In-silico study of biotic and abiotic stress-related transcription factor binding sites in the promoter regions of rice germin-like protein genes

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Abstract

Germin-like proteins (GLPs) are involved in biotic and abiotic stress tolerance in different plant species. Rice (Oryza sativa L.) genome contains about 40 GLP family member proteins in nine chromosomes. Although some of the rice GLP (OsGLP) promoters have been studied through in silico analysis as well as experimentally, studies regarding the distribution pattern of the biotic and abiotic stress associated transcription factor binding sites (TFbs) in the promoter regions of OsGLP genes have not been attempted thoroughly. Several transcription factors (TFs) namely NAC, WRKY, bHLH, bZIP, MYB and AP2/ERF act as major TFs concerned with biotic as well as abiotic stress responses across various plant species. In the present study the in silico analysis was carried out using the 1.5 kilobases (kb) promoter regions from 40 different OsGLP genes for the presence of NAC, WRKY, bHLH, bZIP, MYB and AP2/ERF TFbs in it. Among various OsGLP gene promoters, OsGLP8-11 was found to contain highest number of tested TFbs in the promoter region whereas the promoter region of OsGLP5-1 depicted least amount of TFbs. Phylogenetic study of promoter regions of different OsGLP genes revealed four different clades. Our analyses could reveal the evolutionary significance of different OsGLP gene promoters. It can be presumed from the present findings as well as previous reports that OsGLP gene duplications and subsequent variations in the TFbs in OsGLP gene promoter regions might be the consequences of neofunctionalization of OsGLP genes and their promoters for biotic and abiotic stress tolerance in rice.
Introduction

Germin-like proteins (GLP) belonging to cupin superfamily are evolutionary conserved plant glycoproteins [1]. Germin protein was reported for the first time in germinating wheat grain and it was thought to be a marker gene for germination [2]. Later on GLP proteins have been identified in other angiosperms, gymnosperms as well as mosses [3] and their nomenclature have been given due to their structural similarity with germin proteins as well as their presence in the same superfamily like germin. Further studies established that GLP proteins possess unique biochemical as well as physiological functions across plant species [1]. Majority of the GLP family members have been involved in biotic as well as abiotic stress responses [1, 3–5] in diverse plant.

Rice (Oryza sativa L.) is used as a major staple food for more than half of the world’s population. Since the availability of genomic sequence information in public database, several studies have been conducted to characterize individual GLP family members from rice and other crops [1]. An interesting study on rice reported 12 OsGLP gene families as quantitative traits loci (QTL) associated with blast disease and all of those genes were present on chromosome number 8 [6]. Additionally, RNAi-mediated gene silencing of rice germin-like protein1 (OsGLP1) depicted susceptibility of OsGLP1-down regulated plants to sheath blight and blast diseases of rice compared to untransformed control plants [7]. Further study in a heterologous system of tobacco revealed that over-expression of OsGLP1 gene improved resistance to Fusicarium solani infestation in the transgenic tobacco lines [8]. In addition to biotic stresses several GLP genes have been found to be involved in abiotic stress mechanisms also. RNAi-mediated down-regulation of OsGLP1 has documented improved salt tolerance of transgenic lines during seed germination and early seedling growth compared to untransformed plant [3]. Another study has identified the expressional changes of 31 different OsGLP genes upon exposure to drought, salt, cold and heavy metal stresses [9].

In association with different GLP genes, their promoters have also been found to be involved in biotic and abiotic stress responsiveness. A rice GLP gene (OsRGLP2) promoter showed wound inducible property as well as abiotic stress (dehydration and salt stress) responsiveness [10]. Another detailed study on transgenic potato plants harboring promoter::GUS fusion construct containing 1107 base pair (bp) promoter region of OsRGLP2, depicted expressional up-regulation of GUS fusion protein in response to two fungal pathogens namely F. solani and Alternaria solani [11]. Study has been conducted to analyze the OsGLP1 and a putative germin A promoter region from five Pakistani rice varieties and it revealed that the TATA box binding protein could also recognize regions other than the (-30) TATA box element on the promoter sequences [12]. In addition to rice, the GLP gene promoters have also been characterized in other crops. In an earlier study, a GLP gene (PcGER1) promoter was cloned from Pinus caribaea and the 1520 bp upstream promoter region was characterized in tobacco Bright Yellow 2 (BY-2) cells upon exposure to different phytohormones [13]. Maximum promoter activity was detected in day 4 upon exposure to 2,4-D and BA [13]. In barley (Hordeum vulgare), eight different germin-like protein (GER4) gene promoters have been analyzed and it has been found that different promoters showed diverse response upon pathogen attack preferably due to the presence of W-box domain in the TATA-box proximal promoter region [14]. Another study on barley at evolutionary point of view suggested that in case of barley GER4 gene, the promoter region rather than the coding DNA sequence (CDS) region have been diversified after duplication of the genomic region to subsequently acquire new function [4].

The adaptive strategy of plants under biotic and abiotic stress conditions include expression as well as utilization of several transcription factors (TFs) which eventually regulate a number
of pathogenesis related genes (PR genes) or signaling genes after binding with their promoter regions [15–16]. The adequate expression of a gene under a particular promoter is governed by the presence of appropriate transcription factors binding sites (TFbs) in the promoter region. Some major TFs like NAM/ATAF1/CUC2 (NAC), N-terminal WRKY domain containing TF (WRKY), basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), myeloblastosis viral oncogene homolog TF (MYB) and APETALA 2/ethylene-responsive element binding factor (AP2/ERF) are very well documented in plants for their abiotic and biotic stress responses. In addition to these, TFs like C2H2, Zinc finger protein and MADS box are also having well established role in plant immunity [17–19].

