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Molecular Characterization of Germin-like Protein Genes in *Zea mays* (*ZmGLPs*) Using Various *In Silico* Approaches

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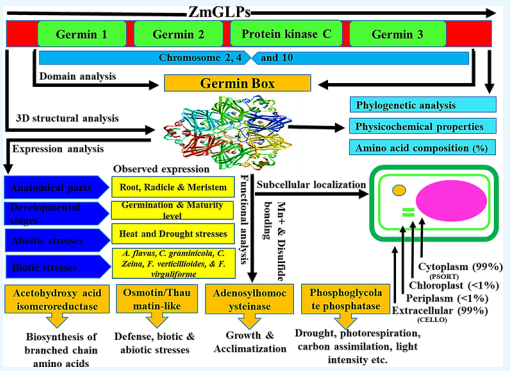
ABSTRACT: Germin (GER) and germin-like proteins (GLPs) play an important role in various plant processes. *Zea mays* contains 26 germin-like protein genes (*ZmGLPs*) located on chromosomes 2, 4, and 10; most of which are functionally unexplored. The present study aimed to characterize all *ZmGLPs* using the latest computational tools. All of them were studied at a physicochemical, subcellular, structural, and functional level, and their expression was predicted in plant development, against biotic and abiotic stresses using various *in silico* approaches. Overall, ZmGLPs showed greater similarity in their physicochemical properties, domain architecture, and structure, mostly localized in the cytoplasmic or extracellular regions. Phylogenetically, they have a narrow genetic background with a recent history of gene duplication events on chromosome 4. Functional analysis revealed novel enzymatic activities of phosphoglycolate phosphatase, adenosylhomocysteinase, phosphoglycolate phosphatase-like, osmotin/thaumatin-like, and acetohydroxy acid isomeroreductase largely mediated by disulfide bonding. Expression analysis revealed their crucial role in the root, root tips, crown root, elongation and maturation zones, radicle, and cortex with the highest expression being observed during germination and at the maturity levels. Further, *ZmGLPs* showed strong expression against biotic (*Aspergillus flavus*, *Colletotrichum graminicola*, *Cercospora zeina*, *Fusarium verticillioides*, and *Fusarium virguliforme*) while limited expression was noted against abiotic stresses. Concisely, our results provide a platform for additional functional exploration of the *ZmGLP* genes against various environmental stresses.



1. INTRODUCTION

Germins (GERs) and germin-like proteins (GLPs) are ubiquitous plant glycoproteins. They play an important role against diverse environmental stresses. Initially, GER was identified in wheat embryos as a germination-specific marker, but later, they were classified as a homohexamer glycoprotein with oxalate oxidase (OXO) activity,[1,2] while proteins showing an average similarity of 50% with it were called germin-like protein (GLPs). GLPs play an important role in diverse plant processes via oxalate oxidase (OXO), superoxide dismutase (SOD), glucophosphodiesterase, and polyphenol oxidase (PPO) enzymatic activities. GLPs can be found in all plant groups with their number varying greatly among different plant species.[3] Till now, around 254 GLP genes have been reported in almost 53 plant species from numerous databases[4] with their number constantly increasing with a recent report of 350.[5] Many of them have been confirmed for providing potential resistance against various biotic and abiotic stresses.[6] However, still, their function is not fully understood. GLPs are expressed in all types of tissues (flowers, seeds, roots, stems, leaves, and embryos) by playing a pivotal role against diverse environmental stresses.[6,7] Previously, among abiotic stresses, they were found effective against heavy metals,[8,9] drought,[10,11] salt,[12,13] desiccation,[14] heat,[15,16] abscisic acid (ABA),[17] and UV radiation acclimation[18] mediated by methylation in the promoter region as observed in rice.[19] Among biotic stresses, they were active against herbivore-induced damage,[20] *Sclerotinia sclerotiorum* (*S. sclerotiorum*),[21] *Verticillium* and *Fusarium* wilt,[22,23] and *Blumeria graminis* f. sp. *tritici*,[24] etc.

Recent advancements in various techniques of computational biology such as next-generation sequencing (NGS) and the development of various plant-specific databases and their associated tools provide an opportunity to explore and unravel the hidden properties of plant genomes. Such tools include Ensemble Plants (https://www.ensembl.org/index.html?redirect=no), National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), Geneious (https://www. geneious.com/), Rice XPro (https://ricexpro.dna.affrc.go.jp/), Blast2Go (https://www.blast2go.

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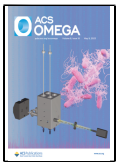

com/), LibD3C (http://lab.malab.cn/soft/LibD3C/weka.html),[25] Pse-in-One 2.0 (http://bliulab.net/Pse-in-One2.0/),[26] BioSeq-Analysis (http://bliulab.net/BioSeq-Analysis2.0/home/),[27] and Max-Relevance-Max-Distance (MRMD) (http://lab.malab.cn/soft/MRMD/index_en.html).[28]

Structural or functional characterization of genes and their associated proteins using various *in silico* approaches is a valuable practice in plant biotechnology.[29] In recent years, the functional assessment of GLPs through computational approaches is a subject of wider interest among the scientific community. Previously, 43 *Oryza sativa* germin-like protein genes (*OsGLPs*) and their promoters were analyzed using computational tools, which gave significant novel insights into their function and regulation[30,31] with recent reports on *OsGER4* and *OsGLP3-7*.[32,33] Similarly, an analysis of *VvGLP3* in *Vitis vinifera* predicted its role against powdery mildew.[34] A sequence of *TaGLP-2b* was analyzed with different tools to predict its functional and structural properties.[35] Similarly, 258 GLPs were identified in wheat, and their function was confirmed against *Blumeria graminis* f. sp. *tritici* (Bgt).[24] Recently 70 *StGLPs* were identified in potatoes, and their roles were confirmed against various stresses.[36] A similar report suggested the crucial role of *AsGLP1* (*Astragalus sinicus*) in symbiotic nodulation by interacting with an outer membrane protein of Rhizobia.[37]

The *Zea mays* genome contains 26 GLP genes[38] among which *ZmGLP1* regulates the development of young and whorled leaves at the late-whorl, tassel, and silk stages[39] and regulates pathogen resistance via oxidative burst and JA signaling pathway activation.[40] However, the rest are functionally unexplored. So, keeping in view the importance of GLPs and their wider role in various stresses, the current study was designed to investigate various structural and functional properties of the 26 *ZmGLP* genes using the latest computational tools. In the future, they can be utilized against diverse stresses in commercially important plants.

2. METHODS

2.1. Sequence Retrieval. DNA and protein sequences of the 26 *ZmGLP* genes were retrieved from the Plant Ensemble database (release 52-Dec 2021, Copyright EMBL-EBI)[41] (http://plants.ensembl.org/index.html accessed on December 2021). The cupin domain of the protein sequence was confirmed with the NCBI conserved domain (CD) search tool[42] (http://www.NCBI.nlm.nih.gov/structure/cdd/wrpsb.cgi accessed on December 2021). The chromosomal locations of the *ZmGLP* genes were mapped through MapGene2Chrom (http://mg2c.iask.in/mg2cv2.1/, accessed on 03 February 2022).

2.2. Alignment and Motive Analysis. The protein sequences were aligned using the Clustal Omega program[43] (https://www.ebi.ac.uk/Tools/msa/clustalo/ accessed on February 2022), and the GER motives, peptide signals, etc. were identified. The sequences were further searched for 5 possible motives through "Multiple Em for Motif Elicitation" (MEME) (http://meme.nbcr.net/meme/cgi-bin/meme.cgi accessed on March 2022) software (version 5.4.1),[44] and their functions were predicted with Motif scan (https://myhits.sib.swiss/cgi-bin/motifscan/software) and Motif search (https://www.Genome.jp/tools/motif/) servers.

2.3. Protein Analysis. Physicochemical properties of the selected proteins were investigated with the ExPASy-ProtParam server (operated by SIB or Swiss Institute of Bioinformatics)[45] (http://web.expasy.org/protparam/, accessed on March 2022). Subcellular localization was predicted with CELLO (subCELlular LOcalization predictor ver. 2.5)[46] (http://cello.life.nctu.edu.tw/, accessed on March 2022) and WoLF PSORT[47] (https://www.genscript.com/wolf-psort.html, March 2022). N-glycosylation and phosphorylation sites (N- and P-sites) were predicted with NetNglyc (ver. 1.0)[48] (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetPhos (ver. 3.1)[49] (http://www.cbs.dtu.dk/services/NetPhos/), respectively (accessed on April 2022). The peptide signal was predicted with "SignalP 4.1"[50] (http://www.cbs.dtu.dk/services/SignalP/). Structural models (3D) were built with a Swiss modeling server[51] (http://swissmodel.expasy.org/interactive) and further authenticated with the Rampage server (RPA) (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) accessed on April 2022. Their phylogeny was investigated with the Molecular and Evolutionary Genetics Analysis tool (MEGA10) using neighbor-joining tree-making methods.[52] Tajma's neutrality test of selection and percentage (%) amino acid were deduced with the same software. Each analysis was performed twice for authentication.

2.4. Functional Analysis. Each gene was investigated for its possible function and expression pattern with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING ver. 11), which considers its homology and coexpression pattern with the previously reported genes.[53] Functional values for ZmGLP1, 4-2, 10-2, and 10-5 were obtained on higher stringency (medium confidence 0.40), while the rest were obtained on a low stringency (low confidence 0.150) level.

2.5. Expression Analysis. *In silico* expression analysis was carried out in different anatomical parts, at different developmental stages, and under various biotic and abiotic stresses using the online server Genevestigator[54] (https://genevestigator.com/ accessed on May 2022). The server utilized an mRNA expression data set (ZM_RNAseq_MAIZE_GL-0) of *Zea mays* across 4064 experiments. The results were obtained as a heat map, which shows the percentage of the expression potential (log2 scale) via the hierarchical cluster analysis tool.

2.5.1. Anatomical Parts and Developmental Stages. The expression profile of the 26 *ZmGLP* genes across 82 different anatomical plant parts was investigated. Parts with no expression were omitted. Similarly, their expression profile was also investigated at 8 developmental stages (germination, seedling stage, stem elongation, inflorescence formation, anthesis, fruit formation, dough stage, and at the maturity level), and the results were presented as heat maps.

2.5.2. Abiotic Stresses. *In silico*-based expression analysis of the *ZmGLP* genes was conducted for plants grown under auxin, drought, heat, and plant density stresses.

2.5.2.1. Auxin. For auxin treatment, plants were grown in a greenhouse under 16 h light/8 h dark cycles at 20−25 °C, and their embryos were isolated from CAL caryopses 10−12 days after pollination, cultured on N6 medium containing 1.5 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin) in darkness at 27 °C for 1, 2, 4, 6, and 8 days, and then sampled.

