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Genomic Exploration: Unraveling the Intricacies of Indica Rice *Oryza sativa* L. Germin-Like Protein Gene 12-3 (*Os*GLP12-3) Promoter via Cloning, Sequencing, and *In Silico* Analysis

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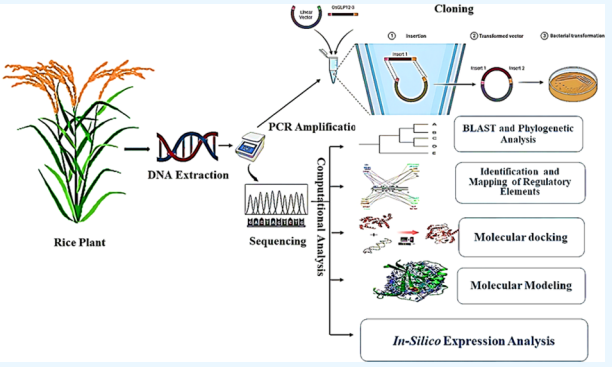
ABSTRACT: Germin and Germin-like proteins (GLPs) are a class of plant proteins that are part of the Cupins superfamily, found in several plant organs including roots, seeds, leaves, and nectar glands. They play a crucial role in plant defense against pathogens and environmental stresses. Herein, this study focused on the promoter analysis of *Os*GLP12-3 in rice cultivar Swat-1 to elucidate its regulation and functions. The region (1863bp) of the *Os*GLP12-3 promoter from Swat-1 genomic DNA was amplified, purified, quantified, and cloned using Topo cloning technology, followed by sequencing. Further in silico comparative analysis was conducted between the *Os*GLP12-3 promoters from Nipponbare and Swat-1 using the Plant CARE database, identifying 24 *cis*-acting regulatory elements with diverse functions. These elements exhibited distinct distribution patterns in the 2 rice varieties. The *Os*GLP12-3 promoter revealed an abundance of regulatory elements associated with biotic and abiotic stress responses. Computational tools were employed to analyze the regulatory features of this region. In silico expression analysis of *Os*GLP12-3, considering various developmental stages, stress conditions, hormones, and expression timing, was performed using the TENOR tool. Pairwise alignment indicated 86% sequence similarity between Nipponbare and Swat-1. Phylogenetic analysis was conducted to explore the evolutionary relationship between the *Os*GLP12-3 and other plant GLPs. Additionally, 2 unique regulatory elements were modeled and docked, GARE and MBS to understand their hydrogen bonding interactions in gene regulation. The study highlights the importance of *Os*GLP12-3 in plant defense against biotic and abiotic stresses, supported by its expression patterns in response to various stressors and the presence of specific regulatory elements within its promoter region.



1. INTRODUCTION

Oryza sativa L., commonly known as rice, holds pivotal status as a primary cereal crop. Global rice production for the year 2015−2016, as reported by the United States Agriculture Department (USAD), reached 469.32 million metric tons. Acknowledging its significance as a model crop plant, Devos and Gale emphasize its utility in elucidating fundamental traits like productivity, yield, hybrid vigor, disease resistance, and stress tolerance due to its fully sequenced genome.[1] Despite *O. sativa* maintaining a diploid genome structure ($2n$ = 24), certain Oryza species exhibit tetraploid genomes ($4n$ = 48). Noteworthy is rice's distinction among food crops for possessing a relatively compact genome, with repetitive sequences constituting approximately half of its genetic content.[2] The rice genome comprises 12 chromosomes, displaying genetic diversity across both cultivated and wild species.[3,4] In-depth genomic analysis,[5] reveals extensive genetic diversity, with the identification of over 50,000 genes within the *O. sativa* genome.

Germins and germin-like proteins (GLPs) belong to the cupin superfamily of plant proteins[6] and play an important role in the plant's defensive mechanism against osmotic and homeostasis stresses.[7] Additionally, they are involved in enhancing the plant's resistance to oxidative stress caused by both abiotic and biotic stimuli,[8−10] have provided a comprehensive description of the germins and GLPs, highlighting their occurrence in plants and their association with several developmental processes.[10,11] Additionally, it has been reported that the genome of *O. sativa* has a considerable collection of GLPs. As a result, *O. sativa* serves as a model

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plant for investigating the structural and functional characteristics of germins and GLPs. Several studies have documented the presence and manifestation of GLPs in different plant tissues,[10,12,13] as well as their response to various unfavorable environmental conditions.[14−16] GLPs have a wide range of expression patterns. Certain developmental stages, including fruit ripening, blooming initiation, and embryogenesis.[17,18] Several biotic and abiotic stress situations, including attacks by pathogens,[13,19] herbivores,[20] and osmotic stress,[13] induce different GLPs. It was found that pathogens activate the barley GER4c gene promoter.[21] In a continuation study,[22] used expression profile data to select an expressed-sequence tag (EST) encoding a putative GLP that is expressed in leaves to functionally analyze the *Eg*GLP gene promoter from *Eucalyptus grandis*. They also confirmed the promoter activity through reverse transcription polymerase chain reaction (PCR) analysis. Additionally, two GLP gene-promoters from two conifers were studied (LmGER1 from hybrid larch and PcGER1 from *Pinus caribaea*).[17] The PcGER1 promoter was shown to be active throughout cell growth,[23,24] whereas the LmGER1 promoter was found to be responsible for reporter gene expression in developing embryonic root caps and cotyledons.[17]

The main goals of this study are the amplification of the *Os*GLP12-3 promoter region from rice genomic DNA, cloning it into a cloning vector, its sequencing, and the use of computational tools to characterize the regulatory elements and potential transcription-factor binding sites. Furthermore, an analysis was carried out on the interplay between the transcription factors and associated regulatory elements. Two transcription factors, the MYB binding site (MBS) and gibberellin responsive elements (GARE), which have a major impact on plant growth and the response to drought stress, respectively, were selected from the several transcription factors found in the *Os*GLP12-3 gene promoter to assess protein−DNA interactions. The presence of regulatory elements in matching promoters and the expression profile of the *Os*GLP12-3 gene in response to different abiotic and biotic stresses indicate that this protein is vital for plant defense against pathogens and abiotic stress conditions.