Although, a large number of GLPs are available in rice, their exact number and proper nomenclature have been found to be highly confusing [9, 20]. A recent study documented the existence of 43 OsGLP members from rice and that particular study revealed expression analysis of different OsGLP members upon exposure to various abiotic stresses at differential developmental stages [9]. Beside the in silico promoter analysis of two rice GLP promoters namely OsRGLP1 and OsRGLP2; functional characterizations of some OsGLP gene promoters have also been conducted [10–11, 21–22]. It is presumed that the promoter regions of these multi-gene family members play crucial role regarding the concerned gene expression under differential biotic and abiotic stresses as detected in other crops [4, 14]. In the present in silico study, the upstream 1.5 kilobases (kb) region of 40 OsGLP gene promoters have been analyzed for putative availability of the major biotic and abiotic stress-related transcription factor binding sites (TFbs). Additionally, the phylogenetic study of OsGLP gene promoters, their evolutionary significance and the expression of OsGLP genes under respective gene promoters have also been interpreted here.

Materials and methods

Data retrieval of rice GLP sequences

Protein sequences of OsGLP genes were collected from Rice Genome Annotation Project Database (https://rapdb.dna.affrc.go.jp/) and from National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/). All of the mRNA sequences for each OsGLP were retrieved from NCBI database. The coding DNA sequences (CDS) of each OsGLP were blasted at NCBI using Ref Seq Representative Genome database and their corresponding DNA sequence was identified from different rice chromosome. As the length of 5’ UTR (untranslated region) of different OsGLP mRNA were found to differ in length [9], the upstream 1500 bp region from CDS were treated as promoter region for uniformity. The region analyzed as promoter was comprised of 5’ UTR (if any) along with the core promoter region as well as distal regulatory elements. All sequences were based on O. sativa Japonica Group cultivar Nipponbare.

Phylogenetic analysis and chromosomal organization

Phylogenetic analysis of 40 different promoter regions of OsGLP genes (i.e. 1.5 kb upstream regulatory region from CDS) was conducted through Neighbor-Joining method using Molecular Evolution Genetic Analysis 7 (MEGA 7) software [23]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) has been shown next to the branches. Construction of Models with the lowest BIC scores (Bayesian Information Criterion) were considered to describe the substitution pattern in a comprehensive manner. The BIC value was 82388.402 and the AICc (Akaike Information Criterion) value was 81718.828 involving 40 nucleotide sequences of 1.5 kb OsGLP gene promoters. All positions containing gaps and missing data were eliminated during evaluation. There were a total
of 801 positions in the final dataset. Tajima’s neutrality test was conducted using the above mentioned software for finding out nucleotide diversity. During selection of the model, nucleotide frequencies and rates of base substitutions for each nucleotide pair were considered in MEGA 7. Chromosomal organization of 40 OsGLP genes were performed with the help of Rice Database Oryzabase-Shigen (https://shigen.nig.ac.jp/rice/oryzabase/) using the Chromosome Map Tool (http://viewer.shigen.info/oryzavw/maptool/MapTool.do). Different OsGLP genes which were separated by a maximum of five genes were designated as duplicated genes in accordance to published protocol [9]. The presence of OsGLP genes on positive or negative strand of the DNA, were counted for considering them as tandemly or inverse tandemly duplicated genes, respectively.

Analysis of the TFbs

Promoter regions of 40 OsGLP genes were analyzed using PlantPAN 2.0 software (http://plantpan2.itps.ncku.edu.tw/) for identification of transcription factor binding sites (TFbs) in OsGLP gene promoters. Multiple promoter analysis programme (http://plantpan2.itps.ncku.edu.tw/gene_group.php#multipromoters) was used and occurrence of same TFbs in different promoter regions were identified. TFbs for different TFs involved in biotic and abiotic stresses namely NAC, WRKY, bHLH, bZIP, MYB and AP2/ERF were studied in 40 OsGLP gene promoters. A total of 1.5 kb upstream sequence was analyzed for each GLP member and it was divided into three regions namely proximal promoter region (500 bp upstream from the start codon), median promoter region (501 bp to 1000 bp upstream from the start codon) and distal promoter region (1001 bp to 1500 bp upstream from the start codon).

Expression study of OsGLP genes

OsGLP gene expression data was extracted by GENEVESTIGATOR from O. sativa database using Affymatrix Rice Genome Array platform (OS_AFFY_RICE-0) and ‘Perturbations’ tool was used to find out the differential gene expression under biotic and abiotic stresses. The fold change in gene expression was detected using filter 2.0 fold as benchmark. The fold changes in the expression of OsGLP genes under different biotic and abiotic stress conditions (S2 and S3 Files) were used to generate gene expression heatmap using Heatmapper online tool (http://www1.heatmapper.ca/expression/) using Red/Green colour scheme where “Red” colour shows down-regulation and “Green” colour shows up-regulation of respective genes. The microarray dataset used for OsGLP gene expression study using GENEVESTIGATOR has been presented in S2 and S3 Files.

