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BIOINFORMATICS STUDIES OF *OSGLP*8-12 GENE FROM *ORYZA SATIVA* (JAPONICA) REVEAL ITS ROLE IN CONFERRING RESISTANCE AGAINST DISEASE AND STRESSES

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Abstract

Plants, from *Myxomycetes* to Gymnosperms has been found to contain ubiquitous proteins namely Germins. These proteins are found to express mainly during plant development; that is specifically confined, to specific organs including seeds, roots, shoots, fruits and nectar glands. Additionally, these proteins are, also found to express in response to different biotic and abiotic stress conditions. The current study was conducted by exploiting different bioinformatics tools in order to analyze *OSGLP*8-12 gene sequence for various parameters. Phylogenetic analysis of this gene reveled the clustering of this gene with other homologous genes in: group 2A and that was very close to another Germin like protein gene namely Germin like protein gene 8-3, the disease resistance gene. The *OsGLP*8-12 gene along with other homologous genes was selected from phylogenetic tree and subject to Genevestigator in order to find/ predict the functions associated with *OsGLP*8-12 gene. The gene under study was found to express in various plant organs variably, with high expression in mid early stage of plant development in different developmental stages. With reference to the induction of this gene by different biotic and abiotic factors, the gene was found to show strong expression in response to both factors. These findings suggest the involvement of this gene in development as well as biotic and abiotic stresses.

Key words: Germin like proteins, Abiotic and biotic stress responsive gene expression, Rice.

Introduction

Plants experience various stresses; both biotic and abiotic, when exposed to an open environment, and accordingly respond in a variety of ways involving different molecular mechanisms, those are poorly understood yet. Bioinformatics and recombinant DNA technology offers efficient tools for deciphering molecular mechanisms that are involved in stress problems.

Germins are a huge and diverse group of proteins that are present mainly in cereals that may include rye, wheat, barley, rice etc. (Lane, 2002). First Germin identified, was identified in germinating wheat embryos and was labeled as germination marker, and hence was named as Germin (Thompson & Lane, 1980). Further characterization of this protein revealed that this protein was cell wall associated, had homopentameric structure, glycoprotein nature and had oxalate oxidase activity (Faye & Chrispeels, 1988, Jaikaran *et al.*, 1990, Lane *et al.*, 1993). Still, further characterization of this protein reflected it to be homohexameric protein comprising of six β-jellyroll monomers forming a trimer of dimers with all dimers locked together to form a stable structure. With reference to amino acid sequence of all Germin proteins, all of them contain a characteristic peptide sequence (PHIHPRATEI) named Germin box (Lane *et al.*, 1991). All proteins found in plants other than cereals, that had Germin box were later on termed as Germin like proteins; GLPs (Dunwell & Gane, 1998). In context of "expression pattern", the expression of Germin like protein genes has been found during development, in one instance, by specific developmental signals variably, in different developmental stages including somatic embryogenesis and early development (Becerra *et al.*, 2006, Mathieu *et al.*, 2006) or floral induction (Staiger, 1999).

However, reports are also present that show expression of Germin like proteins in response to biotic and abiotic factors. The biotic factors include herbivore attack (Lou & Baldwin, 2006), infection with pathogens, disease resistances (Schweizer *et al.*, 1999, Park *et al*., 2004., Park, 2004., Park, 2004. , Christensen, 2004., Zimmermann *et al*., 2006) and fungal inoculation (Banerjee & Maiti, 2010). Abiotic factors like; salt stress has been also found to induce the expression of Germin like Protein genes (Hurkman & Tanaka, 1996; Nakata *et al*., 2002). Like Germins, most of the Germin like proteins were reported to be found in extracellular matrix including cell wall and apoplast as reported by (Nakata *et al.*, 2002, Segarra *et al.*, 2003). This bioinformatics investigation of *OsGLP*8-12 gene sequence was focused to investigate and predict the functions associated with an uncharacterized gene the *OsGLP*8-12.