2. MATERIALS AND METHODS

2.1. Plant Material and Their Cultivation. The seeds of Rice *O. sativa* L. cultivar Swat-1 were obtained from the National Agriculture Research Centre (NARC) Pakistan. After the surface sterilization, these were cultivated on MS basal media hydro-phonically under aseptic conditions.

2.2. Primer Designing and PCR Amplification. For the amplification of the *Os*GLP12-3 gene-promoter region from the selected rice variety, a specific set of primers was designed from available sequences in GenBank of NCBI (National Center for Biotechnology Information) with the assistance of primer 3 (version 0.4.0), and the sequence of primers is given below;

Forward Primer: 5′-CACCACCTTGACTTGTTGTCAG-3′

Reverse Primer: 5′-CATGTTAAGTTGATG-GAACTTTTG-3′

Genomic DNA was extracted from the leaf tissues by the Cetyltrimethyl Ammonium Bromide (CTAB) method described by.[29] Extracted DNA was used for the amplification of the *Os*GLP12-3 gene promoter (1863bp). For the amplification of the *Os*GLP12-3 gene promoter region, a 200 μL PCR reaction mixture was prepared using Dream Taq Green Master Mix 2X (Thermo Scientific) 100 μL, with 72 μL of PCR grade Water provided with Master Mix, 20 μL of template DNA and 4 μL of each 25 Pmol forward and reverse primers, and the reaction was set to run: the conditions were pre-denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 1 min. The final extension cycle was set at 72 °C for 7 min.

2.3. Cloning, Sequencing, and Computational Analysis of the Sequence. *2.3.1. Cloning.* Amplified PCR products were purified using Monarch DNA Gel Extraction Kit (New England Biolabs) as per the manufacturer's instructions. The amplified promoter sequence was cloned to a cloning vector using the Invitrogen pCR 2.1 Topo cloning kit as per the manufacturers' instructions.

2.3.2. Sequencing. The gel-purified PCR products were sequenced by acquiring services from Macrogen Seoul, South Korea.

2.3.3. Computational Analysis of the Promoter Sequence. Various bioinformatics tools, sequence searches, and databases were used for the analysis of the sequencing data. The basic local alignment search tool (BLAST) was used to detect the similarity between sequences. Nucleotide Blast (BLASTn) was applied to find the sequence's resemblance with previously submitted sequences in the sequence databases (http://blast.ncbi.nlm.nih.gov).[25−28] The sequences which show a high level of similarity were selected for the phylogenetic analysis which includes the sequences from the *Sorghum bicolor*, *Setaria italica*, *Zea mays*, *O. sativa* Japonica group and *Panicum hallii*, *Dichanthelium oligosanthes*, *Triticum uratru*, *Hordeum vulgare* subspp. Vulgare, *Aegilops tauschii* subspp. Tauschii, *Triticum aestivum*, and Japonica Rice PAC (PI Artificial Chromosome) clone which also belongs to the monocot group of plants. The software tool CLUSTAL-X downloaded from (https://www.clustal.org), and is available offline, was utilized to align the promoter's sequence with other GLP promoters to perform phylogenetic analysis and multiple sequence alignment. The homology and differences between *Os*GLP12-3 and other GLP promoters were determined by using the MEGA 7 software program, which can be downloaded from (https://www.megasoftware.net/active_download), phylogenetic tree was constructed to determine the degree of similarity between *Os*GLP12-3 and other GLPs promoters as well as to trace back the evolutionary history of genes.[16,25] The promoter region was searched for several *cis*-acting regulatory elements using the bioinformatics tool Plant CARE, which can be accessed at http://bioinformatics.psb.ugent.[30] A separate DOG 2.0 program was utilized to map various *cis*-acting regulatory elements downloaded from (www.mybiosoftware.com/protein-sequence-analysis). Additionally, an online information tool called WEB 3DNA, which can be accessed at http://w3dna.rutgers.edu/, was utilized to model the *cis*-acting regulatory elements located in the promoter region. PDB was used to derive the structural information about the regulatory components of the potential interacting pairs. Missing residues, atomic resolution, and mutation were the selection criteria used to determine which template should be used for further DNA−protein docking study.[31] The HADDOCK web server, which is accessible online at http://haddock.chem.com, was used to perform protein−DNA docking. PyMol, the Molecular Graphic System, which is accessible online at (http://www.pymol.com), was used to visualize the graphic analysis of every model.[25] Additionally, the TENOR "The Encyclopedia of

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Rice" database, available online at (http://tenor.dna.affrc.go.jp/),[32] was used to predict and understand gene's regulatory characteristics, in terms of gene expression, which is governed by *cis*-acting elements that are the Transcription-factor binding sites located on the promoter. The information available, with context to the expression of the gene, governed by its promoter, during development and in response to abiotic and biotic stresses, were retrieved from the TENOR database.[33] For data retrieval from the TENOR database different parameters; including different abiotic stress conditions, and development, along with the time period taken, for the expression of the *Os*GLP12-3 gene, were used.[16,25]

3. RESULTS AND DISCUSSION

3.1. Amplification by PCR. The promoter region was amplified by PCR and resolved as indicated in Figure 1 on a TBE-agarose gel.

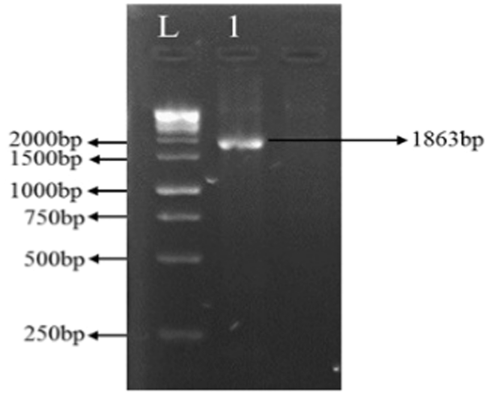

Figure 1. Amplified *Os*GLP12-3 is represented by Lane 1 and the 1KB + DNA Ladder is represented by Lane L.