Results and discussion

Chromosomal organization of OsGLP gene promoters

Characteristics of 40 OsGLP gene promoters of O. sativa retrieved from NCBI database have been depicted in Table 1 and their location on each chromosomes have also been presented (Fig 1). The name of the OsGLP protein, the alternate name of some of the genes coding OsGLP (given in parentheses), their corresponding protein ID (accession number), the promoter regions of each OsGLP genes studied here and chromosomal locus of the respective OsGLP gene has been described (Table 1 and S1 File).

Out of these 40 OsGLP genes, chromosome 1 and 2 were found to possess four OsGLP genes in each and 8 OsGLP genes were observed to be located in chromosome 3 (Fig 1). Chromosome 4, 5 and 11 were detected with single OsGLP gene in each while chromosome 8 was found to possess highest number of OsGLP genes (14) and 3 OsGLP genes were identified in
chromosome 9 (Fig 1). Although a recent study documented 43 different OsGLP genes from rice [9], the promoter regions of only 40 OsGLP members have been characterized here. The CDS sequence of OsGLP5-2 belonging to locus LOC_Os05g19670 [9] showed 100% homology with Oryza sativa japonica Group OsGLP5-1 (accession number XM_015782936). Additionally, the protein sequence of OsGLP1-5 with locus number LOC_Os01g72300 [9] was used to

| GLP name  | Protein id | Promoter region       | Locus on chromosome | Strand |
|-----------|------------|-----------------------|---------------------|--------|
| OsGLP1-1  | XP_015622834.1 | 10170426–10168927 Os01g0284500 | - strand            |        |
| OsGLP1-2  | XP_015616392.1 | 29230400–29231899 Os01g0705100 | + strand            |        |
| OsGLP1-3  | XP_015644058.1 | 41913324–41914823 Os01g0952000 | + strand            |        |
| OsGLP1-4  | XP_015644512.1 | 41916366–41917865 Os01g0952100 | + strand            |        |
| OsGLP2-1  | XP_015624078.1 | 17167497–17165998 Os02g0491600 | - strand            |        |
| OsGLP2-2  | XP_015624077.1 | 17165917–17167416 Os02g0491700 | + strand            |        |
| OsGLP2-3  | XP_015626751.1 | 17173722–17175221 Os02g0491800 | + strand            |        |
| OsGLP2-4  | XP_015622959.1 | 19599980–19598481 Os02g0532500 | - strand            |        |
| OsGLP3-1  | XP_015632295.1 | 4156170–4157669 Os03g0179100 | - strand            |        |
| OsGLP3-2  | XP_015630224.1 | 25314197–25315696 Os03g0651800 | + strand            |        |
| OsGLP3-3  | XP_015629955.1 | 27781471–27779972 Os03g0693700 | + strand            |        |
| OsGLP3-4  | XP_015630078.1 | 27786081–27784582 Os03g0693800 | - strand            |        |
| OsGLP3-5  | XP_015629893.1 | 27790413–27789194 Os03g0693900 | - strand            |        |
| OsGLP3-6  | XP_015629049.1 | 27793400–27791901 Os03g0694000 | - strand            |        |
| OsGLP3-7  | XP_015628931.1 | 33584974–33586473 Os03g0804500 | + strand            |        |
| OsGLP3-8  | XP_015630140.1 | 33589695–33591194 Os03g0804700 | + strand            |        |
| OsGLP4-1  | XP_015634976.1 | 3196595–3195906 Os04g0617900 | - strand            |        |
| OsGLP5-1  | XP_015638422.1 | 11465690–11467189 Os05g0277500 | + strand            |        |
| OsGLP8-1  | XP_015649422.1 | 5185375–5186874 Os08g0188900 | + strand            |        |
| OsGLP8-2 (Germin-like protein 16) | XP_015648581.1 | 5206934–5208433 Os08g0189100 | + strand |        |
| OsGLP8-3  | XP_015650201.1 | 5220775–5222274 Os08g0189200 | + strand            |        |
| OsGLP8-4 (OsGer1) | XP_015650202.1 | 5227380–5228879 Os08g0189300 | + strand |        |
| OsGLP8-5  | XP_015650206.1 | 5232364–5233863 Os08g0189400 | + strand            |        |
| OsGLP8-6  | XP_015650204.1 | 5237572–5239071 Os08g0189500 | + strand            |        |
| OsGLP8-7  | XP_015650203.1 | 5241098–5242597 Os08g0189600 | + strand            |        |
| OsGLP8-8  | XP_015648995.1 | 5247290–5248789 Os08g0189700 | + strand            |        |
| OsGLP8-9  | XP_015648901.1 | 5252864–5254363 Os08g0189850 | + strand            |        |
| OsGLP8-10 (OsRGLP2) | XP_015651068.1 | 5258764–5260263 Os08g0189900 | + strand |        |
| OsGLP8-11 (OsRGLP1) | XP_015651069.1 | 5262817–5264316 Os08g0190100 | + strand |        |
| OsGLP8-12 | XP_015648080.1 | 7997312–7995633 Os08g0231400 | - strand            |        |
| OsGLP8-13 | XP_015649499.1 | 22555500–22554001 Os08g0459700 | - strand |        |
| OsGLP8-14 (OsGer5, OsGLP1) | XP_015648639.1 | 22560443–22558944 Os08g0460000 | - strand |        |
| OsGLP9-1  | XP_015611452.1 | 22697583–22696084 Os09g0568500 | - strand            |        |
| OsGLP9-2  | XP_015612249.1 | 22699889–22693390 Os09g0568600 | - strand            |        |
| OsGLP9-3  | XP_015612248.1 | 22702365–22700866 Os09g0568700 | - strand            |        |
| OsGLP11-1 | XP_015618049.1 | 19584185–19585684 Os11g0373350 | + strand            |        |
| OsGLP12-1 | XP_015619653.1 | 2691258–2689759 Os12g0154700 | - strand            |        |
| OsGLP12-2 | XP_015619655.1 | 2694943–2693444 Os12g0154800 | - strand            |        |
| OsGLP12-3 | XP_015619657.1 | 2698714–2697215 Os12g0154900 | - strand            |        |
| OsGLP12-4 | XP_015619658.1 | 2701472–2699973 Os12g0155000 | - strand            |        |