Materials and Methods

Plant material: Rice (*Oryza sativa japonica)* cultivar *Nipponbare* was used for the study and seed were a generous gift from NARC Islamabad.

Surface sterilization and germination: The seed obtained from NARC were surface sterilized at room temperature as described by Durrani *et al*., 2015. For seeds to be germinated they were inoculated on sterile N6 medium supplemented with vitamins, contained in sterile test tubes and kept to 25±1°C and 16-hour photoperiod conditions.

Genomic DNA isolation form japonica rice cultivar nipponbare: The genomic DNA extraction was extracted from fresh and healthy leaves isolated from newly *In vitro* raised plants, using CTAB method (Richards, 1997) with a slight modification in the basic protocol as described by (Durrani, 2015).

Amplification of *OsGLP*8-12 gene

Oligo design: Sense and antisense oligos pair were designed manually, using *Oryza sativa* CV Nipponbare chromosome 8 Sequence with Genbank accession number: NM_001067838.1 for the amplification of *OsGLP*8-12 gene. Properties and feasibility of annealing of oligos for specifically binding to its template was checked using online bioinformatics tools, the IDT oligo analyzer available online at http://idtdna.com/analyzer/applications/ oligoanalyzer and NCBI primer blast respectively available online at https://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Polymerase chain reaction using *Pfu* ploymerase: A 50 µl polymerase chain reaction was performed, for the amplification of *OsGLP*8-12 gene, using *Pfu* polymerase from Thermofisher Scientific to prepare PCR product for further use in sequencing, using above designed Oligo pair (Table. 1). The reaction mixture contained PCR Buffer 1X, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM each, sense and antisense oligos 0.5 *p*M, DNA Template 1 µg, 2.5 U *Pfu* DNA polymerase and PCR water to 50 µl.

The reactants have undergone 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 2 minutes. Thirty-five cycles preceded three minutes of initial denaturation at 95ºC and proceeded to the final extension of 15 minutes at 72ºC.

Table 1. Pair of oligos used for amplification of *OsGLP*8-12 gene.

Oligo	Sequence
Sense	5/-ATGGCCTCCTCTTCCTTATTTC-3/
Antisense	5/-TCAGTAGTTGTTCTCCCAGAAC-3/

Adapted from (Durrani, 2015)

Purification of the amplicon: After PCR completion, the PCR reaction was resolved on 1% TAE agarose gel along with 1 KB DNA ladder (Thermofisher Scientific Cat # SM0313). Gel piece containing specific 995 bp band was excised and removed from the remaining gel and put into 1.5 ml microfuge tube for further purification. The specific amplicon was purified using Perfectprep® Gel Cleanup kit Cat # 7955152051 from Eppendorf, according to manufacturer's guidelines.

Sequencing of amplicon: The purified sample was first resolved on 1 % TAE agarose gel along with 1 KB ladder to confirm the target 995 bp size and subsequently examined using Nano-drop as well, to check quality and quantity of purified amplicon product. The purified samples were got sequenced by acquiring services from Macrogen Incorporation Korea.

Sequence analysis: Using sequence obtained, the amino acid sequence of *OsGLP*8-12 was deduced using the ExPASy translation tool available online https:// web.expasy.org/translate/ similar protein sequences of *Oryza sativa*, Soybean; *glycine max*, *Zea mays* and *Arabidopsis thaliana*, obtained from NCBI public database, were aligned by using Clustal X2 program. The multiple sequence alignment was subject to a determination of the degree of similarity between aligned proteins and construction of phylogenetic tree using The MEGA 5 program. The multiple sequence alignment also helped in determining putative functions associated with *OsGLP*8-12 protein.

Analysis of expression during development: For this analysis, Genevestigator program® was used. The *OsGLP*8-12 gene expression along with other homologous genes was selected from the phylogenetic tree, and after obtaining their sequences rom public database they were subject to comparative analysis of expression during different developmental stages of plant development.