3.2. Confirmation of Cloning. To determine whether the inset was present in the retrieved putative-recombinant plasmids, PCR was performed using the plasmids as a template. This was done using insert-specific primers and resolved on a TBE-agarose gel, as shown in Figure 2.

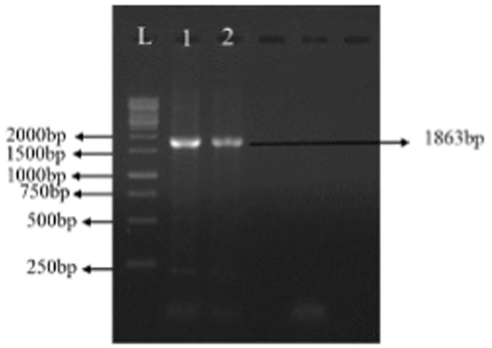

Figure 2. Using gene-specific primers, the clone was confirmed.

3.3. Sequencing. The PCR-amplified product of the *Os*GLP12-3 gene promoter was used for the sequencing. The obtained sequence data for the *Os*GLP12-3 gene-promoter region from Rice variety Swat-1 exhibited 86% similarity, with the *Os*GLP12-3 promoter sequence from *O. sativa* variety Nipphonbare, which is taken as a standard. The sequence was finally submitted to the Genbank database, and accession number MK946667.1 was obtained.

3.4. Comparative Phylogenetic Analysis and Multiple Sequence Alignment. For building a phylogenetic tree, multiple sequence alignments and phylogenetic analyses were applied to determine the evolutionary relationship between *Os*GLP12-3 and other GLPs from various plants. Using phylogenetic analysis and MSA, two major clades are formed. While the sequences in Clade-2 came from the Japonica Rice PAC (PI Artificial Chromosome) clone, which is also a member of the monocot group of plants, the sequences in Clade-1 came from monocot plants, such as *S. italica, S. bicolor, Z. mays, O. sativa Japonica and P. hallii, T. uratru, D. oligosanthes, H. vulgare* subspecie *Vulgare*, and *Tauschii Aegilops tasuschi* subspecie *Titicum aestium*. Two sub-groups, which were subsequently sub-divided into two sub-groups, were formed from the further division of Clade-1. Two sub-groups were created out of group two. However, it was discovered that sub-group 1, and sub-group 2 had a cluster of GLP12-2 branches that belonged to *D. oligosanthes* and a cluster of additional sub-groups that contained sequences of GLPs from different plants. But as Figure 3 illustrates, *Os*GLP12-3 was grouped in Sub-group 1B, which also included the hypothetical protein PAHAL F01217 from *P. hallii* and another GLP protein, PHAL F01217 from Japonica rice. The *Os*GLP12-3 Promoter from Indica rice cultivar Swat-1 is closely connected to the Japonica group of rice according to the phylogenetic study.

3.5. Identification and Mapping of the *cis*-Acting Regulatory Elements. In the two rice cultivars, Swat-1 and Nipponbare (taken as a standard for comparison), the *Os*GLP12-3 gene promoter region revealed to include many *cis*-acting regulatory elements.[25] It was discovered in this investigation that these two varieties contain 24 significantly distinct *cis*-acting elements. Twenty of these regulatory elements, which are listed in Table 1, were found to be unique in one variety with distinct copy numbers from the remaining elements being discovered to be common in both. Thirteen distinct elements were found in Swat-1, whereas seven were found in Nipponbare. In Swat-1, two unique regulatory components were reported. Every regulatory element's location was plotted on the *Os*GLP12-3 promoter. Figure 4 provides a map of the *cis*-acting elements present on the promoter. A detailed comparison of the several *cis*-acting regulatory elements present in the two rice varieties' *Os*GLP12-3 Promoter regions; Nipphonbare and Swat-1 are denoted by V1 and V2, respectively is given in Table 1.

3.6. Transcription Factor Interactions with Regulatory Elements. Studying the interactions between DNA and proteins is crucial to the understanding of gene expression regulation.[16] In the current study, two TFs (MBS and GARE) were chosen to dock for the detection of DNA protein interactions as indicated in Figure 5 and Figure 6 based on the buried surface area, HADDOCK score, and cluster size as provided in Table 2. Before being chosen for docking, the aforementioned transcription factors were modeled to identify DNA−protein interactions. Structural information regarding protein−DNA docking complexes was analyzed in terms of Hydrogen bonding residues. The transcription factor GARE was docked with the *cis*-acting element 5′ AACCTAA 3′ forming hydrogen bonds between them as illustrated by the dotted lines in Figure 7. Its Hydrogen bonding interactions between the protein−DNA residues along with the bond

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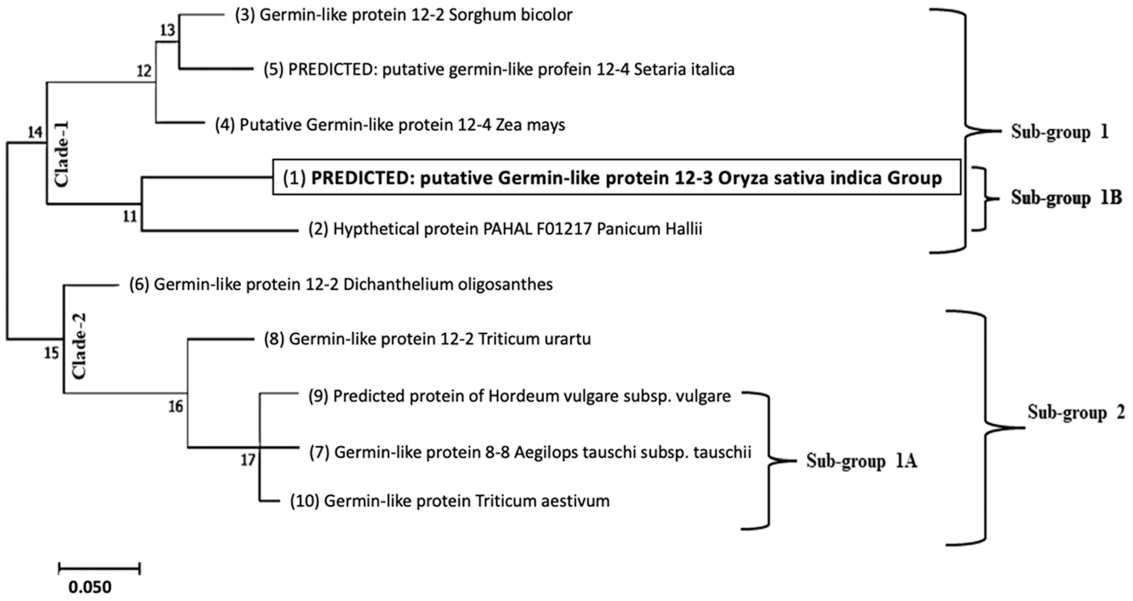

