due to developmental differences between generations (data not shown).

Because of the close relationship of the *OsGLP* family members, and because silencing of multiple gene family members with one construct had not been widely reported, we confirmed

the specificity of silencing to closely related gene members. The silencing construct co-silenced three chr 12 genes (*OsGLP12-1,2,3*) that Kitaake expressed in the T_1 plants (Supplemental Fig. S3C), but did not suppress more distantly related chr 3 oxalate oxidase genes (data not shown).

OsGLP Suppressed Transgenic Plants are More Susceptible to Two Different Diseases

Disease phenotype was assessed using a detached leaf spot inoculation assay (Jia et al., 2003). This assay reliably measured quantitative resistance to *Mg* Che86061 in Kitaake, the host for our silencing studies, as compared to the susceptible control Nipponbare (Supplemental S4A). The quantitative resistance of Kitaake was also confirmed by spray inoculation, a widely used inoculation method for rice blast studies (Supplemental Fig. S4B) (Valent et al., 1991).

Nineteen independent T_0 plants with differential gene silencing and confirmed presence of the transgene were inoculated with *M. oryzae,* and disease phenotypes, ranging from susceptible to resistant, were observed (Fig. 3A, B). In the T_1 generation, 60 plants were first screened by inoculation with *M. oryzae,* and 19 plants that exhibited a range of disease phenotypes were pre-selected based on extreme phenotypes and evaluated for gene silencing and transgene presence (Fig. 3C). All the plants pre-selected by phenotype contained the transgene (Supplemental Fig. S3B).

In both the T_0 and T_1 generations, the transgenic plants with more *OsGLP* gene members silenced were more susceptible to infection with *M. oryzae* (Fig. 3, Supplemental Fig. S3). To test the collective effect of *OsGLP* gene expression, rice blast disease scores for the transgenic silenced plants were correlated with the sums of *OsGLP* band intensity ratios (transgenic line/wild type) in T_0 and T_1 generations (Fig. 4). Significant negative relationships ($p=0.0002$) indicated that as the total amount of silencing increased, more susceptibility to *M. oryzae* was observed in T_0 and T_1 plants.

To test the relationships of each chr 8 *OsGLP* gene silencing to blast disease in the T_0 and T_1 plants, single linear regressions comparing disease scores to band intensity ratios were computed (Table I). Negative relationships ($p<0.05$) repeated for *OsGER4* subfamily members (*OsGLP8-5,6,7,9,11*) in both generations. Estimates of relative contributions of individual family members as predictors were tested using multiple regressions (full models) incorporating all *OsGLP* band intensity ratios (Table I). These tests, however, showed few significant relationships due to high collinearity among independent variables. Collinear variables convey repetitive information (Farrar and Glauber, 1967), suggesting co-regulated expression and functional redundancy among these gene family members. The overall p-values for both full models were significant (Table I), similar to the total *OsGLP* regressions (Fig. 4). This supports the hypothesis that the cluster of chr

8 *OsGLP* genes contributes collectively to disease resistance. Among all statistical tests, relationships between gene silencing and blast disease occurred mostly for *OsGER4* subfamily members, in particular *OsGLP8-6,* which likely contributes more to disease resistance against *M. oryzae* than other family members as it showed the lowest p-value in both generations.

The hypothesis of collinearity of expression/silencing patterns among *OsGLP* genes was tested by pairwise correlation analyses (Table II). In T_0 plants, expression patterns for six *OsGLP* genes significantly predicted the expression of five or more other genes. Likewise, in T_1 plants, expression patterns of five genes predicted expression at least four other *OsGLP*. Only correlations between *OsGER4* family members *OsGLP8-5, 6, 9* and *11* repeated in both T_0 and T_1 generations. The consistent pairwise correlations among the *OsGER4* suggest co-silencing, and, therefore infer co-regulated expression of these particular *OsGLP* genes. These results confirm the collinearity of independent variables in the multiple regressions (Table I).

Gene expression of *OsGER4* members that showed high (*OsGLP6, 7, 9,* and *11*) and low correlation (*OsGLP8-2*) to blast resistance in gene silencing studies were evaluated in wild type Kitaake rice plants at 0, 12, 24, 48 h after inoculation with *Mo.* Consistent with predictions from the silencing results, three (*OsGLP8-6, 7* and *11*) of the four *OsGER4* gene members tested were upregulated after *Mo* inoculation. Inoculation with *Mo* did not induce the *OsGLP8-2* or *OsGLP8-9* gene expression, but there was a measurable basal level of *OsGLP8-9* at all time points (Fig. 5).

Silencing of chr 8 *OsGLP* members also correlated with disease susceptibility to another major rice disease, sheath blight caused by *R. solani.* Thirty T_1 progeny were inoculated with *R. solani,* and 10 plants containing the transgene and showing contrasting phenotypes were tested for silencing of *OsGLP* family members (Supplemental Fig. 5A,B). The more *OsGLP* family members silenced, the more susceptible the transgenic lines were to sheath blight (Fig. 6). Single linear regressions of disease index on individual gene band intensity ratios showed that *OsGLP8-6, 7, 9* and *11,* contribute most to sheath blight resistance (Table III). These are among the same *OsGER4* subfamily members that contribute most to rice blast resistance (Table I) with one exception (*OsGLP8-5*; $p=0.184$). Similar to observations in the rice blast experiments, expression patterns of the five *OsGER4* subfamily members in the sheath blight dataset were correlated for all pairwise combinations ($p < 0.15$; p values ranged from 0.0004-0.13).