https://doi.org/10.1371/journal.pone.0211887.t001

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blast at NCBI protein database and it depicted 100% homology with OsGLP1-4 protein sequence. Nevertheless, another GLP (OsGLP3-9) belonging to the locus LOC_Os03g58990 mentioned in earlier report [9] depicted maximum homology with a hypothetical protein. Consequently, the CDS of OsGLP3-9 showed maximal homology with CDS sequence of OsGLP3-8 gene of Oryza sativa japonica group and no other mRNA from japonica group exhibited significant homology with that GLP. Hence, only 40 unique OsGLP gene promoters have been considered here. These large numbers of OsGLP gene promoters might have been generated following the destinies of duplicated OsGLP genes through neofunctionalization of the promoter region as revealed in barley GLP gene cluster [4, 14]. Due to the presence of related sequences in close proximity and their availability in both the DNA strands on chromosome 8, it was speculated that eleven of the OsGLP (OsGLP8-1, -2, -3, -4, -5, -6, -7, -8, -9, -10 and -11) genes exhibited tandem duplications while OsGLP8-13 and OsGLP8-14 exhibited inverse tandem duplications (Fig 1 and Table 1). Additionally, GLP gene cluster were available as both tandem and inverse tandem duplications in chromosome number 2 and 3 whereas, in chromosome number 12 and 9, only inverse tandem duplications were prevailed. On the other hand, in chromosome 1 only tandem duplication was revealed (Fig 1). An earlier study highlighted the significance of tandem duplications in OsGLP gene families [9], however, specific reports of inverse tandem duplication was not discussed. During the course of evolution the multigene families have been developed through genome wide duplication, deletion, as well as translocation [24]. Several studies revealed that some tandemly duplicated genes in human as well as other genomes are expressed in opposite orientation from its nearby duplicated genes [25]. Mutation study in Salmonella enterica revealed that tandem inversion duplication may arise in the population due to selection pressure [26] and such type of duplication is known as reverse tandem duplication or inverted tandem duplication [27]. Based on the direction of transcript expression OsGLP genes were classified as tandemly or inverse

Fig 1. Chromosomal distribution of 40 different rice GLP (OsGLP) genes. Tandem and inverse tandem duplication of OsGLP on different chromosomes are shown in green and red color, respectively.

https://doi.org/10.1371/journal.pone.0211887.g001
tandemly duplicated genes which were present in two different DNA strands. Gene duplication has evolutionary significance and tandem duplication especially in chromosome number 8 played major role in rice to withstand biotic and abiotic stresses [6, 10]. Earlier studies on abundance of duplicated genes and their novel roles in biotic and abiotic stress management resembled similar findings [14]. In addition to rice, a number of GLP genes have also been reported in other crops viz. Arabidopsis, peanut, tomato as well as barley [1] and these multigene family proteins have been involved in basal host resistance [28].

Analysis of the TFs

In rice as well as other crop species the TF families (NAC, WRKY, bHLH, bZIP, MYB and AP2/ERF) have been categorized on the basis of their involvement in ABA-independent and ABA-dependent pathways [29]. In the following section the frequency and importance of studied transcription factors in the OsGLP gene promoter regions have been discussed elaborately considering their correlation with biotic and abiotic stress management.

NAC. A total of 58 NAC TFbs were recognized in 40 different OsGLP gene promoter regions (Fig 2A). The proximal promoter region was found to possess 20 NACbs in both the strands. The median region constituted of maximum number of NACbs (23) whereas relatively less number of NACbs (15) were observed in the distal promoter region. Bioinformatics analysis depicted that in the present study NAC TFs were found to recognize CATGTG sequence on DNA which were in accordance with the earlier literature [30]. In our study it was disclosed that in some of the OsGLP gene promoters (OsGLP3-2, OsGLP3-3, OsGLP3-6, OsGLP3-7, OsGLP3-8, OsGLP4-1, OsGLP5-1, OsGLP8-4, OsGLP8-8, OsGLP8-10 and OsGLP8-14) NACbs were absent (S1 Table). Maximum number of NACbs was detected in OsGLP3-1 which contained a total of 5 numbers of NACbs distributed throughout the regions. NAC TFs play major role in biotic and abiotic stress tolerance mechanism in plants and most of the NAC genes are induced by various abiotic stress signals [31]. Expression study in rice through microarray depicts that around 45 NAC genes are up-regulated upon exposure to abiotic stresses while more than 26 NAC genes show up-regulation in response to biotic stresses [32]. Additionally, in that study almost six different NAC genes depict differential expression in response to rice stripe virus and rice tungro spherical virus infestation. Over-expression of a NAC gene has been found to enhance drought resistance in transgenic rice at the reproductive stage, and also improve drought as well as salt tolerance in the vegetative stage [33]. In another study OsNAC5 has been over-expressed in transgenic rice under root-specific promoter (RCc3), which depicted significantly higher yield compared to the non-transgenic lines under drought stress condition [34]. Similarly in another experiment, over-expression of OsNAC9 under root-specific RCc3 promoter exhibited increased grain yield in exposure to drought stress [35]. According to certain reports, over-expression of NAC in rice also improved tolerance to dehydration and high salinity, although with growth retardation and low reproductive yields [30, 36].