Figure 3. Phylogenetic tree showing the evolutionary relationship of *Os*GLP12-3 with GLPs from different other plants.

distances among them are shown in Table 3. Furthermore, the second TF, MBS was also docked to the regulatory element 5′ CGGTCA 3′ the Hydrogen bonds formed are shown by the dotted lines in Figure 8. Its Hydrogen bonding interactions between the protein−DNA residues along with the bond distances among them are shown in Table 4. Previous studies have demonstrated that hydrogen bonds between the sequences of proteins and DNA are the causes of TFs DNA interactions.[16,58]

3.7. DNA−Protein Docking Analysis. Further computational analysis was performed for the two important transcription factors, MBS and GARE, to better understand how the transcriptional regulation of *Os*GLP12 3 gene expression works.[59] Structural information regarding protein−DNA docking complexes was analyzed in terms of Hydrogen bonding residues. As shown by the dotted lines in Figure 7, a typical model generated by HADDOCK, the transcription factor GARE was docked with the *cis*-acting element 5′ AACCTAA 3′ creating hydrogen bonds between them. N7 exhibited the greatest quantity of hydrogen bonds in the protein DNA docking data along with THR20, SER70, ILE69, GLY68, and ASN66. Additionally, as Table 3 below shows, Hydrogen bonds bound O1P and LYS18, C2 and ALA21, N and LYS63, O2P and GLY68, and C2N3 and GLY13. As observed in Figure 8, a second TF MBS was also affixed to regulatory element 5′ CGGTCA 3′ by the use of dotted lines to illustrate hydrogen bonding. Stronger connections between the protein and DNA were indicated by this TF's smaller gap and fewer dots between residues. In particular, O2P created the most hydrogen bonds with ARG 126, AGR223, AGR180, AND LEU181. Furthermore, hydrogen bonds were observed between O1P and ARG126, O3 and ARG126, O4 and ALA120 and LUC119, N6 and PR O121 and ALA120 and C5 AND GLY AND PRO123 as shown in the Table. 4 below.

3.8. *In Silico* Expression Analysis of *Os*GLP12-3 Gene. *In silico* analysis for the expression data of the *Os*GLP12-3 gene was retrieved from the TENOR database. Parameters for data retrieval included development and abiotic stress conditions including hormones, along with the time taken for the expression of the *Os*GLP12-3 gene.[33]

3.8.1. Expression of OsGLP12-3 Gene during Development. The *Os*GLP12-3 gene can be observed to be involved in plant development, mainly in roots. Maximum expression level was observed in the first 3 h after germination and was found to be continuously expressed after the first expression. These results are in agreement with former findings by,[60] in which they observed the involvement of the *Os*GLP-1 gene in root elongation when it was transgenically overexpressed in Tobacco.

3.8.2. Expression of OsGLP12-3 Gene in Response to Hormonal Stimuli and Abiotic Stresses. The data gathered about the response of the *Os*GLP12-3 gene to abiotic stress conditions included: high and low concentrations of phosphorus and cadmium; drought (dry), high salinity, flood; cold treatment; osmotic stresses; and hormones, which indicate that all abiotic factors, including hormones, can induce the *Os*GLP-12-3 gene. Examples of these factors include abscisic acid[30,60,39,32] and jasmonic acid.[61−64,39,65] For examination, two different tissue types, the roots and shoots, were chosen.

Five days subsequent to the initial exposure of the plant to cadmium stress in its roots, high level of gene expression in response to heightened cadmium stress was observed. Intriguingly, the commencement of expression ensued within 1 h postexposure to elevated cadmium concentrations, sustaining at moderated levels across the plant's development. Noteworthy is the observation in Figure 9, illustrating the expression of the *Os*GLP12-3 gene in shoots subjected to elevated cadmium stress. The graphical representation in Figure 9 delineates the profile of gene expression, with initiation observed no earlier than 12 hours postexposure to elevated cadmium stress and an exponential surge maintained for a discrete period of 5 days. Under various abiotic stress conditions, including osmotic stress (flood-induced), dry conditions, flooding, and varying cadmium concentrations, the expression levels of *Os*GLP-12-3 exhibit distinct patterns. Modest expression is observed in response to osmotic and dry stresses, while lower moderate expression is noted under flood and low cadmium stresses. It is noteworthy that under high cadmium concentrations, the gene predominantly responds in