DISCUSSION

We demonstrate that a *OsGLP* gene cluster, which physically co-localizes to a rice blast resistance QTL, functions as a complex locus in disease resistance in rice. Furthermore, this

resistance is effective against two distinct important rice pathogens. Field evaluations of rice line BC116 that contains the major effect chr 8 QTL confirm that presence of the QTL correlates with enhanced resistance to rice blast disease for over 7 yr of planting (14 cropping seasons). Thus, the resistance provided by the chr 8 QTL, which contains the *OsGLP* cluster, is broad-spectrum and trends to date suggest that the resistance will be effective for a long time.

While correlation of presence of the chr 8 *OsGLP* cluster with resistance is useful for plant selection purposes, it does not constitute proof of function because BC116 has other introgression segments from the donor of resistance (Liu et al., 2004). Given the multigenic nature of this QTL and the inherent difficulty of making isogenic lines with and without *OsGLP*, we conducted gene silencing of all 12 related chr 8 *OsGLP* members using a highly conserved region. We observed co-silencing of closely related gene family members to variable degrees with a single highly conserved trigger sequence. The phenomenon of silencing multiple gene family members to different levels with a single construct was demonstrated previously for another rice gene family the *OsRac* family (Miki et al., 2005).

In some suppressed T_0 and T_1 plants, the level of expression of some *OsGLP* genes was higher than the expression in the untransformed wild type plants (Fig. 3, Supplemental Fig. S3). This could be due to induction of some *OsGLP* family members as a compensatory measure for the suppression of others (Kafri et al., 2006), or because of developmental variation in expression. These options were not explored. We observed considerable variation in *OsGLP* mRNA levels among T_0 and T_1 generation plants (Fig. 3, Supplemental Fig. S3), possibly due to plant to plant variation rather than gene suppression. However, expression of *OsGLPs* in Kitaake wild type plants showed that variation among biological replications is very low (Fig. 5). This is most obvious for the expression of *OsGLP8-2*, which is not induced after inoculation with *Mo* and remains the same during the time course of infection (Fig. 5). Thus, expression variation among transgenic plants is likely due to the presence of the transgene and suppression of the *OsGLP* members rather than plant to plant variation.

By determining the number of genes co-silenced and the relative amounts of silencing in both T_0 and T_1 lines, we demonstrated that the chr 8 *OsGLP* genes contribute collectively to disease resistance because as more genes were co-silenced, the amount of disease increased (Figs. 3, 4, 6). Closely related *OsGLP* family members on chr 12 were co-silenced in some lines, however, their silencing did not alone increase rice blast susceptibility, suggesting their contributions to resistance are negligible if any (data not shown). It is possible that genes other than the *OsGLP* that reside within the QTL interval on chr 8 may also contribute to the resistance phenotype. However, because suppressed expression of the chr 8 *OsGLP*, and particularly the *OsGER4*

family members, rendered the plants more susceptible to both rice blast and sheath blight infection, we conclude that these genes are major contributors for disease resistance, and may explain resistance governed by the chr 8 QTL.

Contributions of individual chr 8 *OsGLP* gene family members to the resistance phenotype varied, as shown in regression analyses, with certain gene family members contributing more than others. Orthologous GLP members in barley and grapevine are implicated in basal defense responses (Zimmermann et al., 2006; Godfrey et al., 2007). Indeed, in rice, *OsGER4* subfamily members were consistently major contributors to resistance against rice blast when compared to *OsGER3* subfamily members, because their level of silencing was most significantly correlated to increased susceptibility in the transgenic plants. The importance of the *OsGER4* subfamily was also observed for sheath blight. Interestingly, most of the *OsGER4* genes that were correlated with resistance against rice blast were also correlated with resistance against sheath blight, with the exception of the *OsGLP8-5* (Table I, III).

It is not clear from wild type expression data whether resistance depends on constitutive or induced expression of *OsGER4* genes. Transgenic plants used for experiments to identify *OsGLP* genes important for resistance were not inoculated. Therefore, our experiments measured the silencing of constitutive gene expression. In separate experiments using wild type plants inoculated with *Mo,* some *OsGER4* genes identified as important by silencing (*OsGLP8-6, OsGLP8-7*and *OsGLP8-11*) were induced above basal levels after infection with *Mo* (Fig. 5). On the other hand, *OsGLP8-9* showed basal expression, but was not further induced after inoculation. Basal levels of gene expression could be important for resistance by creating a preformed resistant state in the plant. Furthermore, activation of the constitutively expressed enzymes could result in increased production of H_20_2, which has been shown to induce *HvGLP*s in barley (Zimmermann et al., 2006).