WRKY. In the present study, a total of 118 WRKY TFbs were detected covering all the OsGLP gene promoter regions (Fig 2B). The proximal promoter region was found to possess 43 WRKYbs in both the strands. The median region detected with having highest number of bs (49), whereas relatively lesser number of WRKYbs (26) were present in the distal promoter region. Interestingly, in case of OsGLP promoters in chromosome number 1, all the four members were devoid of any WRKYbs in the distal region (Fig 2B). However in case of OsGLP promoters in chromosome number 2 all the members lacked WRKYbs in the proximal region except OsGLP2-1, where not a single WRKYbs were found in the distal region of the promoter. OsGLP3-6 promoter was the single one to have WRKYbs only in the median region. Among
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the rice OsGLP gene promoters the highest number of WRKYbs was found in the promoter region of OsGLP8-4 followed by the promoter region of OsGLP8-7 with the availability of 9 and 8 bs, respectively (S1 Table). WRKY is a conserve class of plant specific transcription factors responsible for defense and abiotic stress management in rice [37]. In the present study, WRKY TFs were expected to be recognized by TTGACN sequence in OsGLP gene promoters where N stands for any DNA bases. Generally the WRKY TFs recognize and attach with the W box ([T][T]TGAC[C/T]) of target gene promoters to modulate transcription [38], but according to certain reports WRKY TFs may bind to TTGACA [39] as well as TTGACG [40] motifs in promoter region. In rice, the role of WRKY as pathogenicity related systemic acquired resistance signaling transcription factors are well established [40]. Moreover, OsWRKY03 regulates cascade of signaling pathways mediated by jasmonic acid and salicylic acid for protecting plants from bacterial and fungal infections [41]. Along with the biotic stress involvement, a number of WRKY family members are also associated with abiotic stress responses in rice [38, 42]. The upregulation of some members of WRKY TFs have been found to implicate salt tolerance, drought tolerance and heat tolerance in plants [43, 44].

In the present study a total of 292 bHLH TFbs were recognized (Fig 3A). The proximal, median and distal regions of OsGLP promoters were detected with 107, 92 and 93 bHLHbs, respectively in both the strands. Among the OsGLP gene promoters the highest number of bHLHbs was found in OsGLP8-11 having 20 bs, while OsGLP5-1 was devoid of any bHLHbs (S1 Table). Present study revealed several bHLHbs in OsGLP gene promoters and some of their consensus sequences were ATANN[A/T], NNNCG and [C/A][A/G]TATN. Earlier study revealed the binding of bHLH with canonical E-box sequences (CANNTG) for regulating gene expression [45]. Although some of the OsGLP gene promoters like OsGLP1-4, OsGLP3-5, OsGLP3-6 and OsGLP8-13 were found to possess “CANNTG” bs, most of them were found to have “CANNTN” sequence and it was recognized as probable bHLHbs. Previous findings confirmed the role of bHLH in inducing ABA-dependent signaling for management of cold stress [46]. Moreover, the involvement of bHLH in influencing the salicylic acid and jasmonic acid biosynthesis genes for conferring defence response has also been discussed in crop plants [47]. Additionally, bHLH TFs are involved in different abiotic responses as well as reactive oxygen species scavenging mechanisms [48]. In rice, some members of bHLH have been identified as an essential regulator of iron uptake and utilization [49] whereas; some other members of this TF family have been associated with regulating anthocyanin and anthocyanidine biosynthesis [50].

bZIP. It was found that a total of 232 bZIP TFbs were detected to be distributed throughout the studied promoter regions covering both the strands (Fig 3B). Most of the bZIPbs were located in the proximal region with relatively highest number of bs (upto 42%) followed by the median region revealing around 30% of bZIPbs availability. Among 40 OsGLP gene promoters, the highest number of bZIP specific bs (21) was observed in OsGLP8-11 promoter, while the least bZIPbs (2 in each) were found in OsGLP1-3, OsGLP3-8 and OsGLP12-2 gene promoters (S1 Table). Earlier studies have depicted that plant bZIPs bind to the A-box (TAGCTA), C-box (GACGTC) and G-box (CACGTG), but there are also reports of nonpalindromic binding
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sites for bZIPs [51]. In the present study, a number of A-box and G-box were detected in OsGLP gene promoters which might be acted as bZIPbs. In plants, bZIP transcription factors represent a divergent family of TFs which regulate several processes including light signaling, seed maturation, pathogen defence, flower development and abiotic stress signaling [51–52]. Extensive studies have confirmed the role of bZIP in ABA signaling in rice and it has been found to respond upon osmotic stress during vegetative growth [53]. Nevertheless, some bZIP have also been reported to induce salt stress resistance in Arabidopsis through interfering proteolysis and translocation from the endoplasmic reticulum to the nucleus and consequently up-regulation of salt stress genes [54]. Beside abiotic stress management, bZIP TFs regulate salicylic acid and glutathione S-transferase 6 (GST6) gene signaling pathway and thus enable enhanced biotic stress tolerance in rice [55]. Earlier, it was observed that bZIP regulated accumulation of active oxygen species for facilitating programmed cell death and hypersensitive response [56].