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Table 1. Comparison of the Several *cis*-acting Regulatory Elements Present in the Two Rice Varieties *Os*GLP12-3 Promoter Regions; Nipponbare and Swat-1, Denoted by V1 and V2, Respectively

			copy number		position		
Sr.,#	name of the regulatory element	sequence of the element	V1	V2	V1	V2	function of regulatory element
1	G-Box	CACGTTCACACATGGAACACATGG	2	0	56−58	0	involved in light responsiveness[34]
2	CAAT-Box	CAATCCAATCAAATCAATTCCAATCCAATCAATTGGCAAT	7	15	128−483	105−686	common cis-acting element in promoters, enhancer region[35]
3	Box-4	ATTAATATTAATATTAAT	3	3	352−400	273−390	part of a conserved DNA module involved in light responsiveness[36]
4	TATA-Box	ATATATTATATATAATAATTATATATAAATTTTATATATAATATATACCTATAAATTTAATATCTATATATT	26	32	42−642	30−729	core promoter element around −30 of transcription initiation region[37]
5	I-Box	GTATAAGGCCGTATAAGGCCGATAGGG	1	0	291	0	part of light-responsive element[38]
6	W-Box	TTGACCTTGACC	2	2	125−560	113−548	linked to gibberellin signaling repression and pathogen-related gene induction[39]
7	5UTR Py-rich stretch	TTTCTTCTCT	2	2	79−114	555−591	element conferring high transcription levels[40]
8	O2-Site	GATGACATGG	1	1	117	552	element involved in Zein metabolism regulation[41]
9	Box-W1	TTGACC	2	2	125−560	113−548	fungal elicitor responsive element[42]
10	TATCCATC-Motif	TATCCAT	1	1	108	564	gibberellin induction element[43]
11	CE3	GACGCGTGTC	0	1	0	551	element involved in ABA and VP1 responsiveness[44]
12	TCCC-Motif	TCTCCCT	1	0	288	0	part of a light-responsive element[45]
13	MBS	CGGTCACAACTG	0	2	0	112−755	MYB binding site involved in drought inducibility[46]
14	AE-Box	AGAAACAT	1	1	309	362	part of a module for light responsiveness[47]
15	C-Repeat/DRE	TGGCCGAC	1	1	408	263	regulatory element involved in cold and dehydration responsiveness[48]
16	AAGAA-Motif	GAAAGAAGGTAAAGAAA	2	0	486−469	0	regulatory element involved in plant development[49]
17	GARE-Motif	AAACAGA	0	1	0	119	gibberellin responsive element[50]
18	GAG-Motif	GGAGATG	2	0	286−521	0	part of a light-responsive element[51]
19	GATA-Motif	GATAGGG	1	0	291	0	part of a light-responsive element[52]
20	RY-Element	CATGCATG	1	0	386	0	element involved in seed-specific regulation[53]
21	LAMP-Element	CTTTATCA	1	0	297	0	part of a light-responsive element[54]
22	Skn-1-Motif	GTCAT	1	0	373	0	element required for endosperm expression[55]
23	ATCT-Motif	AATCTAATCC	1	1	354	342	part of a conserved DNA Module involved in light responsiveness[56]
24	P-Box	CCTTTTG	1	2	550	662	part of a light-responsive element[57]

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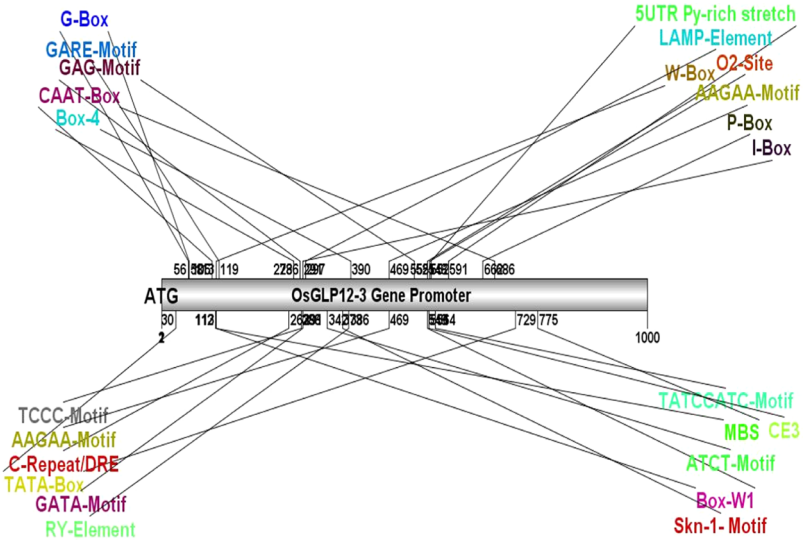

Figure 4. Localizations and Arrangements of various *cis*-acting regulatory elements in the *Os*GLP12-3 Promoter.

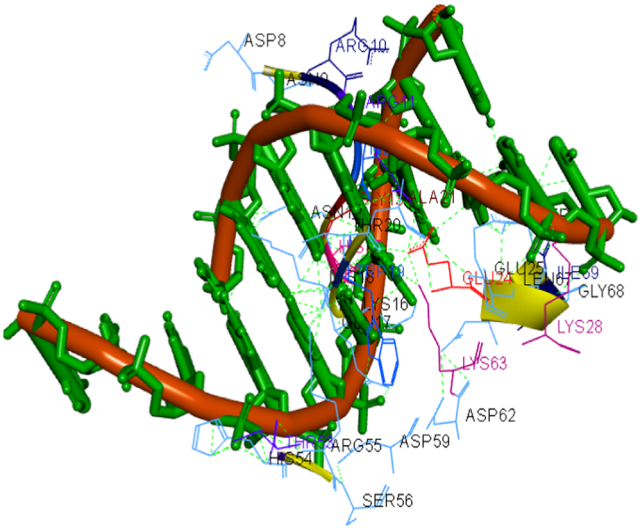

Figure 5. Unveiling TF GARE interactions with AACCTAA *cis*-acting element: Showing the hydrogen bonding through green-dotted lines.