2.5.2.2. Drought Stress. For drought stress, B73 plants were grown in a greenhouse condition without water limitation (the soil moisture content was maintained close to the field capacity) during drought,[55−57] till the onset of silk emergence; then, irrigation was withheld for 3 days. At the end of the 3-day drought period, the plants were hand-pollinated and kept for another 24 h without watering (4 days of drought stress in

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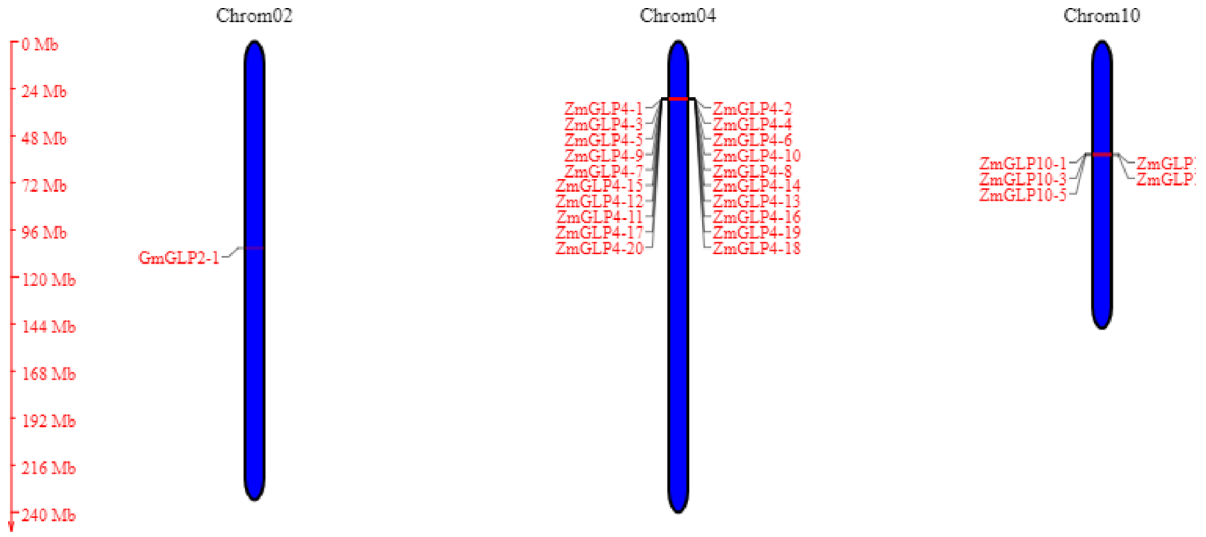

Figure 1. Chromosomal locations and distributions of the *Zea mays* germin-like protein genes (*ZmGLP*) in the maize genome. The chromosomal numbers are shown at the top of each chromosome. The map was built with MapGene2Chrom (http://mg2c.iask.in/mg2cv2.0/) software (accessed on 03 February 2022).

total; at the end of this period, the leaf relative water content was 66.5%). The basal meristematic tissue of the three youngest leaves was then harvested. Plants were grown in 10 L pots containing a mixture of peat, vermiculite, perlite (1:1:1), 6 g of pulverized limestone, 35 g of $CaSO_4$, 42 g of $FeSO_4$, and 1 g of trace fritted element; before the drought stress, a general purpose fertilizer (15-16-17; Scott-Sierra Horticultural Products) was supplied once a week.

2.5.2.3. Heat Stress. For heat stress, different samples of R1-stage B73 plants were grown in a phytotron under 14 h light/10 h dark cycles at 25 °C and 65% relative humidity and then exposed to 38 °C for 2 and 48 h. Ear leaf and the third leaf above the ear were collected from Xianyu 335 plants (at grain filling) grown under high planting density. A control was used in each experiment.

2.5.2.4. High Plant Density. In this study, RNA-seq analysis of leaves at different nodes of the maize hybrid Xianyu 335 under different (low and high) plant densities were conducted to explore the mechanisms of responding to high plant density.

2.5.3. Biotic Stresses. The *in silico*-based expression profile of the *ZmGLP* genes in maize was obtained against *Aspergillus flavus* (*A. flavus*), *Colletotrichum graminicola* (*C. graminicola*), *Fusarium verticillioides* (*F. verticillioides*), *Cercospora zeina* (*C. zeina*) (late-stage GLS disease), and *Fusarium virguliforme* (*F. virguliforme*).

2.5.3.1. Aspergillus flavus. For *A. flavus* infection, B73 plants were grown in field conditions at the Central Crops Research Station near Clayton, NC, USA. Ears were hand-pollinated and covered with paper bags. After pollination (21−22 days), the ears were inoculated with the *Aspergillus flavus* strain NRRL 3357 by dipping a 3 mm needle into a conidial suspension and inserting it into the caryopsis crown (approx. 13 fungal conidia were introduced into the endosperm of a caryopsis). Caryopses were sampled 4, 12, 24, 48, and 72 h after inoculation. At sampling, no fungal mycelia were visible in the caryopses.

2.5.3.2. Colletotrichum graminicola. For *C. graminicola*, 4-days-old B73 plants were inoculated with the *Colletotrichum graminicola* wild-type strain M2 (CgM2) by placing 10 μL droplets of a spore suspension (10^6 spores/mL, in 0.01% Tween 20) on a horizontally oriented third leaf (counting from the shoot base). The inoculated plants were incubated at 25 °C with high humidity for 24, 48, and 120 h (the penetration phase of the fungus), and the inoculated leaf segments were then harvested.

2.5.3.3. Fusarium verticillioides. For *F. verticillioides*, B73 plants were grown in field conditions at the Central Crops Research Station near Clayton, NC, USA. Ears were hand-pollinated and covered with paper bags. After pollination (21−22 days), the ears were inoculated with the strain n16 by dipping a 3 mm needle into a conidial suspension and inserting it into the caryopsis crown (approx. 13 fungal conidia were introduced into the endosperm of a caryopsis). Caryopses were sampled 4, 12, 24, 48, and 72 h after inoculation. At sampling, no fungal mycelia were visible in the caryopses. A control was used in each experiment.

2.5.3.4. Cercospora zeina. For *C. zeina* (late-stage GLS disease), B73 plants were grown for 77 days (planted in December 2012) in a field at Hildesheim Research Station, Pannar Seed Pty Ltd., Greytown, KwaZulu-Natal, South Africa. The plants developed symptoms typical of gray leaf spot (GLS) disease caused by the fungal *Cercospora zeina*. The middle parts of the lower leaf (leaves located at the second and third internodes below the internode bearing the ear shoot) blades showing late-stage GLS disease symptoms (big rectangular lesions) were harvested for analysis.

2.5.3.5. Fusarium virguliforme. For *F. virguliforme*, E13022S seeds were surface sterilized, incubated in sterile water for 24 h in the dark, and germinated for 5 days in darkness at 21 °C in a Petri dish between two sterile, water-soaked sheets of 100 mm Whatman filter paper. Germinated seedlings were thoroughly sprayed with *Fusarium virguliforme* Mont-1 isolate inoculum (10^5 macroconidia/1 mL sterile H_2O), incubated for 1.5 h (after the first 30 min of incubation, the excess inoculum was removed), and then moved to distilled water-moistened plastic pouches (CYG germination pouch, 16.5 cm × 18 cm, Mega International). The pouches with the seedlings were kept for 2, 4, 7, 10, and 14 days in a growth chamber under 14 h light (140 μmol photons m^{-2} s^{-1})/10 h dark cycles at 12 °C (watering with sterile distilled

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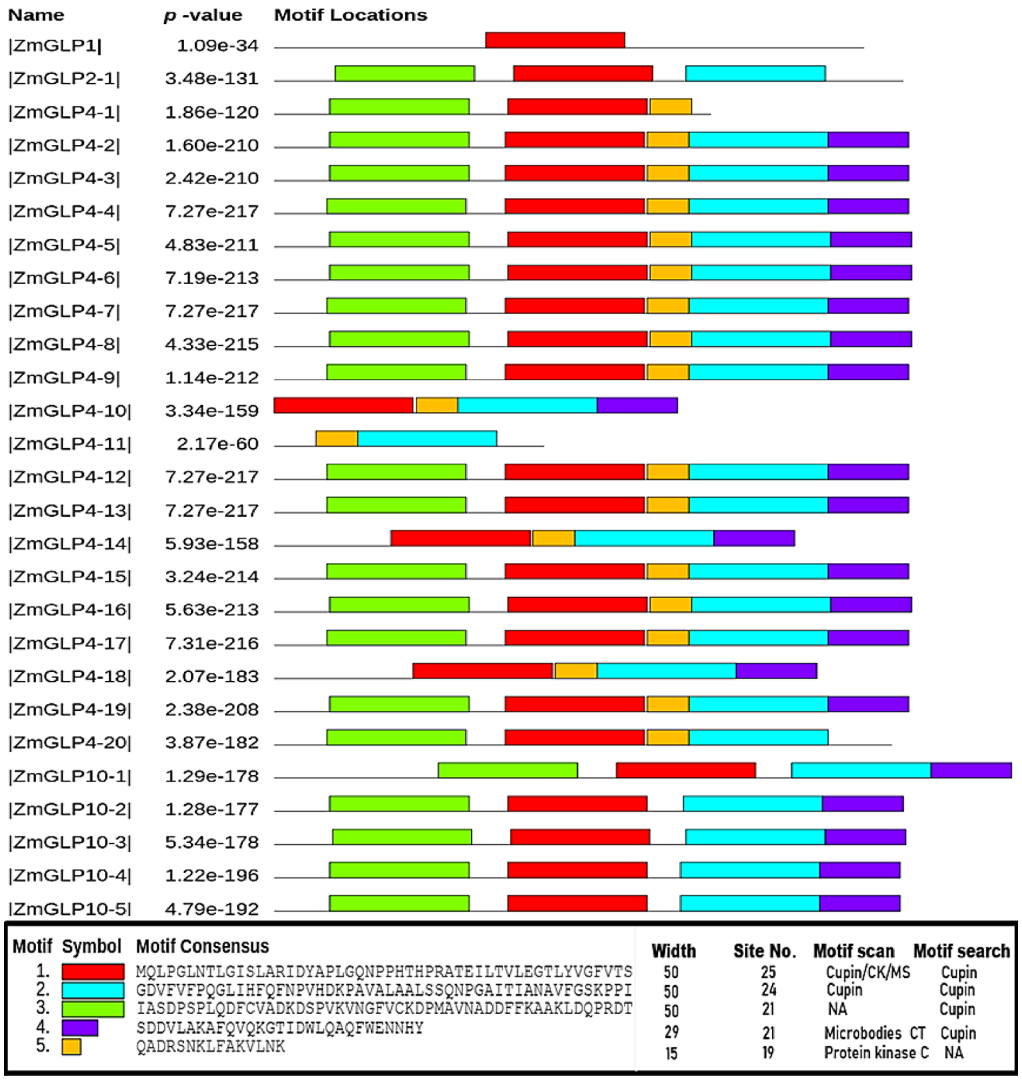

Figure 2. Motive analysis of the *Zea mays* germin-like protein (*ZmGLP*) genes. Each motif is represented with a distinct color. The analysis was conducted with Multiple Em for Motif Elicitation (MEME) suite (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). The function of the domain was found with Motif scan and Motif search servers. CK: casein kinase II phosphorylation site, MS: N-myristoylation site, Microbodies CT: microbodies C-terminal targeting signal, Protein kinase C: protein kinase C phosphorylation site, and NA: not available.

water as needed), after which the 4 cm-long original inoculation site of the primary root was sampled.