**MYB.** A total of 308 MYB TFbs were recognized in 40 OsGLP gene promoter regions (Fig 4A). The distal portion had the highest number of bs (120) followed by the proximal region with 96 bs. The highest number of MYB TFbs was observed in OsGLP3-6 followed by OsGLP8-11 and OsGLP12-1 containing 14, 13 and 13 bs, respectively (S1 Table). MYB members are considered as largest families of TFs in several crop plants. Previous studies indicated that consensus sequence like A(A/C)C(A/T)A(A/C)C, acted as MYB recognition elements (MREs) [57]. However, some previous report indicates WAACCA, TAACTG, CNGTTR, YAACKG, GGATA and CAACTG sequences as cis-acting regulatory elements for MYB binding in drought inducible gene expression [58]. In the present study it was revealed that MYB recognized GGATT and AGATT consensus sequences in the promoter regions of the studied OsGLP genes and that corroborated the earlier finding of plant MYB-related TF binding [59]. MYB members have been found to be involved in abiotic stress management in rice upon dehydration, salt and cold stresses [60]. Members of this TF also influenced ABA, PEG and SA-signaling pathways conferring response towards biotic and abiotic stresses [61]. Several functional genomics studies depict the involvement of MYBbs (G-box and H-box) in governing tissue specific expression and expressional up-regulation upon virus as well as bacterial infestation in plants [57, 62].

**AP2/ERF.** A total of 3238 bs were recognized in context to AP2/ERF TFs(Fig 4B). Grippingly all the AP2/ERFbs were found to be distributed throughout the promoter regions covering both positive and negative strands of proximal, median and distal portion. However, the distal portion was detected with highest number of AP2/ERFbs in comparison to other two portions. Interestingly, all of the studied OsGLP gene promoters had AP2/ERF binding sites. The highest number of AP2/ERFbs was observed in case of OsGLP8-11 followed by OsGLP12-3 comprised of 122 and 120 numbers of bs, respectively. OsGLP8-13 exhibited least number of AP2/ERFbs (23) among all the 40 OsGLP gene promoters (S1 Table). Among the six TFs studied in the present context, the occurrence of AP2/ERFbs was the highest in the promoters of OsGLP gene families of rice. The relation of ERF family members with several stress responsive mechanism in plant species possibly increased its frequencies and distribution among stress-
In-silico study of stress-related transcription factor binding sites in the promoter regions of rice GLP genes
related gene promoters [29]. In the present study within the promoters of OsGLP gene families, AP2/ERF recognized several sequences as cis-acting elements (G/CTCTA, ATCTT/G/C, ATCAA etc.). In the earlier reports it was validated that ERF families could bind with two cis-acting elements viz. GCC box and CRT elements, involved in ethylene responsiveness related to PR genes and expression of cold and dehydration responsive genes, respectively [55]. An interesting review documented several binding sequences of AP2/ERFs [63] and some of those bs were available in OsGLP gene promoters. Several studies reported improved drought, salinity, water use efficiency, heat and cold response without compensating yield losses in transgenic rice plants through over-expression of AP2/ERF genes either in root-specific or in constitutive manner [64–65]. Additionally, some members of ERF families up-regulated the expression of gibberellin-deactivating gene under high-salinity stress causing reduction of the endogenous gibberellic acid (GA) level and consequently repressed growth as well as improved stress adaptation [66]. Regarding biotic stress response, AP2/ERFTF families induced the synthesis of ethylene, salicylic acid and jasmonic acid and increased the expression of PR genes after invasion by pathogen for enabling better resistance mechanism against pathogens [55]. It is worthy to mention that, according to certain reports up-regulation of genes by AP2/ERF members enhanced resistance to specific biotic and abiotic stresses and corrected growth defects [67–68].

In the present study on six TFbs availability among 40 OsGLP gene promoters, largest number of TFbs was detected in OsGLP8-11 followed by OsGLP12-3, while least number of bs was identified in OsGLP5-1 followed by OsGLP1-2 (Fig 5 and S1 Table). Although, systematic analyses of most of the OsGLP gene promoters were not executed earlier in regards to biotic and abiotic stress-related TFbs availability, certain in silico studies were found to be in corroboration with our findings. Our analysis regarding locus identification revealed that OsRGLP1 is similar to OsGLP8-11 (Table 1), the highest TFbs possessing promoter. In silico promoter analysis by another group has demonstrated that the availability of a TFbs "ACGT" in OsRGLP1 is far higher compared to OsRGLP2 (equivalent to OsGLP8-10) promoter region [22]. According to earlier study, TFbs “ACGT” has been found to be involved in drought and senescence response [69] and it could be expected that OsGLP8-11 might be highly responsive to drought stress. Further experimentation is needed with the high and low TFbs possessing OsGLP promoters to unravel their biological significance.