Many *OsGLP*, particularly the *OsGER4* members, showed correlated expression/silencing patterns among transgenic plants, as indicated by the multiple regression and pairwise correlation analysis (Table II). This suggests co-regulation and functional redundancy, as has been speculated for barley GLPs (Zimmermann et al., 2006). The close proximity and redundancy of the *OsGLP* gene family members, as well as their high sequence similarity are suggestive of gene amplification through duplication followed by diversification (Kafri et al., 2006). In other studies, we have shown that the *OsGLP* exhibit different induction patterns during development, wounding and pathogen invasion, and that the most commonly shared promoter motifs occur among *OsGER4* family members (R. Davidson, unpublished data). Alternatively, diversification

of coding sequence could have created gene members that encode proteins with different enzymatic properties and protein activation and localization differences.

While our results show that the *OsGLP* genes confer broad spectrum resistance, how they function to inhibit pathogens remains unknown. The different contributions observed for some of the *OsGER4* gene family members against rice blast and sheath blight may result from tissue-specific induction of these genes rather than pathogen-specific induction, considering that both pathogens have different infection strategies with respect to tissue specificity. However, both pathogens directly penetrate the plant cuticle with distinct structures that may release general elicitors that will activate common defense responses (Marshall and Rush, 1980; Talbot, 2003).

Defense responses to these two pathogens share pathways, as shown in large scale expression profiling experiments in rice (Venu et al., 2007; Zhao et al., 2008). We hypothesize that the chr 8 *OsGLP* genes contribute to resistance through enhancement of basal defense responses (Chisholm et al., 2006). Although rice enzyme function has not been tested, the *OsGLP* on chr 8 are predicted to encode enzymes with superoxide dismutase (SOD) activity based on high amino acid similarity to the barley *HvGER4* member (*HvOXOLP*) (Fig. 2) and the wheat *TaGLP4* gene (Christensen et al., 2004) (data not shown). These SODs are proposed to be involved in basal defense responses, specifically through H_2O_2 generation (Christensen et al., 2004; Zimmermann et al., 2006).

The chr 8 *OsGLP* genes are highly related in sequence, structure and organization with *GLP* genes in divergent cereals such as barley and wheat (Druka et al., 2002). In rice, seven of 12 putative *OsGLP* are tightly clustered on chr 8 (R. Davidson, unpublished data). The orthologous barley *HvGER4* subfamily contains at least nine clustered duplicated gene members including *HvGERa b,c,d,e* (Wei et al., 1998; Druka et al., 2002; Zimmermann et al., 2006). The chr 8 *OsGER4* family members which contribute most clearly to disease resistance are the closest related rice members to the barley *HvGER4,* which are associated with defense responses in fungal-barley interactions (Wei et al., 1998). By comparing markers reported in different studies, we have found that the *HvGER4* colocalize with barley QTL for fungal resistance (Chen et al., 2003), and the barley markers flanking this QTL were physically mapped to rice chr 8 (data not shown). Taken together, the evidence from rice, barley and wheat implicates these cereal genes as contributors to an ancient plant basal defense mechanism (Lane, 2002; Christensen et al., 2004; Zimmermann et al., 2006; Godfrey et al., 2007). Coordinated function among members of the gene family could be an evolutionarily advantageous strategy by providing a stepwise, flexible response in proportion to severity of infection.

The fact that several chr 8 *OsGLP* genes function together to confer resistance supports the emerging concept that QTL may not necessarily resolve to a single locus, but instead may be controlled by several contiguous loci with small additive effects. QTL are predicted to provide broad-spectrum resistance, or resistance against multiple types of the same pathogen and/or diverse pathogen types. Consistent with this, the chr 8 QTL was originally identified in multi-location trials in China and the Philippines, with vastly different populations of *M. oryzae* (Liu et al., 2004), suggesting that it confers resistance to many races of *M. oryzae*. Additionally, *OsGLP* suppressed plants are more susceptible to sheath blight, which is particularly significant, because so far no simply inherited resistance has been identified for sheath blight. The broad-spectrum nature of the *OsGLP*-containing complex QTL may be responsible for the highly effective resistance observed in the deployed rice line BC116. Overall, the identification of multiple *OsGLP* loci conferring quantitative resistance has broad implications in the deployment of defense genes in breeding. If multiple loci are involved, single-gene transformation experiments may lead to the erroneous conclusion that the gene is not important for resistance. Future selection may need to take into consideration the allelic states of multiple loci in a gene family and selection for a specific *OsGLP* cluster may be necessary to capture the collective effect of the specific gene family members.

MATERIALS AND METHODS

Field Studies

Five QTL from cultivar SHZ-2, including the major effect chr 8 QTL that is associated with *OsGLP* genes, were introgressed into the susceptible cultivar TXZ-13 using marker-assisted selection, to develop the line BC116 (Liu et al., 2004). The field experiment (natural infestation) was performed for 7 years with two cropping seasons per year (total, 14 cropping seasons). Plots used a randomized complete block design and three replicates. The experiment was replicated in a second rice blast disease nursery at Conghua, Guangdong Province, China, with similar results (data not shown). Panicle blast symptoms were evaluated on each rice line using IRRI Standard Evaluation System for Rice (http://www.knowledgebank.irri.org/ses/SES.htm).