3. RESULTS AND DISCUSSION

3.1. Sequence Retrieval. The *Zea mays* genome has 26 *ZmGLP* genes (details given in Table S1) among which 20 were located on chromosome 4 while 5 on 10 and 1 on chromosome 2 (Figure 1). GLPs play an important role in growth and development, but their number varies greatly among different plant species. For example, *Arabidopsis* contains 32, rice 43, barley 48, soybean 69, and wheat 258 GLP genes. Mostly, *ZmGLPs* are located on the chr 4 suggesting their origin through tandem duplication events. Clustering and duplication on specific chromosomes are common in GLP families. Previously, most of the *OsGLPs*[30,58] and *HvGLPs*[59] were found on chr 8, while in *Sorghum bicolor* and *Brachypodium distachyon*,[38] they can be found on chr 7 and 3, respectively. The same is true for soybean (*GmGLPs*) where most are located on chr 16, 19, and 20.[60] Similarly, most of the *TaGLPs* can be found on chr 4b.[24] Sometimes, these clustered loci offer incredible resistance against a wide range of biotic and abiotic stresses.[38] The high duplication rate may be due to ectopic recombination, transposable elements, and epigenetic effects, which offer more diverse structural and functional roles within the changing environment.[30] The highest number of nucleotides (690) was shown by *ZmGLP4-5*, *ZmGLP4-6*, *ZmGLP4-8*, and *ZmGLP4-16*, while the smallest (438) was by *ZmGLP4-10*. Similarly, the largest peptide sequence (229 aa) was shown by *ZmGLP4-5*, *ZmGLP4-6*, and *ZmGLP4-16*, while the smallest (97 aa) by *ZmGLP4-11*. It may be due to high sequence similarity and common origin that *ZmGLPs* showed little variation in their sizes, which indicated their functional redundancy.[59] Similarity indicates their origin through recent tandem duplication events, showing that tandem repeats are important in the implication of the *ZmGLP* gene family.

3.2. Protein Sequence Analysis. *3.2.1. Motive Analysis.* Sequence alignment revealed several conserved regions among *ZmGLPs* showing greater similarity, but certain mutational gaps were also found. The peptide signal (MASS) was found at the start of each sequence, which is important for protein function by helping in targeting the protein to or across the plasma membrane.[30] GER motif 1 (M1) was found ≈25 aa

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Table 1. Computational Analyses Based on Various Structural and Functional Properties of the *Zea mays* Germin-Like Protein (*ZmGLP*) Family[a]

name	M.wt	Pl	−R	+R	EC	II	Al	GRAVY	Pfam domain	subcell prediction (CELLO)	subcell prediction (PSORT)	N-sites	P-site
ZmGLP2-1	24,603.25	6.40	20	19	22,585	32.27	94.51	0.051	cupin domain	cytoplasmic	extracellular	0	0
ZmGLP3-1	16,921.44	6.25	13	12	4595	19.75	96.24	0.088	cupin domain	cytoplasmic	extracellular	6	1
ZmGLP3-3	24,802.43	7.77	17	18	18,575	86.54	92.81	0.022	cupin domain	cytoplasmic	extracellular	8	1
ZmGLP3-4	24,637.19	6.50	19	18	15,595	25.51	92.41	0.034	cupin domain	cytoplasmic	extracellular	6	1
ZmGLP3-5	24,766.29	6.28	20	18	17,085	26.18	94.89	0.054	cupin domain	cytoplasmic	extracellular	8	1
ZmGLP3-6	24,791.30	6.28	20	18	17,085	24.75	92.88	0.015	cupin domain	cytoplasmic	extracellular	8	1
ZmGLP3-7	26,607.17	6.50	19	18	15,595	26.55	93.29	0.046	cupin domain	cytoplasmic	extracellular	9	1
ZmGLP3-8	24,781.37	6.57	19	18	17,085	23.89	94.1	0.06	cupin domain	cytoplasmic	extracellular	9	1
ZmGLP3-9	2464.27	6.89	18	18	17,085	26.12	95.00	0.077	cupin domain	cytoplasmic	extracellular	8	2
ZmGLP3-10	15,851.11	6.96	10	10	15,470	30.30	92.21	−0.064	cupin domain	periplasmic	extracellular	7	0
ZmGLP3-11	10,462.09	10.11	6	11	n-ter.	25.30	106.49	0.069	cupin domain	cytoplasmic	chloroplast	4	0
ZmGLP3-12	24,568.14	6.57	19	18	15,595	24.79	94.12	0.053	cupin domain	cytoplasmic	extracellular	4	1
ZmGLP3-13	24,568.14	6.57	19	18	15,595	24.79	94.12	0.053	cupin domain	cytoplasmic	extracellular	8	1
ZmGLP3-14	20,400.24	6.50	16	15	15,470	20.64	90.27	−0.102	cupin domain	cytoplasmic	extracellular	8	1
ZmGLP3-15	24,597.18	7.01	18	18	15,595	23.82	93.68	0.042	cupin domain	cytoplasmic	extracellular	7	2
ZmGLP3-16	24,793.38	6.57	19	18	17,085	26.11	92.88	0.042	cupin domain	periplasmic	extracellular	8	1
ZmGLP3-17	24,720.33	6.96	19	19	15,595	26.92	93.29	0.029	cupin domain	cytoplasmic	extracellular	10	1
ZmGLP3-18	21,253.25	6.57	18	17	14,105	23.64	87.59	−0.021	cupin domain	cytoplasmic	extracellular	7	1
ZmGLP3-19	24,515.05	6.50	18	17	15,595	24.21	94.56	0.066	cupin domain	cytoplasmic	extracellular	5	1
ZmGLP3-20	23,819.38	6.89	18	18	8605	24.34	96.26	0.118	cupin domain	cytoplasmic	extracellular	7	1
ZmGLP10-1	28,946.06	7.00	25	25	18,575	32.09	90.91	−0.108	cupin domain	cytoplasmic	chloroplast	14	2
ZmGLP10-2	24,685.31	6.18	22	20	21,095	27.33	92.74	0.001	cupin domain	cytoplasmic	extracellular	6	2
ZmGLP10-3	297.3	6.04	23	20	19,605	25.19	94.49	0.000	cupin domain	cytoplasmic	extracellular	6	2
ZmGLP10-4	24,300.90	6.03	19	16	18,575	25.04	94.09	0.146	cupin domain	cytoplasmic	cytoplasmic	7	1
ZmGLP10-5	24,249.82	6.28	18	16	18,575	29.02	94.09	0.163	cupin domain	cytoplasmic	extracellular	8	1

[a]PM: plasma membrane, M.wt: molecular weight, pI: isoelectric point, +R: positively charged residues, −R: negatively charged residues, EC: extinction coefficient, II: instability index, Al: aliphatic index values, GRAVY: grand average of hydropathicity, N-sites: N-glycosylation site, and P-site: phosphorylation site.

while GER motif 2 (M2) was found ≈90 to 100 aa away from the N-terminus of the protein. Similarly, GER motif 3 (M3) was found ≈155 to 165 aa away from the N-terminal end (N-end) of the protein. Motives 1, 2, and 3 are part of the GER box, which is the fundamental signature of GLPs with a consensus sequence of GxxxxHxHPxAxEh (where "x" is any amino acid and "h" is hydrophobic).[61] The putative KGD motif was found upstream of the GER motif 3, which is an important structural feature of the GLPs,[62] playing an important role in protein−protein interactions. Plant proteins with such motifs perform their function by interacting with other membrane proteins by mediating signal transduction in the matrix,[63] which is relevant to the role of these genes in plant pathogenicity and stress responses. Further, *ZmGLP* sequences were scanned with a MEME server to get more insight into their motive structures and functions (Figure 2). It showed that the first 4 motives were part of the basic cupin domain or GER box.[6] The importance of the cupin domain in controlling plant responses against a wide range of biotic and abiotic stresses is well-validated.[7] However, M1 also exhibited casein kinase II phosphorylation and N-myristoylation-related activities. The former plays an important role in various

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environmental stresses and signaling by regulating protein activity and stability in various signaling pathways.[64] The latter regulates plant responses against diverse stresses via membrane targeting and signal transduction.[65] Similarly, M4 is important in microbody C-terminal targeting signals having a role in microbody production. It is considered an alternative to a peroxisome that possesses catalase (H_2O_2-degrading enzymes) as well oxidase activities.[66] M5 showed protein kinase activity, which is important in protein phosphorylation, which controls activities and interactions of the proteins with other molecules. It is also involved in regulatory processes and metabolic activities of the cells including light signaling, pathogenicity, hormonal regulation, nutrient deprivation, heat stresses, etc.[67] All the motives were found in variable numbers and at the different positions where the highest variability in number and position was noted for M4 and M5, which may be due to the high mutation rate and retrotransposon activity. However, GER M1, 2, and 3 showed similar positioning in 88% of the sequences, which represents their importance and evolutionary significance as being part of the core protein structure. Similar results were also reported for GLPs of various other plants such as soybean, rice, and grapes.[5,30,60]