Analysis of expression pattern: In continuation of the previous section, Genevestigator® also investigated expression pattern in different plant tissues and organs including calluses.

Analysis with respect to gene regulation: The program also addressed the regulatory aspect of *OsGLP*8-12 gene expression in response to biotic and abiotic environmental factors/stresses including hormones.

Results and Discussion

PCR amplification of *OsGLP*8-12 gene: The PCR amplification resulted in production of DNA fragment with length near one Kb (995 bp) as shown in Fig. 1.

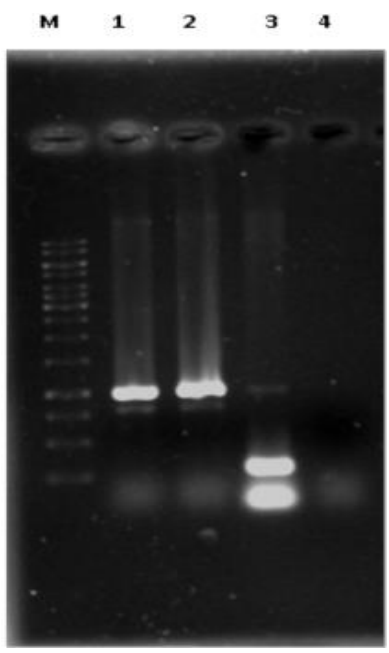

Fig. 1. PCR Amplification of *Os*RGLP8-12 Gene.

Adapted from (Durrani, 2015.)./ thesis dissertation of irfan safdar durrani

Lanes 1 and 2 contain *Os*RGLP8-12 amplified products
Lane 3 is positive control with Ubiquitin Primers
Lane 4 is negative control
M specifies 1 Kb marker (Fermentas Lithuania UAB)

Sequencing of *OsGLP*8-12 gene: The sequence of amplified PCR product of approximately 995 base pairs size is shown in Fig. 2.

Phylogenetic analysis of *OsGLP*8-12 gene: Phylogenetic analysis was performed in order to determine the degree of similarity between O*sGLP*8-12 and other Germin like protein genes from other plants located on different chromosomes.

The phylogenetic tree shows two groups. Group 1 contains sequences from dicot plants including Arabidopsis and soybean, while group 2 contained sequences from monocot plants including *Oryza sativa* and *Zea mays* The GLPs from *Arabidopsis* and soybean can be further clustered into two different subgroups.

Similarly Group 2 comprises of two groups. Subgroup 2 A contains three GLPs from rice including *OsGLP*8-12 while subgroup 2B contains GLPs from *Zea mays* and *Oryza sativa*, however the protein sequences of rice and maize can be seen clustered in separate groups. Phylogenetic analysis shows a close relationship between *OsGLP*8-12 (*Os*08g0232400 01) and *Os*GER2 (*Os*08g0189200 01) on an evolutionary scale, among the selected proteins, and genes for both proteins (Fig. 3).

It was suggested by (Mo Lu, 2010), that the Germin and Germin-like genes of the plant species might be evolved by independent gene duplication events, means all GLP genes have a single common ancestor.

This might be the reason that our phylogenetic analysis results show a significant similarity between *OsGLP*8-12 and *Os*GER2 proteins and variability with other GLPs as compared to *OsGLP*8-12 protein.

In silico characterization of *OsGLP*8-12 gene: In this part of work, homologous genes from the phylogenetic tree were selected and subject to analysis using Genetyx software. Analysis by software led to the construction of the table.2, describing properties associated with *OsGLP*12-8 protein. Looking into the table we can find presences of high percentage of hydrophobic amino acids that leads to the speculation that *OsGLP*8-12 protein is localized in the extracellular matrix of plant tissues/ cells. This speculation confirms previous findings that Germin like proteins are localized to cell walls (Faye & Chrispeels, 1988, Jaikaran *et al*., 1990, Lane *et al*., 1993, Nakata *et al*., 2002, Segarra *et al*., 2003, Lu *et al.*, 2010).