The presence of the chr 8 QTL in BC116 was confirmed using Single Sequence Repeat (SSR) analysis (Temnykh et al., 2000), SNP detection by TILLING analysis (Raghavan et al., 2007), and Single Feature Polymorphism (SFP) analysis using the University of Arizona rice genotyping array (Galbraith, 2006). TILLING analysis was used to detect mismatch between the two different alleles for *OsGLP8-8* and *OsGLP8-9* from SHZ-2 and TXZ-13 by heteroduplex

cleavage, and was performed as described (Raghavan et al., 2007)(Supplemental Fig. S1). Primers for TILLING were *OsGLP8-8*F, CTTGTTCTCCATCACAAGTTTACG; *OsGLP8-8*R, ATGCACGCCAAATAATTGATAGTA; *OsGLP8-9*F, AGAGAAGATAGCAGAAACCCAAAG; *OsGLP8-9*R, AGCTTGCAAGTATGCATAACAAGT.

Bioinformatics and Phylogenetic Analysis

H. vulgare cDNA sequences *HvOXOa* and *HvOXOLP* (accession no: Y142203 and X93171, respectively) were used as queries for tblastx searches (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) using the HTGS database. FGENESH (http://www.softberry.com/berry.phtml) was used to predict putative oxalate oxidase and *OsGLP* from significant rice BAC hits. All nucleotide and inferred amino acid sequences corresponding to different predicted members were aligned using CLUSTAL W. 1.83 (Thompson et al., 1997) in the BCM Search Launcher Interface (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Ambiguous regions were removed, and 214 amino acid characters were used in Bayesian MCMC analysis (Ronquist and Huelsenbeck, 2003) to generate the tree. A mixed amino acid model was specified and four chains were allowed to run for 1.5 X 10^7 generations (repeated 4X), after which 5,000 trees were sampled from each run to determine the final consensus tree and posterior probabilities for each clade.

RNAi Silencing

The *OsGLP* RNAi construct (*pTSi-OsGLP*) was generated by cloning an antisense 500 bp PCR product corresponding to the second exon of *OsGLP8-3*, which is highly conserved among all *OsGLP* gene members on rice chr 8, into *XcmI* -digested *pTSi* vector (Zhao, 2004) (*pTSi-OsGLP*, Supplemental Fig. S2). This fragment was amplified from IR64 genomic DNA using the primers: OXOF2, 5'-TGGGTTTCCTTGCAAGAACC-3' and OXOR2, 5'-TTCTTCTCCACTTGAAATGCC-3'. The two *NheI* restriction sites in the pTSi-1 RNAi vector were used to clone the *pTSi-OsGLP* RNAi construct into *XbaI*-digested pCambia 1305 binary vector (http://www.cambia.org/daisy/cambia/materials/vectors/585.html) and used to transform *Agrobacterium tumefaciens* EHA105. Rice cultivar Kitaake was used for *Agrobacterium* transformation (Hiei et al., 1994; Zhao et al., 2005). The presence of the transgene was confirmed in T_0 plants by PCR amplification with a reverse primer specific to the 2xCaMV35S promoter and a forward primer specific to the transgene (OXOF2R2) (Supplemental Table S3).

Plant and Fungal Growth and Inoculation Methods

For rice blast assays, plants were grown with a photoperiod of 16 h light/8 h dark in a growth chamber with photon flux of 135 $\mu molm^{-2}s^{-1}$ and day/night temperatures of 28°/26°C. *M. oryzae* was grown on oatmeal agar media under constant light at 26°C for 2 wk. T_0 transgenic plants were inoculated two weeks after transfer from tissue culture to soil. The phenotype was validated by inoculation of leaves from the same T_0 at an older growth stage. T_1 transgenic and Kitaake wild type plants were inoculated two-weeks after planting in soil. Disease phenotypes were assessed using a detached leaf spot inoculation assay (Jia et al., 2003) that distinguished QTL-governed resistant and susceptible responses, and that we confirmed to produce results in agreement with a commonly used spray inoculation assays (Valent et al., 1991) (Supplemental Fig. 4). The second youngest rice leaves were spotted with $5x10^4$ spores/ml suspension of *M. oryzae* isolate Che86061, and disease was scored by visual assessment of the amount of mycelia present and colonization at the site (R = little or no mycelia or colonization, score 0-2; MS = moderate levels of mycelia and colonization, score 3-4; S = extensive mycelial growth and colonization, score 5-7) at 7 dpi. Sheath blight assays of T_1 lines derived from T_0 plant 10 were performed in a greenhouse using a micro-chamber screening method (Jia et al., 2007). Plants were inoculated with *R. solani* isolate RM0140-1 at 14 days after seed germination and were scored at 14 dpi (Jia et al., 2007).

For expression experiments of wild type plants after inoculation with *Mo* isolate Che86061, tissue from 21 day old plants was harvested by combining the three most fully expanded leaves pooled from two plants per cultivar. Three rounds of RT-PCR were performed with three independently isolated total RNA samples (from three independent plant inoculation experiments). Plants were inoculated with 5×10^5 spores/ml at 20 psi using an artist's air brush (Valent et al., 1991). Plants were kept in a mist chamber at 100% RH for 24 h after inoculation, and then returned to the growth chamber under the conditions described above.