3.2.2. Physicochemical Properties. Physicochemical properties of the ZmGLPs revealed noticeable variations (Table 1). The size and molecular weight varied substantially ensuing variations in other properties as well. The molecular weight ranged from 297.3 (ZmGLP10-3) to 28946.06 Da (ZmGLP10-1), while the isoelectric point (pI) ranged from 6.01 (ZmGLP1) to 10.11 (ZmGLP4-11). Both are considered important parameters for the estimation of solubility, electrophoresis, and electrophoretic separation of the protein.[68] The extinction coefficient (EC) at 280 nm ranged from 8605 (ZmGLP4-20) to 22,585 (ZmGLP2-1) indicating protein stability in the cellular environment. EC also describes the type and purity of protein. ZmGLPs of chr 2 and 10 have slightly high EC values, which may be due to the presence of a large number of Tyr (tyrosine), Phe (L-phenylalanine), and Trp (tryptophan) residues.[69] The instability index (II) ranged from 19.75 (ZmGLP4-1) to 86.54 (ZmGLP4-3). Protein stability in the cellular environment is crucial for protein function.[70,71] All the ZmGLPs are stable in the cellular environment having an II value lower than 40, except ZmGLP4-3, which may be due to variations in the structural and functional properties. However, sometimes, II values are questionable as protein stability is dependent not only on the intrinsic nature of the protein but also on the condition of the protein milieu.[72] The aliphatic index (AI) ranged from 87.59 (ZmGLP4-13) to 102.12 (ZmGLP1). Thermal stability of the proteins is positively indicated by high AI values as noted for ZmGLP1, ZmGLP4-1, and ZmGLP4-9. However, proteins with lower AI values indicate that their structural flexibility at a wide range of temperatures largely contributed to the presence of aliphatic amino acids (alanine, valine, isoleucine, and leucine) with an aliphatic side chain.[73] It is in accordance with the fact that Ala, Leu, and Val were the most frequent residues in the ZmGLPs, forming 10.70, 9.90, and 8.90% of the total AAs, respectively, while Trp (0.80%) and Cys (0.90%) were the least occurring amino acids (Figure 3). GRAVY ranged from −0.102 (ZmGLP4-14) to 0.163 (ZmGLP10-5). Analysis showed that 22 proteins (84.61%) were hydrophobic in nature as their GRAVY values were positive (above zero), while the rest (ZmGLP4-10, ZmGLP4-14, ZmGLP4-18, and ZmGLP10-1) were hydrophilic in nature showing their better interaction with water molecules.

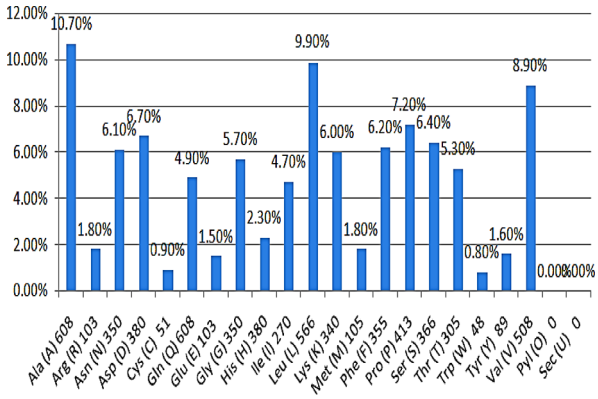

Figure 3. Amino acid composition of the *Zea mays* germin-like proteins (ZmGLPs). The analysis was conducted with the Molecular and Evolutionary Genetics Analysis tool (MEGA10).

3.2.3. Subcellular Localization. At the subcellular level, ZmGLPs are either expressed in the cytoplasm or extracellular regions indicating their role in these regions. It is contradictory to the study of OsGLPs and VvGLPs where a diverse expression pattern was noted.[5,30] However, some ZmGLPs showed either chloroplast- (ZmGLP4-11 and ZmGLP10-1) or periplasm (ZmGLP4-10 and ZmGLP4-16)-specific expression, which may be due to their fundamental role in the photosynthesis or metabolism. Previously, organelle-specific expression was also reported for some members of the *AhGLP* family.[12] Similarly, *OsGLP1-2*, *OsGLP4-7*, and *OsGLP4-1* showed chloroplast-, endoplasmic reticulum-, or mitochondrion-specific expression, respectively.[30] Similar observations were also noted for VvGLPs.[5]

3.2.4. N-Glycosylation and Phosphorylation Sites. ZmGLPs revealed a variable number of N-glycosylation (from 1 to 2) and phosphorylation sites (4−14). The highest N-sites (2) were found in ZmGLP4-9, ZmGLP4-15, ZmGLP10-1, ZmGLP10-2, and ZmGLP10-3, while no sites were predicted in ZmGLP2-1, ZmGLP4-10, and ZmGLP4-11. N-glycosylation is one of the major post-translational modifications. Most of the plant's extracellular proteins are glycosylated by N-linked oligosaccharides, which greatly affect their physicochemical properties and function. It also plays an important role in protein folding and quality control. Despite the limited knowledge of complex N-glycans in plants, their role in protein stability, conformation, cellular activities, cellulose biosynthesis, and growth under stress is well-validated.[74,75] The lowest P-sites (4) were found in ZmGLP2-1, ZmGLP4-11, and ZmGLP4-12, while the highest ones (14) were found in ZmGLP10-1. Protein phosphorylation regulates various cellular processes in plants including differentiation, proliferation, metabolism, and apoptosis, particularly at threonine, serine, or tyrosine residues.[49] The presence of a variable number of N-sites and P-sites predicted the crucial role of ZmGLPs in these processes.

3.3. 3D Structural Analyses. Three structural models were obtained for each ZmGLP (Figure S1 in supplementary data). The best model was chosen based on its global model quality estimation (GMQE) and Qmean (*Z*-score) estimation scores where high values suggest reliable results. The models were further confirmed with RPA, which revealed that on average, each protein has 87.68% of its residues existing in

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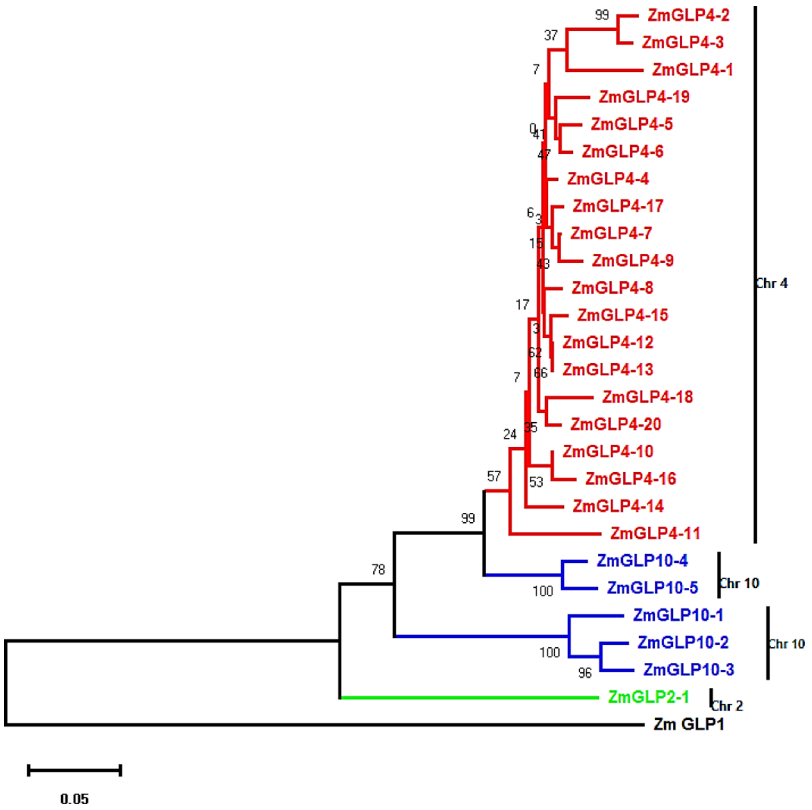

Figure 4. Phylogenetic analysis of the germin-like protein in *Zea mays* (ZmGLPs). The analysis was conducted with the Molecular and Evolutionary Genetics Analysis (MEGA10) tool. The phylogram is divided into various groups. Genes in each group are represented with distinct colors. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 1.34338359 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the *p*-distance method and are in the units of the number of amino acid differences per site. This analysis involved 27 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 270 positions in the final dataset. Chr: chromosome.

favored, 10.39% in allowed, and 93% in outliner regions, which confirmed their good quality. RPA is a standard tool used for the structural analysis of various molecules. Previously, various techniques such as crystallography and nuclear magnetic resonance (NMR) were used for structural studies, but now, they can be predicted by aligning and blasting with the known protein structure using various softwares.[76] Most of the ZmGLPs (20 or 76.92%) showed similarity in the overall structure and position of the cupin domains, which either consist of a single GER monomer each bound by a single Mn^{+2} ion or may combine to form a hexameric structure that is similar to the earlier findings.[38] Similar results were also reported for OsGLPs.[30] Structurally, ZmGLPs are similar due to common evolutionary history, but functionally, they are diverse, which may be due to their better interactive abilities with other proteins to deal with various functions and stresses. On the other hand, ZmGLP2-1, ZmGLP4-10, and ZmGLP10-5 showed significant variation in their structure, which may be due to the absence or presence of certain residues. Similarity indicates their common origin, while variation specifies evolutionary changes caused by environmental constraints. Genes located on the chr 4 and 10 have greater similarity caused by tandem duplication. Despite the resemblance, variation was also observed in the distribution of protein cavities and residue location, which may give rise to their functional diversity. It may be due to variable interactive abilities with the substrate largely affected by protein's surface and cavities.[77] Similarities and differences in protein cavities indicate some clues about their proposed function caused by various evolutionary forces and environmental factors resulting in neofunctionalization or subfunctionalization of the protein. However, it may also have an undesirable effect on protein's function[78] due to which a new function can be gained or lost.