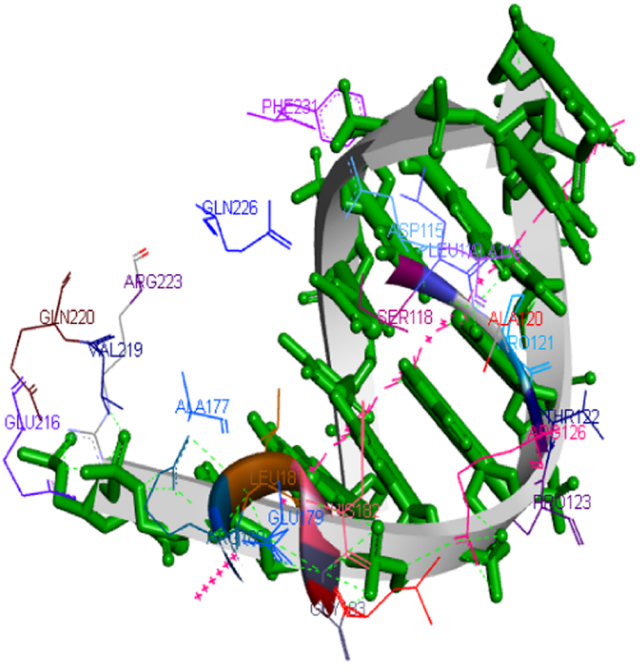

Figure 6. Exploring TF MBS interactions with the CGGTCA *cis*-acting element: highlighting hydrogen bonding via green-dotted lines.

roots with minimal expression in shoots, a behavior observed consistently even at low cadmium concentrations. Under flood stress, the *Os*GLP-12-3 gene demonstrates a lower moderate expression, as depicted in Figure 9. Across all instances, the gene consistently responds to various abiotic stresses, albeit with variable intensity, predominantly in roots. Notably, during strong salinity stress, the gene exhibits activity in both roots and shoots, with roots displaying a 2-fold higher expression level than shoots. The expression pattern in response to hormones, such as jasmonic acid and abscisic acid, is positive in both roots and shoots, but sustained expression is limited to roots. Analyzing *in silico* results, it becomes evident that the *Os*GLP-12-3 gene consistently and primarily expresses itself in roots under abiotic stress conditions, encompassing high and low phosphorus, high salinity, high cadmium, drought, flood, cold, and osmotic stress. Additionally, in response to hormones like ABA and MeJa (methyljasmonate), the *Os*GLP-12-3 gene expression is consistently induced in roots. Hormone-induced increases in *Os*GLP-12-3 gene expression exhibit erratic patterns, reinforcing the predominant expression in roots and comparatively lower expression in shoots, as depicted in Figure 9. Lu et al., reported in their study that indole acetic acid (IAA) stimulates the expression of the soybean GmGER genes. This phenomenon reflects that the expression of germin-like proteins genes is affected by hormones.[66]

Germins and germin-like proteins (GLPs) are ubiquitous plant proteins characterized by their diverse sequences and evolutionary lineage. They perform pivotal functions in response to both biotic and abiotic stressors with their expression detected across diverse plant organs and developmental stages. Furthermore, they actively participate in critical processes and pathways associated with plant development and defense systems.[67] Comparative analysis discloses 86% similarity in the sequence of the *Os*GLP12-3 promoter between Swat-1 and Nipponbare, underscoring a close

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Table 2. Results of HADDOCK Web Server Regarding the Interaction between TFs and *cis*-acting Elements

proteins/DNA	HADDOCK score	cluster size	RMSD (Å)	van der Walls energy (kcal/mol)	electrostatic energy (kcal/mol)	desolation energy (kcal/mol)	restrains violation energy (kcal/mol)	buried surface area
GARE-CGGTCA	−142.0	18	13.1	−85.4	−661.6	28.9	168.1	1883.3
GARE-CGGTCACAACGA	−159.5	23	57.7	−85.8	−615.1	26.8	226.0	I 57.7
GARE- AGAAACAT	−165.6	26	64.6	−84.8	−616.8	28.2	203.6	1852.I
MBS- AACCTAA	−185.7	24	68.0	−62.1	−411.7	25.6	660.7	168-1
MBS-ACCTAAAG	−129.8	16	50.5	−63.2	−557.6	18.5	206.4	1777.0
MBS-CGGTTAA	−114.6	21	45.0	−65.0	−126.6	7.5	432.3	1722.2

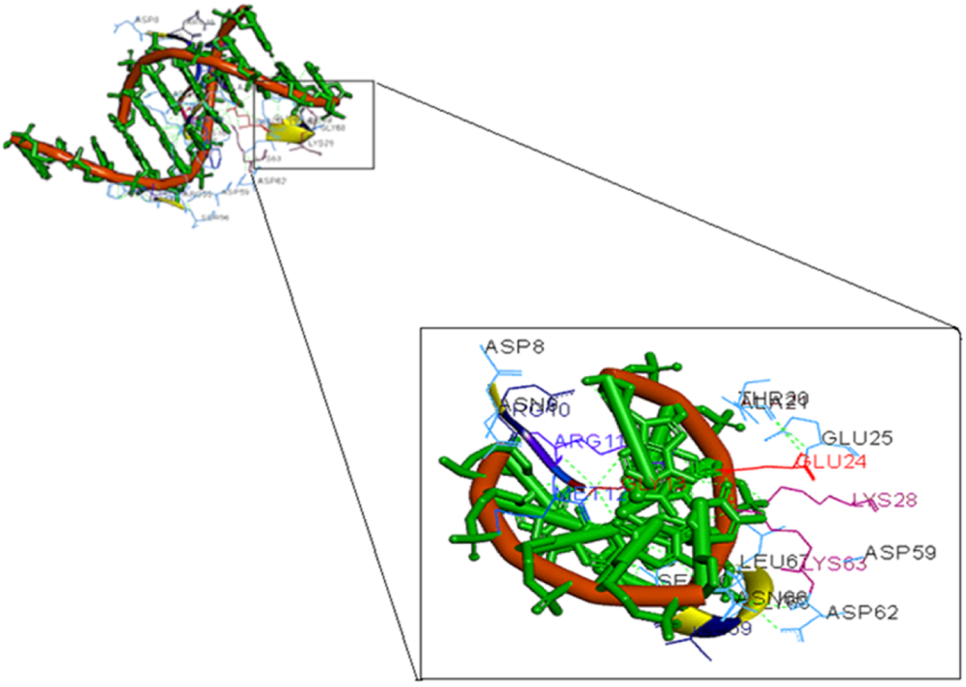

Figure 7. Modeling the interaction of TF GARE with the AACCTAA *cis*-acting element.