DNA and RNA Analyses

Rice leaf genomic DNA was isolated (Murray and Thompson, 1980) and quantified by UV absorbance. Silencing patterns of the *OsGLP* gene family members were determined in independent T_0 and T_1 transgenic lines using reverse transcriptase (RT)-PCR. RT-PCR was performed using gene-specific primers for each *OsGLP* gene family member (Supplemental Table S3) in uninoculated T_0 and T_1 transgenic plants. Leaf tissue for RNA extraction was harvested from T_0 transgenic plants two weeks after transfer from tissue culture to soil, and from two-week old T_1 transgenic plants and Kitaake wild type (WT) plants. Total RNA for RT-PCR

was isolated with Trizol reagent (Invitrogen), and treated with DNase (1 unit/ ug total RNA, Promega). cDNA was synthesized using a Superscript III reverse transcriptase kit (Invitrogen), and was amplified using HotStar Taq DNA Polymerase (Qiagen) and gene specific primers (10 pmol of each primer) for each *OsGLP* gene on chr 3, 8 and 12 (Supplemental Table S3). *EF1-α* and ubiquitin genes were amplified as internal controls (Supplemental Table S3). Optimized cycles for unsaturated PCR reactions, determined by a PCR cycle gradient with internal control primers, were 25 and 30 cycles for ubiquitin and *EF1-α*, respectively (data not shown). Hygromycin primers (Supplemental Table S3) and construct-specific primers (Supplemental Fig. S2) were used to determine the transgene presence. Ethidium bromide stained gels were digitally photographed using a Gene Genius Bioimaging System and associated Gene Tools Gel Analysis software (Syngene). Band intensity values were calculated by subtracting the signal of the negative control in a given gel, and were normalized with the band intensity of the *EF1-α* internal control. Band intensity ratios of transgenic line/wild type were calculated for each constitutively expressed *OsGLP* gene Heat maps were drawn using R (http://www.R-project.org). Single linear and multiple regressions of disease scores on *OsGLP* band intensity ratios were performed using SAS software version 9.1.3 for Windows (SAS Institute Inc., 2003), PROC REG, and correlations of *OsGLP* band intensity ratios were computed using PROC CORR. Time point means (n=3 biological replications) for wild type expression of each gene were compared using PROC GLM and the least significant difference (LSD) method with a Student-Newman-Keuls multiple testing correction.

Supplemental Material

The following materials are available in the online version of this article.

Supplemental Figure S1. Backcross (BC_3) rice line BC116 contains the chr 8 QTL harboring the *OsGLP8* gene cluster from the resistant parent SHZ-2. Confirmation that BC116 contains the QTL region and that these alleles were from the SHZ-2 resistant parent are from: (**A**) Single Feature Polymorphism (SFP) analysis and Single Sequence Repeat (SSR) marker analysis. RM547 locates on chr 8 between 5586058 – 5586291. (**B**) TILLING analysis for two chr 8 *OsGLP* family members, *OsGLP8-8* and *OsGLP8-9* (located on chr 8 between 5,242,446-5,254,949). Template DNA used in TILLING analyses were from BC116, BC116 + SHZ-2 (116/S), and BC116 + TXZ-13 (116/T). Double band (arrow) in 116/T is indicative of heteroduplex formation between the two different alleles from SHZ-2 and TXZ-13. M, molecular marker.

Supplemental Figure S2. RNAi silencing vector pTSi-1 and the *OsGLP* RNAi construct. **(A)** Vector pTSi-1 contains the HBT35S promoter, which includes a minimal CaM35S enhancer plus the 5' UTR of maize *CDDPK* gene (1). The inverted *NOS* carry the antisense sequence of the *Agrobacterium NOS* terminator plus an 81 bp fragment of the 3' end of the *GFP* gene. A pair of *XcmI* sites is used to generate "T" 3' extensions for cloning PCR products. **(B)** RNAi silencing construct pTSi-*OsGLP* was constructed by inserting an antisense 500 bp PCR product of *OsGLP8-3* into the pTSi-1 RNAi vector. *NheI*-digested pTSi-*OsGLP* vector was inserted into *XbaI*-digested pCambia 1305 binary vector.

Supplemental Figure S3. Silencing patterns of the *OsGLP* in independent T_0 and T_1 transgenic plants. RT-PCR was performed using gene-specific primers for each *OsGLP* gene family member (SI Table 3) in uninoculated T_0 **(A)** and T_1 **(B)** transgenic plants. After amplification, band intensities were quantified and compared with the wild type band (WT). *EF1-α* and ubiquitin genes were amplified as internal controls; hygromycin was the selection marker. Seed from T_0 lines 10 and 24 were advanced to T_1. For T_1 analysis, RNA was extracted from the third youngest leaf of 2-wk-old uninoculated T_1 transgenic and Kitaake wild type plants (WT). **(C)** RT-PCR using gene specific primers revealed silencing for the *OsGLP* gene members on chr 12 in the cDNA samples from T_1 plants used in **(B)** 1 Kb ladder (L) and DNA amplification controls.

Supplemental Figure S4. Disease phenotypes of Kitaake and Nipponbare after *Magnoporthe oryzae* (*Mo*) inoculation. **(A)** Spot inoculation of rice detached leaves of Kitaake (resistant) and Nipponbare (susceptible) with *Mo* isolate Che86061. **(B)** Spray inoculation of Kitaake and Nipponbare plants with *Mo* isolate Che86061.