**Phylogenetic analysis of OsGLP gene promoters**

Phylogenetically, 40 OsGLP gene promoters were found to be divided into 4 different clades with bootstrap values of 0 to 100 (Fig 6). Clade I contained 15 sequences of which cluster 1 had 12 sequences (OsGLP8-1, -2, -5, -6, -7, -8, -9, -10, OsGLP12-1, -2, -3 and -4) from chromosome 8 and 12, while the cluster 2 had 3 sequences (OsGLP8-3, -4 and -12) belonging to chromosome 8. Clade II was comprised of 6 sequences having two clusters where, cluster 1 contained 3 sequences (OsGLP3-7, -8 and OsGLP4-1) from chromosome 3 and 4 while the cluster 2 was composed of 3 promoter sequences (OsGLP8-14, OsGLP9-2 and OsGLP11-1) from 3 different chromosomes (chromosome 8, 9 and 11). The clade III was revealed as the smallest clade and
it consisted of only 3 sequences; of which two (OsGLP3-1 and OsGLP3-3) were from chromosome 3 and one (OsGLP1-1) from chromosome 1. Clade IV was identified as the largest clade consisting of 16 promoter sequences of which 8 sequences (OsGLP1-2, OsGLP2-1, -2, OsGLP3-4, -5, -6, OsGLP5-1 and OsGLP8-13) were in cluster 1 and remaining 8 sequences (OsGLP1-3, -4, OsGLP2-3, -4, OsGLP3-2, OsGLP9-1, -3 and OsGLP8-11) were belonging to cluster 2.

OsGLP promoters on the chromosome 12 showed maximum homology among themselves and OsGLP promoters on chromosome 8 also exhibited high level of similarity with each other (Fig 6). Certain promoters on chromosome 8 (OsGLP8-11, -13 and -14) were distantly related to the rest of the OsGLP promoters present in the same chromosome. In addition to the OsGLP promoters of chromosomes 8 and 12, promoters belonging to chromosome 2 (OsGLP2-1, -2, -3 and -4) were also grouped in a particular clade (clade IV). It was observed that on chromosomes 1, one OsGLP promoter (OsGLP1-1) was categorized under clade III while other three promoters (OsGLP1-2, -3 and -4) were grouped in clade IV (Fig 6). Eight different OsGLP promoters available on chromosome 3 were scattered in clade II, III and IV. Nonetheless, two promoters (OsGLP9-1 and -3) of chromosome 9, were grouped in clade IV while another promoter (OsGLP9-2) was grouped under clade II (Fig 6). Rice chromosomes (4, 5 and 11) containing single OsGLP also exhibited diversity in their OsGLP promoter regions (Figs 1 and 6). Tajima’s neutrality test was conducted using 1.5 kb promoter sequences from 40 OsGLP and it was found to be significant ($D = 7.36$ where, $D = $ Tajima test statistic). Additionally, the nucleotide diversity among the tested sequences was 0.696. Most of the OsGLP promoters on chromosomes 8 and 12 shared the highest sequence similarity and this might be due to the duplication of the genomic region including the presence of TFbs. In corroboration to our findings, OsGLP promoters analyzed using 1.0 kb promoter region also depicted the relatedness of the promoters on chromosomes 8 and 12 [70]. Although, according to our study certain promoters on chromosome 8 viz. OsGLP8-11, -13 and -14 were distantly related, earlier study [70] depicted that OsGLP8-11, -12 and -13 promoters were phylogenetically distinct from the rest of the OsGLP
promoters on chromosome 8. This difference with earlier study might be due to the consideration of 1.5 kb promoter region in our study. TFbs analysis revealed that the OsGLP8-14 promoters was devoid of any NACbs and only one WRKYbs was available in the distal promoter region making it unique from rest of the OsGLP promoters of chromosome 8 (Fig 2). Consistent to our finding previous report also depicted that OsGLP8-14 is preferentially expressed in panicle development stage [9], which is unique among the OsGLP genes. Additionally, the availability of fewest numbers of TFbs (Fig 5) in OsGLP8-13 promoter might be the reason of the phylogenetic distinctiveness of this promoter from rest of the OsGLP promoters belonging to chromosome 8. Another study depicts that the promoter region of Os RGLP1 and Os RGLP2 gene have only 21% homology [71] and those genes are same as OsGLP8-11 and OsGLP8-10, respectively (Table 1). Hence, in support to earlier literature [71] our findings can be justified regarding the phylogenetic classification of OsGLP8-10 and OsGLP8-11 promoters in different clade (Fig 6). According to earlier report [9] OsGLP2-1, OsGLP2-2 and OsGLP2-3 were tandemly duplicated; but as in chromosome 2, OsGLP2-1 was in the negative strand while
OsGLP2-2 and OsGLP2-3 were available in positive strand, they were expected to be originated from inverse tandem and tandem duplications, respectively (Fig 1 and Table 1). All of the OsGLP promoters in chromosome 2 were categorized into clade IV possibly due to their origin through duplication in chromosomal region along with their close proximity in same chromosomal arm (Fig 1).