Table 2. Properties of *OsGLP*8-12 Protein.

Feature	Property
Total P L	224 Amino Acids
Found M W	24.58 kilo Dalton
Found I P	5.41
Hydrophobic Amino Acids	125 (55.80 Percent)

Adapted from Thesis Dissertation of Irfan Safdar Durrani

Table Legend: P L= Protein length, M W = Molecular Weight, I P= Isoelectric point

Analysis of expression during development: This analysis was done by selecting homologous genes, downloading their sequences from NCBI public data base and finally subjecting for analysis using Genevestigator® software along with *OsGLP*8-12 gene. Target gene showed moderate expression in mid-early developmental phase of plant development as compared other homologous genes analyzed (Fig. 4), however, in comparison two other homologous genes specifically *Os*08g08970 and *Os*08g08980 were found to show a higher level of expression in initial seedling Stages of plant development. These findings validate previous findings of (Thompson & Lane, 1980) where first identified Germin as wheat germination marker.

ATGGCCTCCTCTTCCTTATTTCTCCTGGGTGCTCTTCTTGTGCTGGCCTCATGGCAGGCCATCGTCGCCT
ATGATCCTAGCCCGCTCCAAGATTTCTGCGTTGCAGACATGAACTCACCTGGTGTGTGTATGTTTTGCA
CTTCACTTAATTGTCTTTTTAAACACAGTACAGACGCAAAACACTTATATACATGCACTCACACTCACC
CCTATAAACCACACGCACCCTACGAGCAGCTGTCATATCTTAATTGTTGTCCTTATCTTTGGTAGCATA
TATATTATCACTGCTGGATCCGAAATTGTATGTTGTTACATGGTGACAGTACTAGAGAAGAATGGTAG
TTGTGATTACTGAAAACTTTGACATGCATATTTAATATTTATTGACCTTTGGAAATATATACATGTTAC
ATTCATTCCTCTATATATGCAAACAGTGCGAGTGAATGGATTTGCTTGCAAGAATCCAATGGATGTGA
GCTCGGAAGACTTCTTCAATGCAGCCAAGTTTGATATGCCAAGGAATACTTTTAACAAGCTCGGGTCC
AACGTCACCAACCTCAACGTCATGGAGTTTCCGGGTCTCAATACCCTTGGCATCTCACTAGCTCGTATC
GACTACGCGCCAATGGGTGTGAACCCACCACACATACATCCCCGTGCTACTGAGCTCCTTACCGTGCTT
GAGGGAACACTCTATGTTGGTTTTGTCACGTCCAACCCAAACAAGCTCTTCTCTAAGGTGGTTTGTAAG
GTGACGTGTTTGTGTTCCCTAAGGCAATGATTCACTTCCAAATGAACCTAGATCATGACAAGCCAGCA
GTTGCCCAATCAGCACTCAGTAGCCAAAACCCTGGAGTTATTACTATTGCAAGCGCGGTGTTTGGCTC
ACAGCCACCGATCTCCGATGATGTTCTGACCAAGGCGTTTCAGGTGGAAAAGAAGCTAATTGATTGGC
TCCAATCTCAGTTCTGGGAGAACAACTACTGA

Fig. 2. The *Os*RGLP8-12 Gene Sequence Obtained from Macrogen Inc. Adapted from Thesis Dissertation of Irfan Safdar Durrani.