Supplemental Figure S5. *OsGLP*-silenced T_1 plants show higher levels of sheath blight disease. **(A)** Sheath blight disease scores of T_1 RNAi transgenic plants assessed using the sheath blight disease index (2). Kitaake (WT, resistant control) and Lemont (LEM, susceptible control). **(B)** Sheath blight disease phenotypes on T_1 silenced plants with different levels of gene suppression.

Supplemental Table S1. Disease ratings for Sanhuangzhan 2 (SHZ-2, donor resistant parent), BC116 (backcross line harboring QTL from SHZ-2) and commercial cultivar Texianzhan

13 (TXZ-13, recurrent susceptible parent) after 7 yr of field evaluation in a rice blast nursery in Yangjiang, Guangdong, China.

Supplemental Table S2. Gene members of the barley germin-like protein (*HvGER*) subfamilies used for the phylogenetic analysis of the *OsGER* subfamilies in rice.

Supplemental Table S3. Oligonucleotide primers used in the study.

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Figure Legends

Figure 1. Rice line BC116 containing the chr 8 disease resistance QTL shows consistent panicle blast resistance over 7 yrs (14 cropping seasons). BC116, TXZ-13 (rice blast susceptible recurrent parent), and SHZ-2 (rice blast resistant parent) were planted in Yangjiang, Guangdong, China two seasons per year over 7 consecutive years. Panicle blast, the most severe form of rice blast, was evaluated in three replicates (60 individuals per replicate), and are presented as the average of two seasons/year. Differences in disease were observed between BC116 and TXZ-13 in all years (t-test, $P<0.001$) except 2004 ($P=0.0035$).

Figure 2. Phylogenetic relationships of germin-box containing proteins from rice and barley. Amino acid sequence similarities among predicted GLP proteins from rice were compared with known barley HvGER proteins (Supplemental Table S2). Rice *GLP* gene members were classified as known subfamilies *OsGER1-6*, based on relationships with the barley HvGER proteins. Inferred amino acid sequences of 60 GLP proteins were aligned using ClustalX version 1.83. The phylogenetic tree was reconstructed using Bayesian MCMC analysis (Ronquist and Huelsenbeck, 2003). Posterior probabilities (scaled to 100) are indicated at nodes.

Figure 3. Rice transgenic plants silenced for chr 8 *OsGLP* gene expression show increased rice blast disease relative to wild type (WT) Kitaake. Silencing of *OsGLP* gene expression in independent uninoculated T_0 (A) and T_1 (C) transgenic plants, as determined by semi-quantitative RT-PCR, is indicated as heat maps. Each square in the heat maps indicates band intensity ratio (transgenic plant/WT) for a single chr 8 *OsGLP* gene family member (row) in an independent transgenic plant (column). Color keys for each map show range of expression (relative to wt, green = maximal suppression; red = maximal expression; - = missing data) and histograms with distributions of data points. Rice blast disease phenotypes for individual plants, S (susceptible), MS (moderately susceptible) and R (resistant) are indicated below heat maps. (B) Range of blast disease symptoms on individual T_0 and WT plants 7 dpi.

Figure 4. Reduced expression of rice chr 8 *OsGLP* gene members correlates with increased rice blast disease in both T_0 and T_1 plants. Rice blast disease score was assessed in individual T_0 and T_1 transgenic
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Figure 5. Induction of *OsGLP* genes after inoculation with *Magnaporthe oryzae* (*Mo*). Three week old wild-type Kitaake plants were inoculated with 105 spores/ml of *Mo* isolate Che86061, and leaves were sampled for RNA at 12, 24 and 48 h post inoculation (x-axis). 0 time plants were not inoculated. Gene expression of selected *OsGLP* genes were screened by RT-PCR, and gel band intensities were quantified and normalized against the reference gene, *EF1α* (y-axis, Relative band intensities are arbitrary units). Time point means (n=3 biological reps) for each

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Figure 6. Reduced expression of rice chr 8 *OsGLP* gene members in individual silenced T_1 plants correlates with increased sheath blight disease. Sheath blight disease index was assessed at 14 dpi as described (Jia et al, 2007), and total relative *OsGLP* mRNA values were determined as in Fig. 4.

Table I. Expression/silencing of *OsGER4* subfamily members correlates with rice blast disease ($P \leq 0.05$, bold).[a]

	T_0 transgenic plants				T_1 transgenic plants			
OsGLP	**Slope[b]**	**R^{2b}**	**P[b]**	***P*[c]**	**Slope[b]**	**R^{2b}**	**P[b]**	***P*[c]**
8-1	-1.30	0.14	0.133	0.382	(-)	(-)	(-)	(-)
8-2	-2.51	0.19	0.074	0.289	-1.51	0.04	0.417	0.189
8-3	-0.92	0.12	0.160	0.257	(-)	(-)	(-)	(-)
8-5	-4.06	0.25	**0.035**	0.587	-5.09	0.41	**0.003**	0.253
8-6	-2.67	0.72	**<.0001**	0.067	-5.79	0.66	**<.0001**	**0.014**
8-7	-4.20	0.62	**0.0001**	0.771	-3.64	0.47	**0.001**	**0.032**
8-8	(-)	(-)	(-)	(-)	-3.95	0.29	**0.018**	0.570
8-9	-4.90	0.48	**0.001**	0.625	-5.61	0.39	**0.004**	0.449
8-11	-3.50	0.39	**0.006**	0.876	-2.38	0.22	**0.043**	0.397
8-12	-1.57	0.11	0.182	0.425	-1.40	0.01	0.697	0.099
Overall P-value for full model:				**0.025**				**0.003**

[a] Regressions of rice blast disease score by *OsGLP* gene band intensity ratio; n=19 individuals per generation; (-), not expressed

[b]Single linear regression

[c]Multiple regression

Table II. Pairwise correlations[a] of *OsGLP* gene expression ratios[b] in silenced transgenic lines (n=19).