In accordance with the previous report [70], in the present study also OsGLP promoters under chromosome 1, 3 and 9 exhibited maximum diversification. Interestingly, OsGLP1-1 and OsGLP1-2 were found to be located at different arms on chromosome 1, and this positional difference might be causing diversity between them. On the other hand, OsGLP1-3 and OsGLP1-4 were found to be located in close proximity and might be generated due to tandem duplication (Fig 1) so, these two promoters were closely related and belonging to same clade (Fig 6). In a similar manner OsGLP3-7 and -8 promoters on chromosome 3 were phylogenetically closer probably due to their origin through tandem duplication in chromosome 3 while OsGLP3-4, -5 and -6 were highly phylogenetically related due to their derivation through inverse tandem duplication (Figs 1 and 6). The phylogenetic differences among the rest of the OsGLP promoters under chromosome 3 and 9 might be due to the diversification in their TFbs as mentioned by earlier researchers also [70]. Another study on barley germin-like GER4 gene cluster revealed that barley genome contains tandemly duplicated genes (GER4a-h) and different GER4 promoters exhibited differential expression in response to biotic stress due to the available changes in their cis-regulatory region [14].

OsGLP gene expression study in response to biotic and abiotic stresses

Expression study by GENEVESTIGATOR using the microarray dataset from rice depicted that in response to biotic and abiotic stresses there is some correlation in OsGLP gene expression and OsGLP gene promoter classification. Gene expression of OsGLP8-3 and OsGLP8-4 depicted retarded gene expression under several biotic stresses while the expression study by microarray demonstrated up-regulated gene expression for OsGLP8-7 and OsGLP8-10 in response to brown plant hopper (Nilaparvata lugens), Agrobacterium tumefaciens and fungal infestations (Fig 7 and S2 File). Similarly the expression of OsGLP8-7 and OsGLP8-10 genes depicted high level of similarity upon exposure to several abiotic stresses like cold stress, salt stress and anoxia (Fig 8 and S3 File). It is to be recalled here that phylogenetic study of OsGLP gene promoters showed that OsGLP8-7 and -10 belong to clade I while OsGLP8-3 and -4 were categorized under clade II (Fig 6). Although OsGLP8-14 and OsGLP11-1 depicted about 3 fold expressional up-regulation upon cold stress (S3 File), due to inadequate data availability no additional significant similarity was revealed among the gene expression of OsGLP3-7, 3−8, 4−1, 8−14, 9−2 and 11−1. Phylogenetic study of OsGLP promoters depicted that all of those 6 promoters were classified in clade II. The gene expression of OsGLP1-1 and OsGLP3-3 depicted that upon several abiotic stresses like cold, drought and heat stresses their expressions were up-regulated (Fig 8). Additionally the gene expression data depicted that in response to A. tumefaciens and fungal infestations, the gene expression of OsGLP1-1 and OsGLP3-3 were down-regulated (Fig 7). Promoters of OsGLP1-1 and OsGLP3-3 were categorized in clade III through phylogenetic analysis (Fig 6). In response to submersion, the expression of OsGLP3-6 as well as OsGLP5-1 genes were found to be down-regulated (Fig 8) and the promoter regions of those two genes belong to clade IV (Fig 6). Interestingly, the expression of OsGLP8-11 and OsGLP2-4 also showed expressional retardation upon submersion and according to our phylogenetic study these two OsGLP gene promoters were also categorized in clade IV. From the present study and earlier literature, it can be enumerated that a number of OsGLPs located in the chromosome 8 revealed as major gene cluster conferring
biotic stress tolerance in rice [6]. Similar kind of defence responses have also been observed in barley and grape by the orthologus GLP members [4, 72].

Although the genome wide duplication as well as neofunctionalization of the OsGLP genes as well as promoter region were taken place during the course of evolution, expression analyses of microarray data from public database revealed certain homology among the expression of OsGLP genes whose promoter regions were classified under same clade. Considering the previous finding [9, 14] and our present study it can be inferred that OsGLP duplication and subsequent variation in TFbs resulted neofunctionalization and these genes as well as their promoters might be involved in tolerance to biotic and abiotic stresses via broad-spectrum resistance or basal mechanism of tolerance in plants.

**Conclusion**

In the present study among 40 OsGLP promoter regions, the presence of different kinds of TFbs was revealed in varied frequencies and some of these promoters were phylogenetically distinct in spite of their presence in the same chromosome. It can be inferred that during the course of evolution these OsGLP promoters were under considerable environmental pressure. In the present study we have considered six important TFs viz. NAC, WRKY, bHLH, bZIP, MYB and AP2/ERF, due to their significant involvement in biotic and abiotic stresses. Although certain OsGLP promoters (OsGLP8-11 and OsGLP12-3) were found to possess larger number of TFbs and some promoters (OsGLP1-2, OsGLP5-1 and OsGLP8-13) had fewer, further study is needed to validate these OsGLP promoters for subsequent utilization in plant genetic engineering as stress inducible promoter. Gene expression data revealed that certain
Promoters belonging to the same clade had similar patterns of gene expression. It can be concluded that OsGLP gene duplication and subsequent variation in TFbs resulted in neofunctionalization of gene and promoter regions to cope up with various biotic and abiotic stresses during the course of evolution.

**Supporting information**

S1 Table. Availability of different transcription factor binding sites (TFbs) in 40 OsGLP promoters.

S1 File. The 1.5 kb promoter sequences of 40 OsGLP genes from 9 different chromosomes of rice.

S2 File. Microarray data of OsGLP gene expression (expressed in fold change) in response to biotic stresses.

S3 File. Microarray data of OsGLP gene expression (expressed in fold change) in response to abiotic stresses.

**Acknowledgments**

The authors would like to acknowledge Indian Institute of Technology Kharagpur and Bidhan Chandra Krishi Viswavidyalaya for providing the basic facilities to carry out this research.
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