3.4. Phylogenetic Analysis. Phylogenetic analysis revealed a narrow genetic background with an overall genetic variation of 0.05 percent (0.05%) showing a recent history of gene diversification events (Figure 4), which is similar to the study of GLPs in *Oryza sativa*[30] and *Vitis vinifera*.[5] Genes located close to each other in the phylogram suggest their common origin. It may be due to gene duplication events on specific chromosomes that result in gene clustering with similar exon structures. Previously, GLP clusters on specific chromosomes were reported in rice,[30,58,79] barley,[59] soybean,[60] cucumber,[17] and wheat.[24] More often, such clusters are associated with disease resistance by offering incredible resistance against various stresses. For example, in rice and barley, GLPs of chr 8 provide protection against rice blast, sheath blight,[79] and *Blumeria graminis* f. sp. *hordei*.[59] GLPs of chr 4 were mostly similar and advanced with recent tandem duplication events. The highest similarity was observed between ZmGLP4-2 and ZmGLP4-3, ZmGLP4-5 and ZmGLP4-6, ZmGLP4-7 and ZmGLP4-9, ZmGLP4-10 and

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Table 2. Functional Analysis of Germin-like Proteins in *Zea mays* (*ZmGLPs*)[a]

s. no.	gene	predicted functional domains
1	*ZmGLP1*	remorin, n-terminal, and cupin
2	*ZmGLP2-1*	remorin, N-terminal, cupin protease, ricin B-like lectin EULS3-like, Clp protease proteolytic subunit, translocation-enhancing protein TepA, Clp ATPase, and ClpP/crotonase-like domain superfamily
3	*ZmGLP4-1*	NA
4	*ZmGLP4-2*	disulfide bond, *S*-adenosyl-L-homocysteine hydrolase, adenosylhomocysteinase-like superfamily, thaumatin family, acetohydroxy acid isomeroreductase, and NAD/NADPH-binding domain
5	*ZmGLP4-3*	disulfide bond, *S*-adenosyl-L-homocysteine hydrolase, adenosylhomocysteinase-like superfamily, thaumatin family, and NAD/NADPH-binding domain
6	*ZmGLP4-4*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
7	*ZmGLP4-5*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
8	*ZmGLP4-6*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
9	*ZmGLP4-7*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
10	*ZmGLP4-8*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
11	*ZmGLP4-9*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
12	*ZmGLP4-10*	NA
13	*ZmGLP4-11*	NA
14	*ZmGLP4-12*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
15	*ZmGLP4-13*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
16	*ZmGLP4-14*	NA
17	*ZmGLP4-15*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
18	*ZmGLP4-16*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
19	*ZmGLP4-17*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
20	*ZmGLP4-18*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
21	*ZmGLP4-19*	disulfide bond, *S*-adenosyl-L-homocysteine hydrolase, NAD/NADH-binding domain, acetohydroxy acid isomeroreductase, thaumatin family, and adenosylhomocysteinase-like
22	*ZmGLP4-20*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
23	*ZmGLP10-1*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
24	*ZmGLP10-2*	*S*-adenosylmethionine decarboxylase, arginine and proline metabolism, zinc finger C_2H_2 superfamily, cysteine and methionine metabolism, polyamine biosynthesis, ornithine/DAP/Arg decarboxylase, spermidine biosynthesis, zymogen, Schiff base, and autocatalytic cleavage
25	*ZmGLP10-3*	disulfide bond, phosphoglycolate phosphatase-like, domain 2, and osmotin/thaumatin-like superfamily
26	*ZmGLP10-4*	disulfide bond, *S*-adenosyl-L-homocysteine hydrolase, NAD/NADH-binding domain, acetohydroxy acid isomeroreductase, thaumatin family, and adenosylhomocysteinase-like
27	*ZmGLP10-5*	PGG domain, germin, and manganese binding site, mostly uncharacterized, incl. domains of unknown function duf2040 and asparaginyl endopeptidase

[a]The analyses were conducted using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). NA: not available; NAD: nicotinamide adenine dinucleotide.

ZmGLP4-16, ZmGLP4-12 and ZmGLP4-13, and ZmGLP4-18 and ZmGLP4-20. However, ZmGLP4-11 and ZmGLP4-14 showed a distant relationship. Similarly, the GLP of chr 10 serves as an ancestral group from which chr 4 GLPs arose through the course of evolution. ZmGLP10-4 and ZmGLP10-5 were highly similar to chr 4 GLPs, which may be due to their shared exon structure probably because of retrotransposon activity. Other similar genes include ZmGLP10-1, ZmGLP10-2, and ZmGLP10-3. A high diversification rate was observed on chr 4 as compared to 10 as represented by their high bootstrap's values, which may be due to the high mutation rate. However, distinctive positions were noted for ZmGLP2-1 and ZmGLP1, which may be due their presence on separate chromosomes. They are the most primitive members of this family among which ZmGLP1 followed a separate lineage while ZmGLP2-1 shared their evolutionary history with chr 4 and 10 GLPs. Chromosomes with the highest number of GLPs represent high duplication rate forming clusters, which may be due to high selection pressure. However, the reason why certain chromosomes are under high selection pressure is still unknown.

3.5. Functional Analysis. The functional analysis predicted various roles for ZmGLPs based on their domain homology and coexpression pattern with other related proteins (Table 2). However, no functional values were recorded for ZmGLP4-1, ZmGLP4-10, ZmGLP4-11, and ZmGLP4-14. STRING obtained values from UniProt, Pfam, InterPro, and SMART protein domain databases. Analysis showed that genes of the same chr have similar functional properties. Among all, 19 showed disulfide bonding, which is important for the proper biological function of the protein. It increases stability as well as protects against the destructive effect of extreme environments and proteolytic degradation.[80] ZmGLP4-4, ZmGLP4-5, ZmGLP4-6, ZmGLP4-7, ZmGLP4-8, ZmGLP4-9, ZmGLP4-12, ZmGLP4-13, ZmGLP4-15, ZmGLP4-16, ZmGLP4-17, ZmGLP4-18, ZmGLP4-20, ZmGLP10-1, and ZmGLP10-3 showed phosphoglycolate phosphatase-like (PGLP), domain 2, and osmotin/thaumatin-like enzymatic activities. PGLP activity is important for photorespiration, carbon assimilation, and allocation[81] as well as improving plant responses to light intensity and drought.[82] Similarly, osmotin/thaumatin is a multifunctional protein family related to plant responses against biotic and abiotic stresses by acting as an osmoregulator.[83] The observation suggested that these genes might act in a coregulated manner to control plant responses against various stresses. Previously, the combinatorial action of GER4 or GER5 subfamilies protects barley epidermal cells from *Blumeria graminis* f. sp. *hordei* infection.[59] In another study, a QTL consisting of 12 *OsGLPs* provides immunity against rice blast disease.[79] Recently the role of *OsGLP3-7* was

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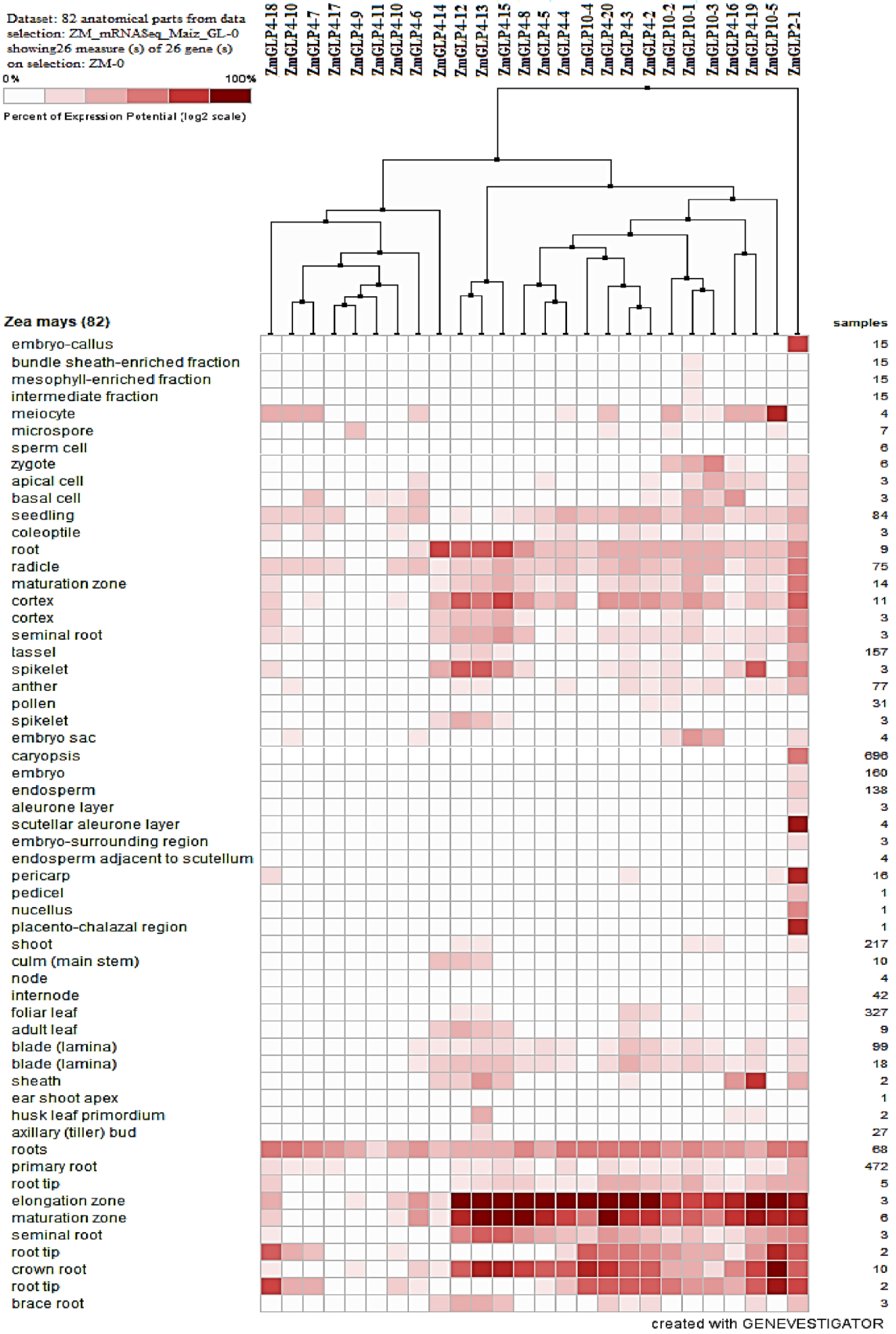

Figure 5. Expression analysis of the germin-like protein genes in *Zea mays* (*ZmGLPs*) in different plant parts. Plant parts with no expression were omitted. The analysis was conducted with Genevestigator (https://genevestigator.com/).