T_0 transgenic plants

8-1	*8-2*	*8-3*	*8-5*	*8-6*	*8-7*	*8-9*	*8-11*	*8-12*	***OsGLP* genes**
1.00	0.22	0.72*	0.61*	0.34	0.64*	0.80**	0.48*	0.63*	***8-1***
	1.00	0.44	0.14	0.50*	0.33	0.43	0.19	-0.18	***8-2***
		1.00	0.20	0.15	0.33	0.61*	0.1	0.26	***8-3***
			1.00	0.52*	0.72*	0.80**	0.88**	0.81**	***8-5***
				1.00	0.81**	0.63*	0.70*	0.37	***8-6***
					1.00	0.86**	0.75*	0.63*	***8-7***
						1.00	0.73*	0.65*	***8-9***
							1.00	0.72*	***8-11***
								1.00	***8-12***

T_1 transgenic plants

8-2	*8-5*	*8-6*	*8-7*	*8-8*	*8-9*	*8-11*	*8-12*	***OsGLP* genes**
1.00	0.08	0.01	0.43	-0.23	-0.03	0.04	0.54*	***8-2***
	1.00	0.81**	0.49*	0.70*	0.70*	0.66*	0.01	***8-5***
		1.00	0.53*	0.74*	0.72*	0.69*	0.11	***8-6***
			1.00	0.26	0.44	0.44	0.48*	***8-7***
				1.00	0.77*	0.69*	0.06	***8-8***
					1.00	0.63*	0.21	***8-9***
						1.00	0.28	***8-11***
							1.00	***8-12***

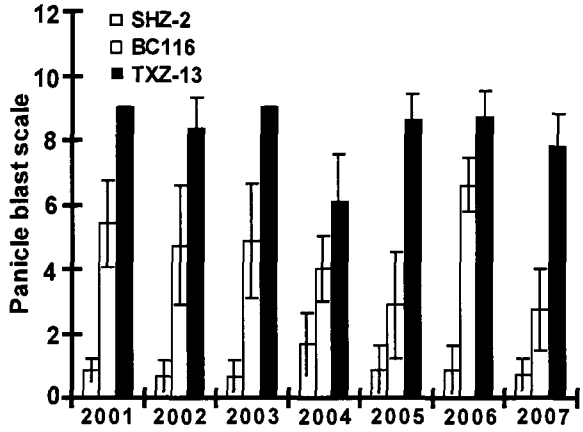
[a]Using band intensity ratios for the T_0 and T_1 plants, correlation tests of all pairwise combinations of *OsGLP* genes were computed using the SAS program, PROC CORR. R^2 values are reported in the matrices, and significant values are indicated. * $P < .05$, ** $P < .0001$

[b]Transgenic gel band intensity / wild type gel band intensity, normalized with the gel band intensity of the internal control EF1-α.

Table III. Expression/silencing of *OsGER4* subfamily members correlates with sheath blight disease in T_1 transgenic plants ($P \leq 0.05$, bold).[a]

OsGLP	Slope[b]	R^2	P
8-5	-1.12	0.21	0.184
8-6	-0.78	0.40	**0.049**
8-7	-1.28	0.55	**0.014**
8-9	-3.07	0.40	**0.048**
8-11	-0.99	0.60	**0.008**

[a] Linear regression of sheath blight disease index by *OsGLP* gene band intensity ratio; n=10 individuals.



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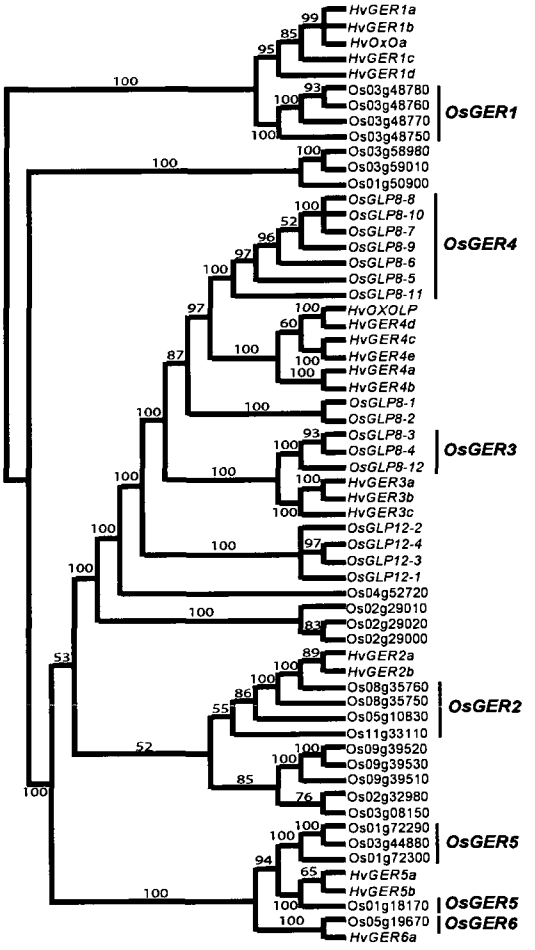