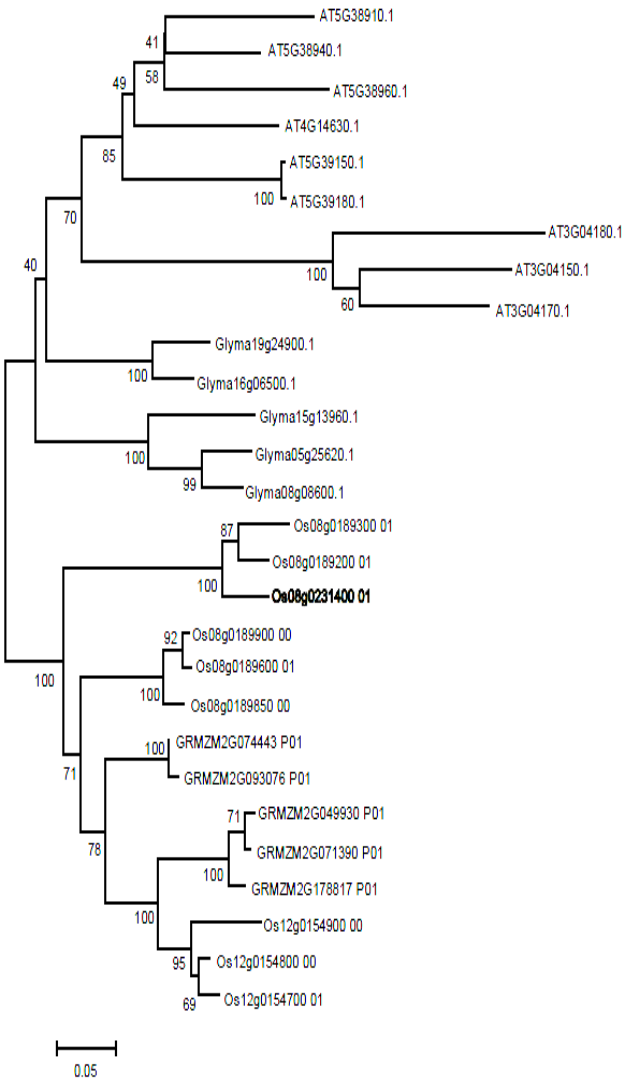

Fig. 3. Phylogenetic Tree of *OsGLP*8-12 Showing Close Similarity between *OsGLP*8-12 and (*Os*08g0232400 01) and *Os*GER2 (*Os*08g0189200 01) on Evolutionary Scale. Both proteins were found to be located on the same chromosome i.e. chromosome 8.

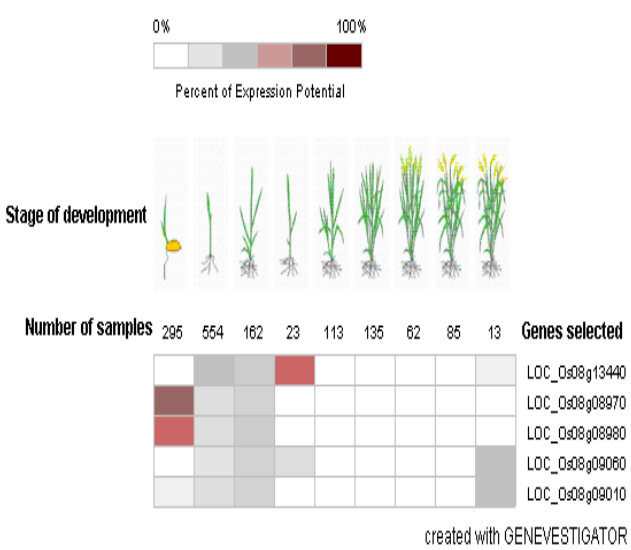

Fig. 4. Comparison of different homologous genes for their expression in different developmental stages of plant development.