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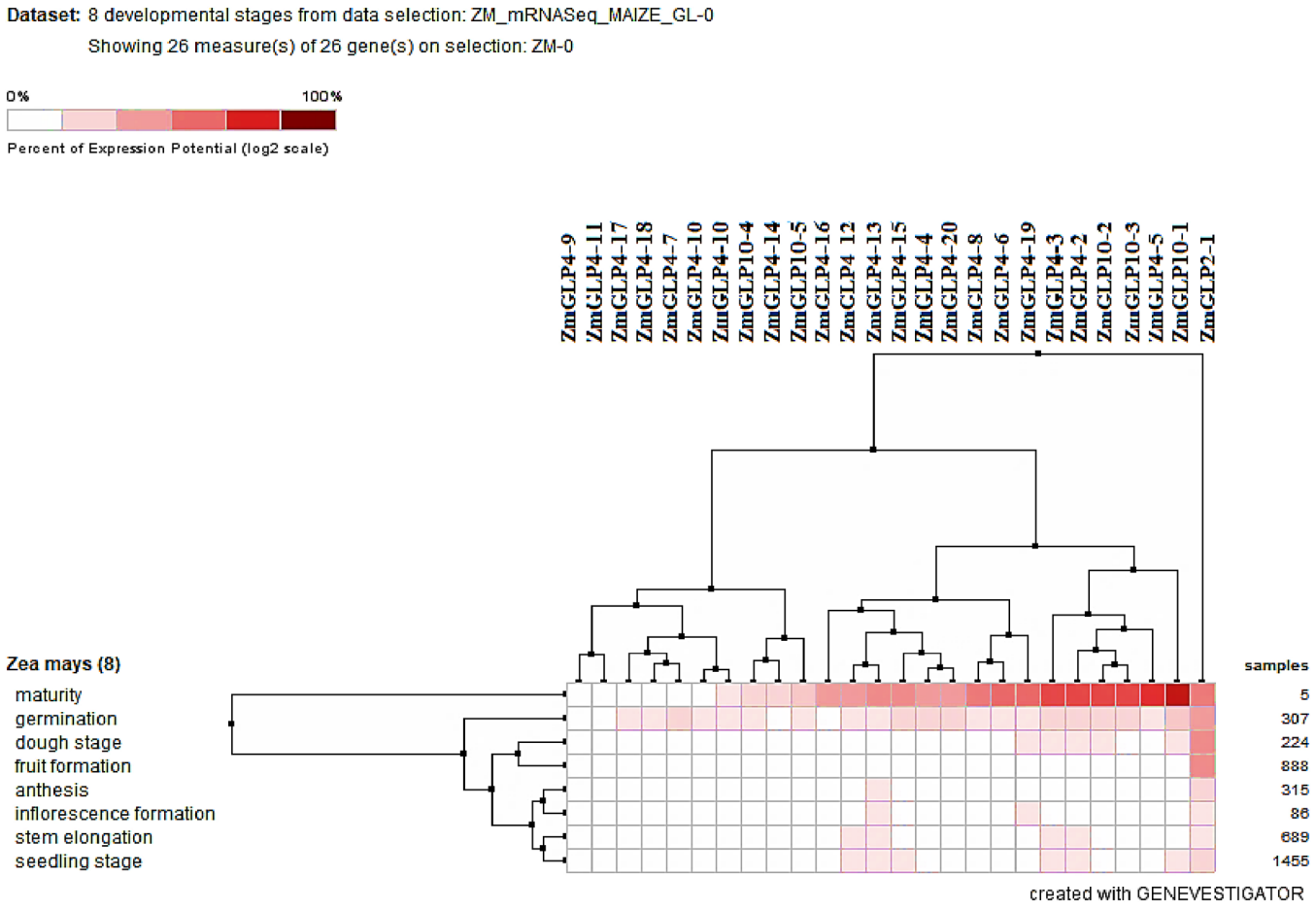

Figure 6. Expression analysis of the germin-like protein genes in *Zea mays* (*ZmGLPs*) at different growth stages of the maize plant. The analysis was conducted with Genevestigator (https://genevestigator.com/). The intensity of the colors represents the expression level of the genes.

confirmed against leaf blast, panicle blast, and bacterial blight by activating JA and phytoalexin metabolic pathways.[32] Similarly, *TaGLPs* located on 4A, 4B, and 4D chr provide potential resistance against *Blumeria graminis* f. sp. *tritici* (*bgt*) infection.[24] ZmGLP4-2, ZmGLP4-3, ZmGLP4-19, and ZmGLP10-4 have similar enzymatic functions related to *S*-adenosyl-L-homocysteine hydrolase (SAHH) and acetohydroxy acid isomeroreductase activities. The former removes *S*-adenosyl-L-homocysteine (SAH), a byproduct of the *trans*-methylation reaction, thereby helping plants to grow and acclimatize to the natural environment.[84] The latter helps in the biosynthetic pathways of branched-chain amino acids, i.e., valine, leucine, and isoleucine. Because of its plant-specific nature, it serves as a potential target for various fungicides and herbicides.[85] Further, these genes also showed thaumatin-like protein (TLP) properties, which is a highly complex protein family linked with host-defense and developmental processes in plants, animals, and fungi.[83] ZmGLP1 and ZmGLP2-1 exhibited remorin-related enzymatic activities, a plant-specific membrane-localized protein having a role in plant growth and development, signaling, and various stress responses.[86] Previously, ZmGLP1 was also found active in young whorled leaves and tassels and cobs by mainly expressing in mesophyllous, phloem, and guard cells mediated by several important regulatory elements in the promoter, which control the expression level and circadian rhythm-oscillated patterns in transgenic *Arabidopsis*.[39] It also regulates resistance against biotrophic PstDC3000 and necrotrophic *S. sclerotiorum* via oxidative burst and JA signaling pathway activation in transgenic *Arabidopsis*.[40] ZmGLP2-1 contains a ricin B-like lectin (RBL) domain, which protects against Fusarium head blight (FHB) caused by *Fusarium graminearum* (*F. graminearum*) in wheat[87] and other fungal infections. It suggests that these genes protect against various pathogens by regulating membrane biology. Other domains that are present in ZmGLPs include the Clp protease proteolytic subunit, which is important in the biogenesis of plastids, protein unfolding response in the chloroplast, metabolism, or cellular proteostasis. Similarly, Clp ATPase serves as a molecular chaperon that falls within the AAA+ superfamily of ATPases involved in protein unfolding and degradation along with a broader range of biological processes.[88,89] Similarly, translocation-enhancing protein TepA [UniProtKB-Q99171 (TEPA_BACSU)] is required for the efficient secretion and translocation of protein across the membrane.[90] Another important domain in ZmGLPs belongs to the ClpP/crotonase-like domain superfamily (IPR029045). Protein crotonylation is an important post-translational modification having an important regulatory role in a wide range of processes.[91] The analysis revealed that ZmGLPs are mostly active in the cellular processes; however, they may also provide protection against various stresses by regulating various internal pathways.

3.6. Expression Analysis. *3.6.1. Anatomical Parts.* Hierarchical cluster analysis of the selected genes showed diverse expression patterns across 82 different anatomical parts of the maize plant (Figure 5). Parts with no expression were

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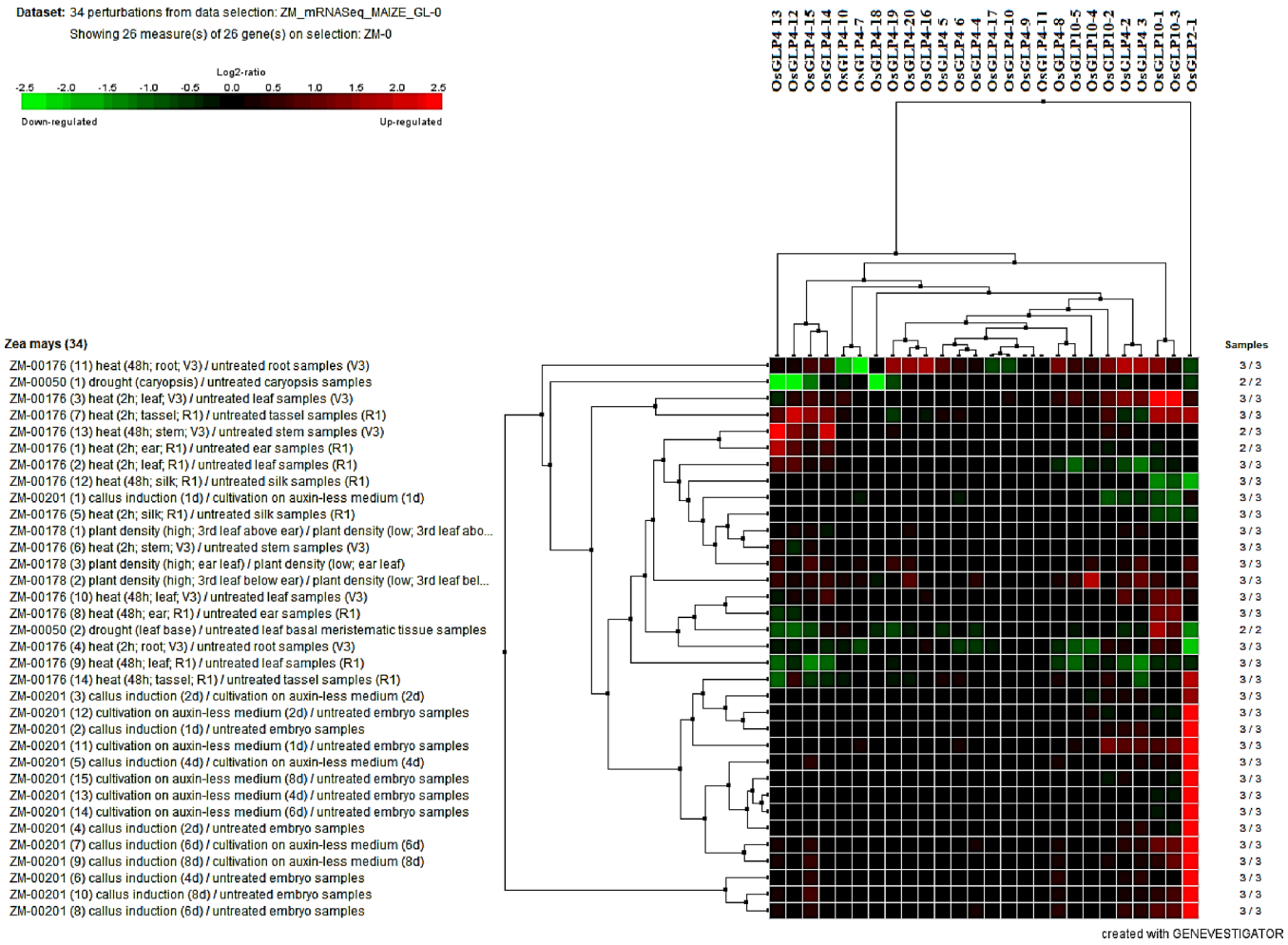