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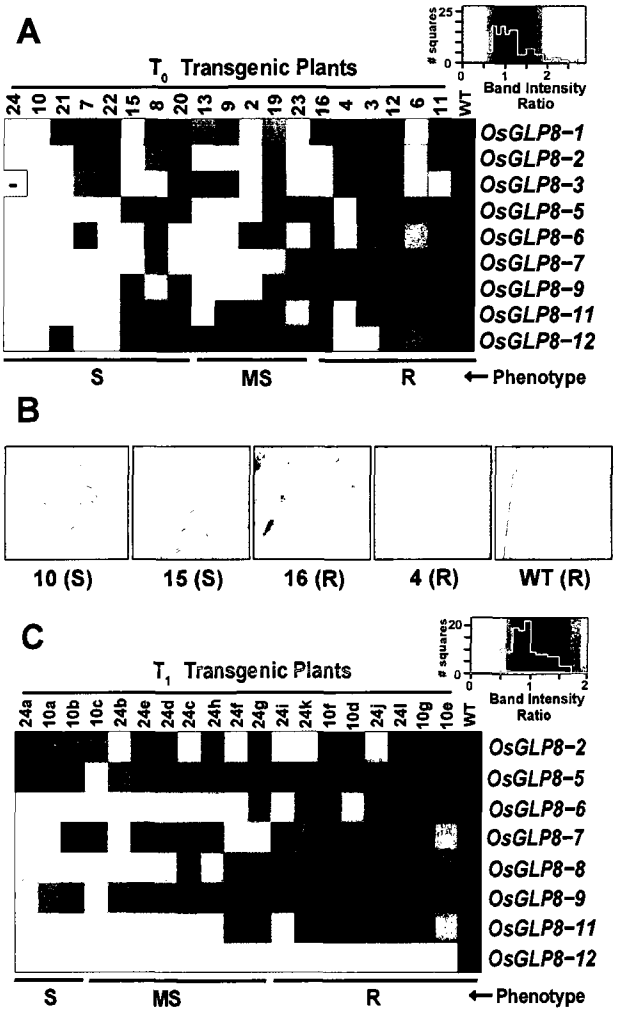

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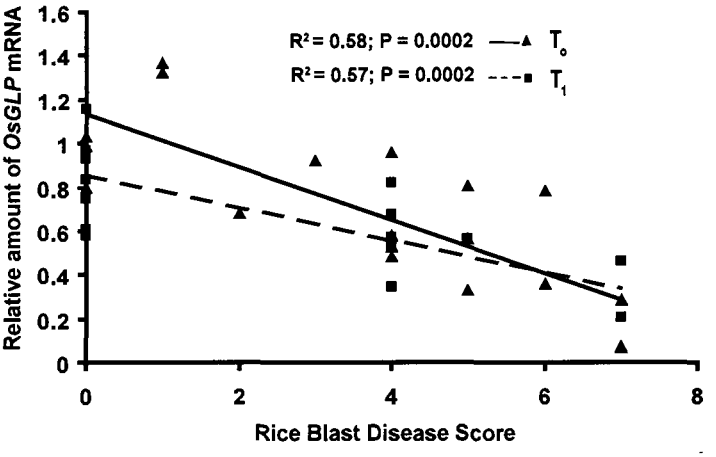

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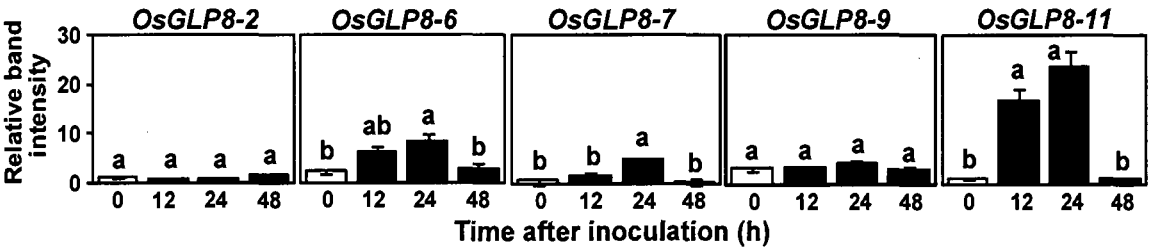

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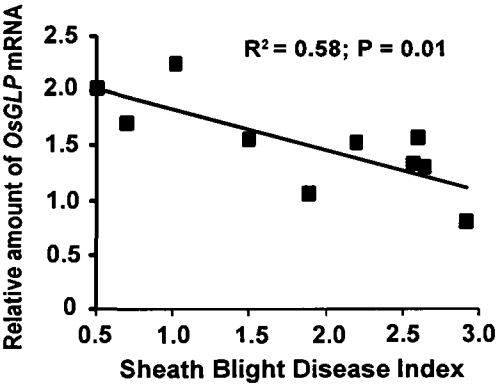

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