Table 3. Hydrogen Bonds between DNA Sequence AAACAGA and the TF GARE

protein residue	protein atoms	amino acid position	DNA residue	DNA atoms	nucleotide position	distance in Å
LYS	HZ2	3	T	O5	11	2.3
LYS	HZ3	3	T	O1P	12	2.0
LYS	HZ1	43	A	O1P	9	2.0
LYS	HZ1	43	A	O5′	10	2.3
LYS	HZ2	63	A	O1P	1	2.0
LYS	HZ1	63	A	H5″	2	2.8
ARG	HN	52	A	O1P	2	1.9
HIS	HE2	53	G	O6	3	2.3
SER	HN	7	G	O1P	17	2.4
SER	HN	7	T	O2P	18	1.9
TYR	HH	40	T	O1P	18	2.1
ASN	HD2	49	T	O2P	19	2.1
TRP	O	47	T	O2P	18	3.6
TYR	HH	8	T	O2P	18	2.0
SER	HE2	50	T	O6	19	2.3
SER	HG	50	T	O2P	18	2.1

relationship within the Indica and Japonica rice groups. Phylogenetic analysis further demonstrates the variable homology in GLPs across distinct plant species. The *Os*GLP12-3 gene promoter from Japonica rice exhibits a link with the Hypothetical protein PAHAL F01217 from *Panicurn hallii*, suggesting independent gene duplication events during evolution.[66] *In silico* analysis of the expression of *Os*GLP12-3's during development corroborates earlier findings in soybean. Regulatory control over the treatment of *Os*GLP12-3 involves hormones such as ABA and jasmonate. The *Os*GLP12-3 promoter harbors *cis*-acting elements TGACG (for MeJA recognition) and GACGCGTGTC (for the ABA response). The gene exhibits varied responses to abiotic stresses, aligning consistently with prior reports in soybean and rice.[14,66] Various *Os*GLP promoter region analyses revealed the presence of abiotic and biotic stress-related transcription factor binding sites. Phylogenetic analysis also confirms the evolutionary significance and expression of *Os*GLP genes govern by their respective promoters.[16] Analysis of the *Os*GLP12-3 promoter region reveals unique gibberellic-acid-responsive elements (GARE) and W-box *cis*-acting elements. The W-box is linked to gibberellin signaling repression and pathogen-related gene induction.[68] The *Os*GLP12-3 promoter features light-responsive I-box elements and two GATA boxes pivotal to light-induced gene expression. In Swat-1, two copies of the *cis*-acting element CGGTCA are identified, binding MYB transcription factors linked to drought inducibility. These findings correlate with the responsiveness of the *Os*GLP12-3's to drought and flood stress conditions. Overall, the promoter encompasses elements associated with diverse stresses, light responses, and

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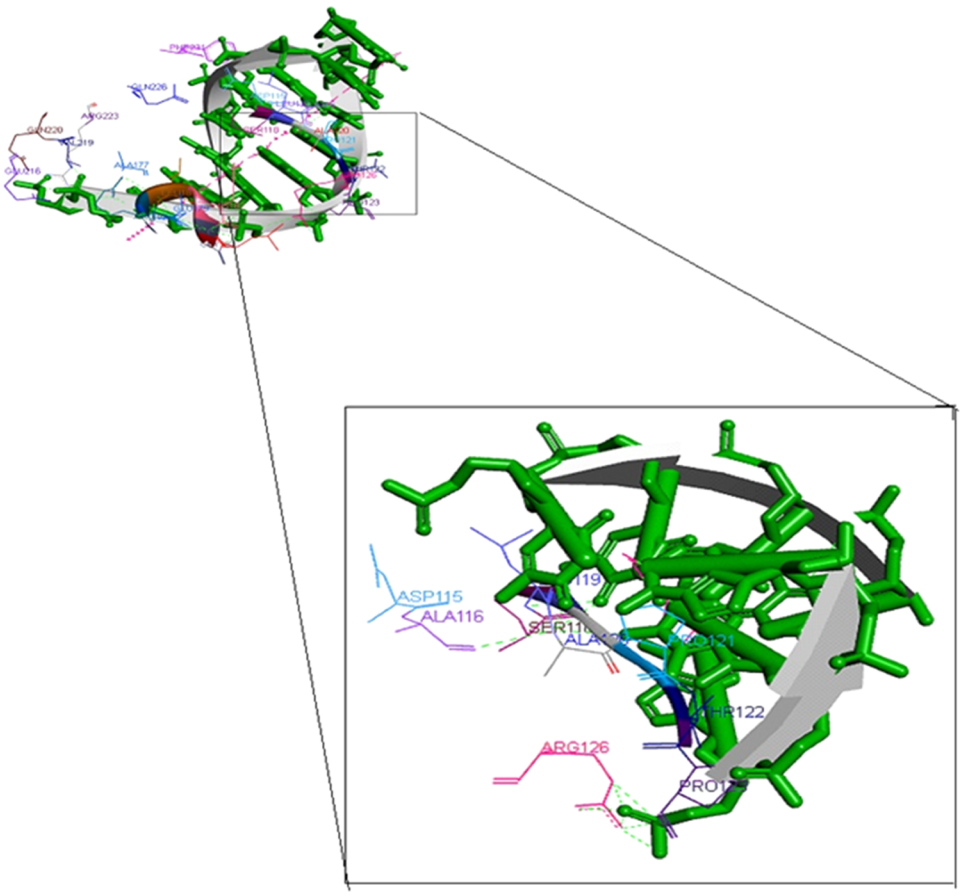

Figure 8. Modeling the interaction of TF MBS with the CGGTCA *cis*-acting element.