Figure 7. *In silico* expression analysis of germin-like protein genes in *Zea mays* (*ZmGLPs*) under different abiotic stresses. For auxin stress, embryos were isolated from CAL caryopses 10−12 days after pollination, cultured on an N6 medium containing 1.5 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin) in darkness at 27 °C for 1, 2, 4, 6, and 8 days, and then sampled. Before embryo isolation, plants were grown in a greenhouse under 16 h light/8 h dark cycles at 20−25 °C. For drought stress, B73 plants were grown in a greenhouse without water limitation (the soil moisture content was maintained close to the field capacity) till the onset of silk emergence; then, irrigation was withheld for 3 days. At the end of the 3-day drought period, the plants were hand-pollinated and kept for another 24 h without watering (4 days of drought stress in total; at the end of this period, the leaf relative water content was 66.5%). The basal meristematic tissue of the three youngest leaves was then harvested. Other conditions: plants were grown in 10 L pots containing a mixture of peat, vermiculite, perlite (1:1:1), 6 g of pulverized limestone, 35 g of $CaSO_4$, 42 g of $FeSO_4$, and 1 g of trace fritted element; before the drought stress, a general purpose fertilizer (15-16-17; Scott-Sierra Horticultural Products) was supplied once a week. For heat stress, different samples of R1-stage B73 plants were grown in a phytotron under 14 h light/10 h dark cycles at 25 °C and 65% relative humidity and then exposed to 38 °C for 2 and 48 h. Ear leaf and the third leaf above ear were collected from Xianyu 335 plants (at grain filling) grown under high planting density along with the control. For high plant density, RNA-seq of leaves at different nodes of the maize hybrid Xianyu 335 under different (low and high) plant densities was conducted to explore the mechanisms of responding to high plant density. A control was used in each experiment.

omitted. ZmGLPs either showed individual or combinatorial expression patterns in different plant parts. The highest was noted in the root, root tips, crown root, elongation and maturation zones, primary root, radicle, and cortex, while other parts showed either moderate or minimal expression. The study revealed that they regulate various plant processes mainly in the root and maturation zones, which is per the prominent role of GER and GLPs in roots.[7,12,92] Previously, a germin-like oxalate oxidase activity was detected in the primary root (epidermal cells) and coleorhiza of barley seedlings.[93] Similarly, the GUS gene controlled by the *OsRGLP1* promoter showed strong activity in the root when tested against drought, ABA, wounding, and salinity.[94] A similar result was also noted for the *OsRGLP2* promoter.[95,96] In the same way, the JA-mediated role of *OsGER4* in crown root development was also confirmed in rice.[33] Likewise, a moderate response was noted in seedlings, anthers, seminal roots, etc., which is similar to the expression of germin-like oxalate oxidase in seedlings of *Hordeum vulgare*[93] and *Beta vulgaris* L.[97] However, foliar leaf, blade, shoot, etc. showed reduced expression, which is comparable to the study of *HvGerB*, which was mainly expressed in developing shoots, while *GerF* showed enhanced expression in seedling roots, developing spikes, and pericarp/testa.[98] Similarly, *HvGLP1* was mainly expressed in young leaves and less abundant in older leaves but not in roots.[99] Currently, the highest expression in the abovementioned parts was noted for *ZmGLP2-1*, *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-5*, *ZmGLP4-8*, *ZmGLP4-12*, *ZmGLP4-13*, *ZmGLP4-14*, *ZmGLP4-15*, *ZmGLP4-16*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, *ZmGLP10-4*, and

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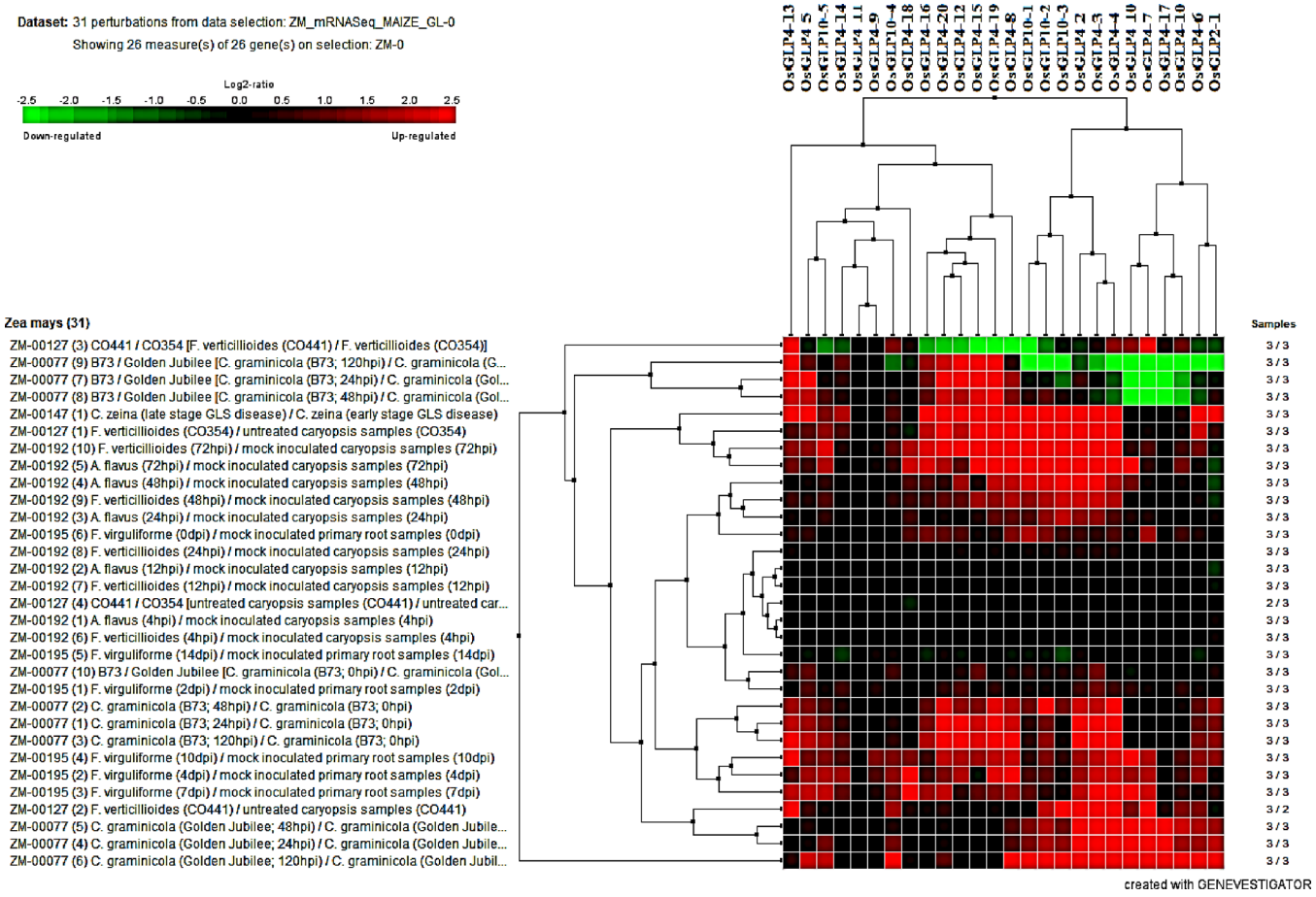

Figure 8. *In silico* expression profile of *Zea mays* germin-like protein (*ZmGLP*) genes against various biotic stresses. For *Aspergillus flavus* (24 hpi), B73 plant ears were hand-pollinated, and after 21−22 days, the ears were inoculated with the *Aspergillus flavus* strain NRRL 3357 by dipping a 3 mm needle into a conidial suspension and inserting it into the caryopsis crown (approx. 13 fungal conidia were introduced into the endosperm of a caryopsis). Caryopses were sampled 4, 12, 24, 48, and 72 h after inoculation. For *Colletotrichum graminicola* (B73; 24 hpi), 4-days-old B73 plants were inoculated with the wild-type M2 (CgM2) strain by placing 10 μL droplets of a spore suspension (10^6 spores/mL, in 0.01% Tween 20) on a horizontally oriented third leaf (counting from the shoot base), incubated at 25 °C and high humidity for 24, 48, and 120 h (the penetration phase of the fungus), and harvested. For *Fusarium verticillioides*, B73 plants were grown in field conditions at the Central Crops Research Station near Clayton, NC, USA. Ears were hand-pollinated and covered with paper bags. After pollination (21−22 days), the ears were inoculated with the strain n16 by dipping a 3 mm needle into a conidial suspension and inserting it into the caryopsis crown (approx. 13 fungal conidia were introduced into the endosperm of a caryopsis). Caryopses were sampled 4, 12, 24, 48, and 72 h after inoculation. At sampling, no fungal mycelia were visible in the caryopses. *Fusarium verticillioides* is a fungus that can colonize maize ears via silk and wounds and grows into ears from infected stalks. It produces fumonisin. A control was used in each experiment. For *Cercospora zeina* (late-stage GLS disease), B73 plant's middle parts of lower leaf blades showing late-stage gray leaf spot (GLS) disease symptoms (big rectangular lesions) were harvested. For *Fusarium virguliforme*, E13022S seeds were surface sterilized, incubated in sterile water for 24 h in the dark, and germinated for 5 days in darkness at 21 °C in a Petri dish. Germinated seedlings were thoroughly sprayed with *Fusarium virguliforme* Mont-1 isolate inoculum, incubated for 1.5 h, and kept for 2, 4, 7, 10, and 14 days in a growth chamber under 14 h light (140 μmol photons m^{-2} s^{-1})/10 h dark cycles at 12 °C after which the 4 cm-long original inoculation site of the primary root was sampled.

ZmGLP10-5, showing that these genes may play important biological roles in these parts. However, other genes showed minimal expression. Such a diverse role was previously reported for *OsGLPs*.[30] The expression pattern suggested that these genes, which are mostly located on chromosome 4, act in a combinatorial manner to regulate various plant processes. Previously, 14 *OsGLPs* located on chromosome 8 acted as a QTL by providing resistance against rice blast and sheath blight.[79] Recently, OsGLP3-7 provides protection against leaf blast, panicle blast, and bacterial blight mainly expressed in leaves and the cytoplasm.[32] A similar response was also reported for *HvGERs* (chromosome 8) against *Blumeria graminis* f. sp. *hordei* in barley.[59] Recently, most of the *Blumeria graminis* f. sp. *tritici* (*Bgt*)-resistant genes (*TaGLPs*) were found on the fourth (4D) homolog chromosome in wheat.[24] In addition to the abovementioned parts, some of the genes were also active in specific parts as well. For example, *ZmGLP2-1* showed enhanced expression in embryo-callus, scutellar-aleurone layer, pericarp, and placenta-chalazal regions. Similarly, *ZmGLP10-5* was active in meiocytes confirming its role in development, while *ZmGLP4-19* was active in spikelets and sheath cells suggesting its role in the development and storage of various materials. Previously, *GerF* was also predominantly expressed in seedling roots, developing spikes, and pericarp/testa.[98] Similarly, a distinct expression pattern was observed for *AhGLPs* in various parts of peanuts.[12] Recently, *AhGLP17-1* was specifically found active in pericarp, while no expression was detected in other parts (leaves, stems,

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roots, flowers, cotyledons, or embryos) of peanuts.[100] Contrarily, some of the genes (*ZmGLP2-1*, *ZmGLP10-1*, and *ZmGLP10*) showed expression in all parts suggesting their role in overall plant development and defense.