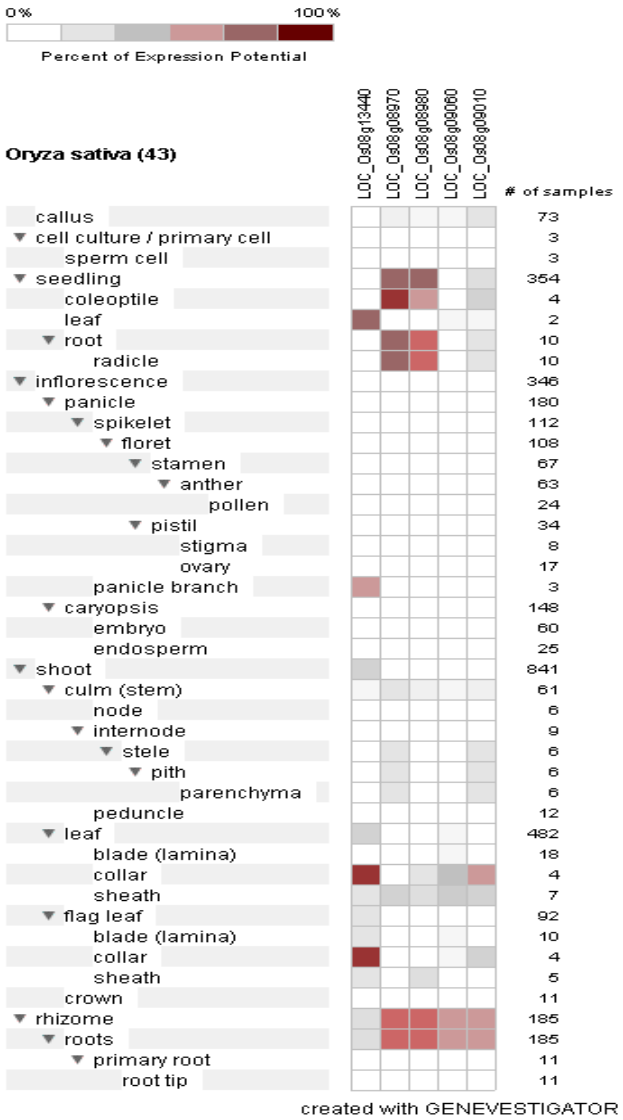

Fig. 5. Expression pattern of *OsGLP9* gene in different plant organs.

As far as the expression pattern of *OsGLP*8-12 in plant organs and tissues is concerned, the expression was found to be active in leaf, seedling, panicle branch, leaf collar and flag leaf collar (Fig. 5). Different groups have reported quite diversified expression pattern in different plant organs involving seeds, flowers, roots, grape skin and pulp, cotyledons, mature leaves etc. (Berna & Bernier, 1999, Crespo *et al.*, 2006, Godfrey, 2007.).

The Genevestigator® program also addressed expression of *OsGLP*8-12 in response to various biotic and abiotic factors. The abiotic factors included hormones induction and different environmental factors including temperature, photoperiod, nutrients, aerobic and anaerobic germination while biotic factors included bacterial and fungal pathogens. The analysis showed strong expression in response to both bacterial and fungal pathogens. In the case of abiotic factors including hormonal germination both aerobic and anaerobic, including variable drought stresses, the program showed strong induction of *OsGLP*8-12 in all cases (Fig. 6). Previous reports demonstrate that on one hand expression of Germin like genes is likely to be induced by developmental signals, floral induction and somatic embryogenesis (Becerra *et al.*, 2006, Mathieu *et al.*, 2006, Dorothee Staiger *et al.*, 1999). In other instances,

GLP gene's induction is also triggered by stresses, both biotic and abiotic (Hurkman, 1996, Schweizer *et al*., 1999, Nakata *et al*., 2002, Park *et al*., 2004., Lou & Baldwin, 2006, Christensen, 2004., Zimmermann *et al*., 2006.). These analyses and findings are further supported by work of (Banerjee & Maiti, 2010) where they report upregulation of rice Germin like protein 1 gene in response to biotic and abiotic stresses. Genevestigator also indicated downregulation of *OsGLP*8-12 gene. The analysis used iron and phosphorus, supplied as nutrients to generate gene's response. Drought stress and some other variable abiotic stresses also contribute to downregulation of *OsGLP*8-12 gene as analyzed by Genevestigator program. Most of the Germin like proteins are reported to possess either oxalate oxidase or superoxide dismutase activity and both of them produce H_2O_2 (Fig. 6).