Table 4. Hydrogen Bonds between DNA Sequence CGGTCACAACTG and the TF MBS

protein residue	proteins atoms	amino acid position	DNA residue	DNA atoms	nucleotide position	distance in Å
LYS	HZ3	7	T	O1P	26	2.2
LYS	HZ3	7	T	02P	27	2.1
ARG	HN11	8	A	02P	25	2.9
ARG	HN11	8	C	O1P	24	2.1
ARG	HE	8	T	02P	26	2.1
ARG	HH21	10	T	02	8	2.4
ARG	HE	10	T	02	26	2.1
ASN	HD22	18	A	O1P	14	2.3
LYS	HN	19	T	O1P	23	2.1
LYS	HZ3	19	C	O1P	24	2.1
LYS	HZ2	17	G	O1P	13	2.1
GLY	HN	20	T	O1P	22	2.1
GLY	HN	20	T	O1P	23	2.2
ARG	HH21	12	G	04	13	2.5
ARG	HN	12	T	O1P	23	2.1
SER	HG	16	C	03	24	2.6
GLY	HN	15	A	O1P	12	2.1
GLY	HN	11	A	04	25	2.4
GLY	HN	11	A	04	24	2.4

developmental processes.[25] The distinctive elements within the promoter render it valuable for applications, such as genetically engineered stress-resistant crops. Comprehending gene regulation necessitates the exploration of DNA−protein interactions and hydrogen bonding, which influences stability and specificity. Transcription factors (TFs) linked to *cis*-acting elements with strong hydrogen bonds play a crucial role in gene regulation.[69] Docking findings reveal that hydrogen bonds stabilize interactions between TFs and regulatory elements, contributing to the regulation of the expression of the *Os*GLP12-3 gene expression.

4. CONCLUSIONS AND OUTLOOK

Germin and Germin-like proteins in different plant species may have originated through distinct gene duplication events. Nucleotide sequences reveal no significant differences between the Indica Rice and Japonica Rice groups in the vicinity of the *Os*GLP12-3 promoter. The promoter region of *Os*GLP12-3 holds economic importance, harboring numerous *cis*-acting regulatory elements that are responsive to both biotic and abiotic stimuli. This characteristic renders it as a valuable tool for developing stress-resistant crops. Stabilization of the connections between regulatory DNA regions and their associated transcription factors involves several potential hydrogen bonds. The expression of the *Os*GLP12-3 gene is regulated by a stable connection between transcription factors (TFs) and their associated regulatory elements. *In silico* findings necessitate further validation through an *in vitro* expression analysis. Furthermore, The *Os*GLP12-3 Promoter may be engineered upstream of the GUS gene and subsequently transfermed in potatoes to assess its activity against biotic and abiotic stresses. If *Os*GLP12-3 imparts resistance to these stress conditions, then it holds promise for integration into agriculturally important crops to enhance resistance against both biotic and abiotic stresses.

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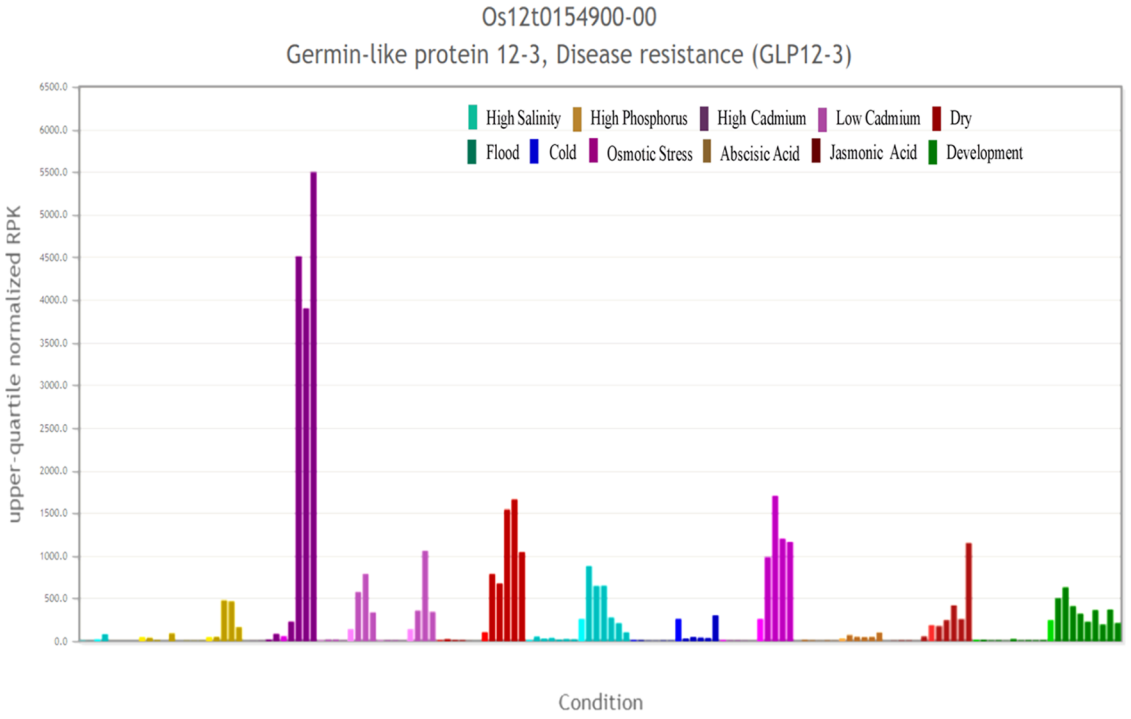

Figure 9. Mapping the expression pattern of the OsGLP12-3 gene: hormonal induction, developmental variation, and diverse stress responses.

■ ASSOCIATED CONTENT

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09670.

Pairwise alignments, the expression of OsGLP12-3 gene during development; expression in response to various abiotic stimuli in detail; specific cis-acting regulatory elements with their proposed functions in detail, CTAB (cetyltrimethylammonium bromide) buffer, TE (Tris−EDTA) buffer, MS (Murashig and Scouge) solution, 10× TBE (tris borate ethyl dimethyl tetra acetic acid) buffer, SOC (super optimal broth with catabolite repression) media composition for 1 L, composition of LB (Luria Broth) media for 1L composition of stock as well as working important solutions which were used during the experimental work, sequence of OsGLP12-3 promoter from Indica rice cv Swat-1. Topo cloning principle, preparation for heat shock competent cells, transformation of heat shock *Escherichia coli* DH5α competent cells (PDF)

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Author Contributions

A.R.: Conceptualization and writing—original draft preparation, I.U.: Writing, U.Z.: Formal analysis, A.S.: Editing, R.A.: Project funding, K.S.S. and D.I.S.: Supervision. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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