Work of (Banerjee & Maiti, 2010), where they overexpressed rice Germin like protein 1 gene, transgenicaly in tobacco, reports overproduction of H_2O_2 that in turn leads to reinforcement of cell wall components and there-by pose resistance to laboratory isolate of *Fusarium solani* inoculation, rendering transgenic tobacco plant with transgene to be resistant against this fungal pathogen. Furthermore, transcriptome analysis of *OsGLP*8-12 shows it to be disease resistant (Fig. 7).

For further analysis regarding expression profile of the gene under study, Online tool TENOR (Transcriptome Encyclopedia of Rice) available at https:// tenor.dna.affrc.go.jp/ was used to retrieve expression profile Japonica rice. Please refer to Fig. 7 for details.

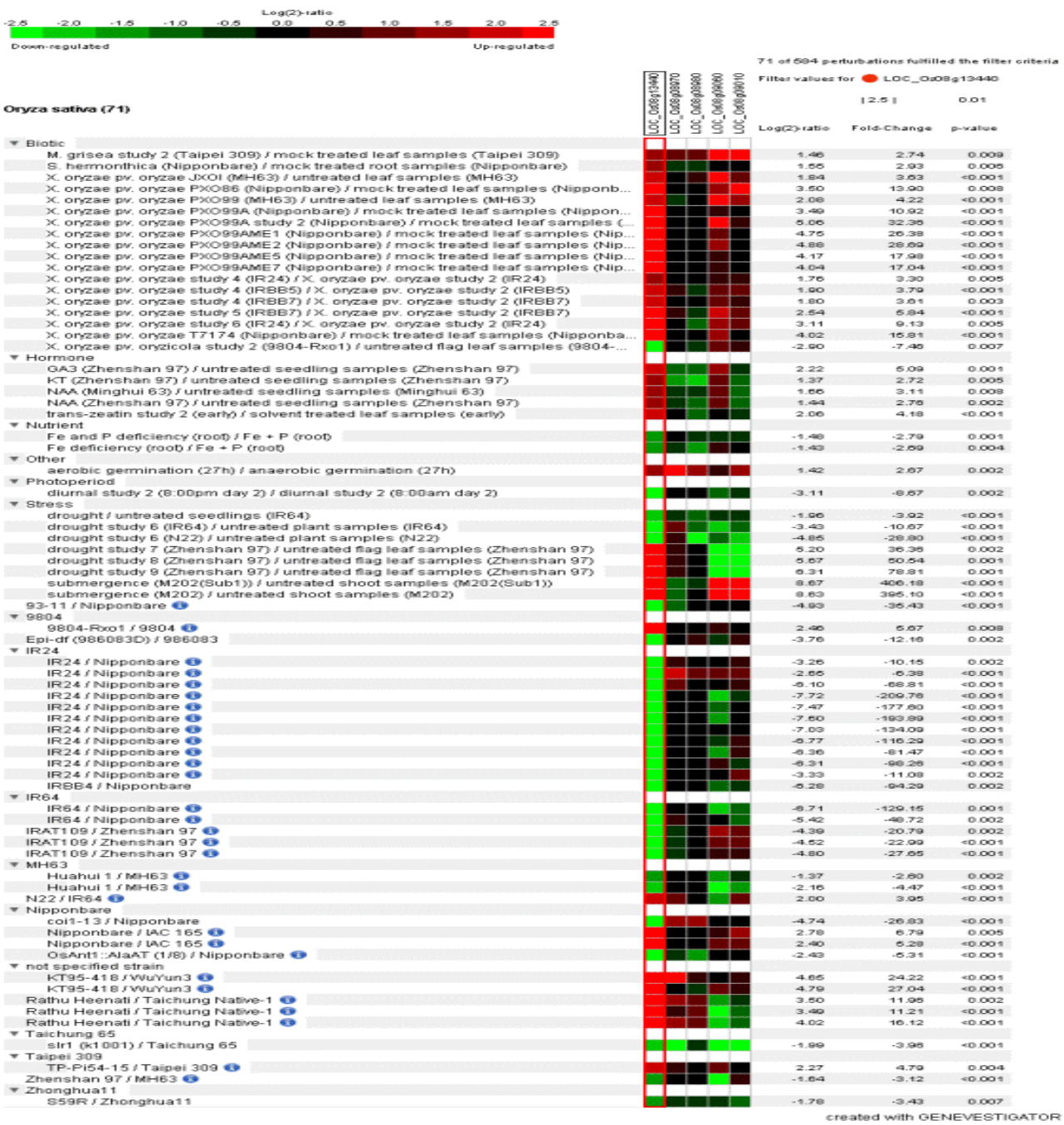

Fig. 6. Regulation of *OsGLP9* Gene in Response to Biotic and Abiotic Stresses.

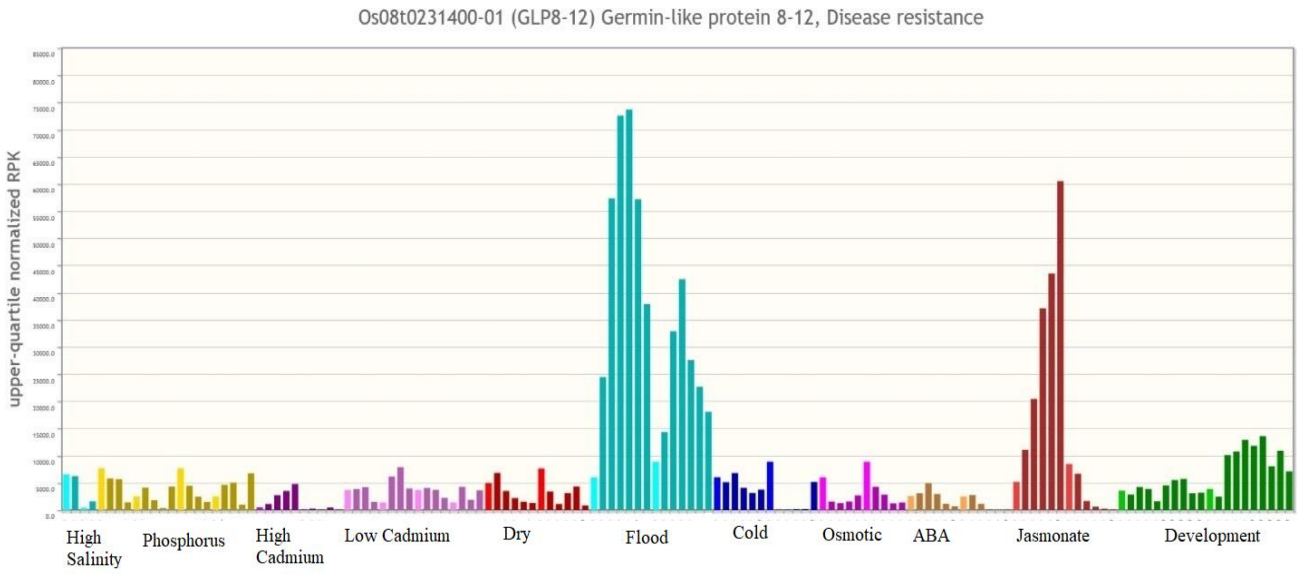

Fig. 7. Expression profile of *OsGLP*8-12 Gene Retrieved from TENOR Database.

This figure shows that *OsGLP*8-12 gene is responsible for conferring disease resistance.

Conclusion

All above findings and discussion conclude that various biotic and abiotic factors trigger the activity of *OsGLP*8-12 gene and thereby may be responsible for conferring resistance to various bacterial and fungal pathogens. However, these *In Silico* findings need further confirmation by using a transgenic approach.

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