3.6.2. Developmental Stages. *ZmGLP* expression was tested at 8 developmental stages of *Zea mays*, namely, germination, seedling phase, stem elongation, inflorescence formation, anthesis, fruit formation, dough stage, and at maturity levels (Figure 6). The highest expression was observed at germination and maturity levels, while other stages showed limited expression, which is closely linked to the crucial role of *ZmGLPs* in seed germination. Previously, *PvGLP1* was active during the early stages of embryo development but showed weak expression at later stages in *Phaseolus vulgaris* L.[101] Another study revealed a striking increase in germin-like oxalate oxidase ("gl-OXO") expression in wheat calli.[102] Among all, *ZmGLP2-1* showed the highest expression followed by *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-13*, and *ZmGLP4-12* showing their role in plant development. However, *ZmGLP2-1* was active at all levels, particularly during fruit formation, dough stage, germination, and maturity, which suggested the importance of these genes. However, others showed stage-specific expression, for example, *ZmGLP10-1* is highly active at the maturity level. Previously, high GLP expression was noted at maturity levels, for example, 2 novel GLPs (*Ps-GLP1* and *2*) were highly active during fruit development and ripening stages of early and late plum cultivars largely regulated by auxin and ethylene production.[103]

3.6.3. Abiotic Stresses. *ZmGLPs* did not show any substantial expression in abiotic stresses (Figure 7), but some of them were either up- or downregulated. The highest expression was noted against heat stress where many genes such as *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-5*, *ZmGLP4-6*, *ZmGLP4-8*, *ZmGLP4-12*, *ZmGLP4-13*, *ZmGLP4-14*, *ZmGLP4-15* *ZmGLP4-16*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, and *ZmGLP10-4* were highly active. Previously, *PsGER1* was highly active in high-temperature stress via SOD activity in the developing pea root nodules.[104] Similarly, *StGLP* protects potatoes against gradual heat stress (GHS) by activating several antioxidant enzymes and heat shock-responsive genes.[15] Recently, the role of 70 *StGLP* genes was confirmed against salt, ABA, and heat stresses among which 19 were highly active in heat stress with more focus on 3 (*StGLP14*, *StGLP17*, and *StGLP30*).[36] In drought, *ZmGLP10-1* and *ZmGLP10-3* showed minor expression, but most of them were downregulated contradicting the study in *CsGLP*, which showed upregulation against drought, salt, and ABA stresses.[17] A similar expression was also shown by *OsGLP8-4*, *OsGLP8-7*, and *OsGLP8-12* in two rice varieties (Super Basmati and KS282) largely mediated by methylation of specific cytosine residues in the promoter of *OsGLPs*.[105] In another study, GLP was highly accumulated in the leaves of *Boea hygrometrica* against drought.[14] In the case of auxin, no observable response was observed where *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-8*, *ZmGLP4-13*, *ZmGLP4-15*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, and *ZmGLP10-4* showed moderate expression toward callus induction on auxin-less medium and in embryo samples, but *ZmGLP2-1* showed the highest expression. Previously, *ZmGLP2-1* (Zm00001d004401) and germin-like protein-1 (Zm00001d008210) were also highly upregulated during transcriptome analysis of *Zea mays* embryogenesis.[106] Contrarily, expression of germin-like OXO was noted during wheat embryogenic callus formation upon auxin treatment.[102] Similarly, the study of *CpGLP1* and *2* showed no expression during callus induction.[107] In the case of plant density, *ZmGLP2-1*, *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-13*, *ZmGLP4-14*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP4-15*, and *ZmGLP10-1* showed minor expression, whereas relatively high expression was noted for *ZmGLP10-4*. Plant density plays an important role in plant−plant interactions under different environmental stresses.[108] The generation of high-density planting (HDP) stress-tolerant cultivars in tropical maize has been previously reported.[109] The current study showed that *ZmGLPs* play an important role in HDP stress tolerance. However, according to our knowledge, no such studies have still far been conducted on such an aspect of GLPs.

3.6.4. Biotic Stresses. 3.6.4.1. Aspergillus flavus. *ZmGLPs* showed better expression in biotic stresses as compared to abiotic stresses (Figure 8). In *Aspergillus flavus*, the highest response was noted at 48 and 72 hpi (hours postinoculation) periods, minor at 24 hpi, but no response was observed at 4 and 12 hpi. The highest expression was noted for *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-8*, *ZmGLP4-12*, *ZmGLP4-13*, *ZmGLP4-15*, *ZmGLP4-16*, *ZmGLP4-18*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, and *ZmGLP10-5*. A similar expression was previously noted for *AhGLP1*, *2*, *3*, *4*, and *5*, which showed significant upregulation after *A. flavus* infection.[12] In another study, germin-like protein subfamily-1 member 17 was found abundant during a proteomic study of the rachis tissue of the susceptible maize inbred line (B17) after *Aspergillus flavus* infection.[110] Similarly, several ZmGLPs showed upregulation during the study of the host/pathogen interaction pathway in the *Zea mays*/*Aspergillus flavus* pathosystem.[111] However, it is contrary to the study of *GhGLP1*, which showed downregulation when cultured ovules were subjected to *A. flavus* infection.[63]

3.6.4.2. Colletotrichum graminicola. In *Colletotrichum graminicola*, the highest response was observed after 120 h of inoculation followed by a 48 and 24 hpi period. Highly active genes were *ZmGLP2-1*, *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-6*, *ZmGLP4-7*, *ZmGLP4-8*, *ZmGLP4-10*, *ZmGLP4-10*, *ZmGLP4-12*, *ZmGLP4-15*, *ZmGLP4-17*, *ZmGLP4-18*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, and *ZmGLP10-4*, while moderately expressed genes were *ZmGLP4-5*, *ZmGLP4-13-*, *ZmGLP4-14*, and *ZmGLP10-5*. However, *ZmGLP4-9* and *ZmGLP4-11* showed minor expression. Recently, several germin-like proteins showed upregulation during a transcriptomic study of the maize leaves infected with *Colletotrichum graminicola*.[112] Similarly, several GLPs were involved in the defense response against *C. graminicola* in maize.[113] In another study, a maize plant inoculated with *C. graminicola* showed 2000 genes being upregulated with a 2.5-fold increase in the transcript level including GLPs.[112]

3.6.4.3. Fusarium verticillioides. In *Fusarium verticillioides*, the highest expression was noted after a 72 hpi period where most of the active genes were *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, *ZmGLP10-4*, *ZmGLP10-5*, *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-5*, *ZmGLP4-8*, *ZmGLP4-12*, *ZmGLP4-13*, *ZmGLP4-15*, *ZmGLP4-16*, *ZmGLP4-18*, *ZmGLP4-19*, and *ZmGLP4-20*. Moderate expression was noted at 48 hpi where *ZmGLP4-2*, *ZmGLP4-3*, *ZmGLP4-4*, *ZmGLP4-8*, *ZmGLP4-12*, *ZmGLP4-15*, *ZmGLP4-19*, *ZmGLP4-20*, *ZmGLP10-1*, *ZmGLP10-2*, *ZmGLP10-3*, *ZmGLP10-4*, and *ZmGLP10-5*

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were mostly active, while no expression was observed at 4, 12, and 24 hpi. Previously, few GLPs showed expression at 7 days postinteraction with *Fusarium verticillioides* in maize.[114] In another study, a few GLPs along with other defense proteins were upregulated after *Fusarium graminearum* inoculation.[115] A similar study conducted in wheat showed GLPs being the main player in defense against *F. graminearum*.[116]

3.6.4.4. Cercospora zeina. In the case of *Cercospora zeina*, all the genes were highly expressed except *ZmGLP4-7*, *ZmGLP4-9*, *ZmGLP4-10*, *ZmGLP4-11*, *ZmGLP4-17*, and *ZmGLP4-18*. Previously, various candidate genes and QTLs have been identified that provide resistance against gray leaf spot (GLS) disease caused by *Cercospora zeina* in maize.[117−119] However, no comprehensive study has still far been conducted using GLPs. The present study provides a clue to test these genes against GLS disease in maize.

3.6.4.5. Fusarium virguliforme. In the case of *Fusarium virguliforme*, the highest expression was shown by all genes except *ZmGLP2-1*, *ZmGLP4-11*, *ZmGLP4-17*, and *ZmGLP10-3*. Similarly, the highest expression was noted at 4, 7, and 10 dpi (days postinfection), while the minimal response was noted at 0 and 2 dpi, but no expression was noted at 14 dpi. Till now, no significant data are available regarding the use of GLPs against *F. virguliforme*, but reports can be found against *Fusarium solani*,[96,120] *Fusarium oxysporum*,[22,121] *Fusarium graminearum*,[115] and *Fusarium verticillioides*,[114] Similarly, *GhABP19* regulates resistance against Verticillium (*Verticillium dahliae*) and Fusarium wilt (*Fusarium oxysporum*) in plants via SOD activity-regulating JA pathways in cotton.[22] A similar observation was noted for 11 *GhGLP* genes against *Verticillium dahliae*-infected *Gossypium barbadense*.[122] Recently JA and phytoalexin-mediated protection was noted for *OsGLP2-7* against leaf blast, panicle blast, and bacterial blight.[32]

4. CONCLUSIONS AND FUTURE RECOMMENDATIONS

Identification and characterization of useful genes with novel functions are among the main challenges for crop improvement in plant biotechnology. The study revealed that *ZmGLPs* seem similar in structure, but functionally, they are much more diverse mainly evolved on chromosome 4 via tandem duplication events. Genes located on the same chr possess similar physicochemical properties, subcellular localization, 3D structures, functional properties, and expression patterns and close phylogenetic relationships. Through the course of evolution, *ZmGLPs* adapted phosphoglycolate phosphatase, adenosylhomocysteinase, phosphoglycolate phosphatase-like, osmotin/thaumatin-like, and acetohydroxy acid isomeroreductase-like enzymatic activities mostly mediated by disulfide bonding. Functionally, *ZmGLP* acts in a coregulated manner remaining active in the root system at a germination stage and maturity level protecting against various biotic stresses mainly expressed in the cytoplasm or extracellular region. In the future, this information can be used to produce economically and agronomically resilient cultivars against a wide range of stresses.

■ 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 supplementary materials.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01104.

(Figure S1) 3D structural analysis of the *Zea mays* germin-like protein (ZmGLP) family; (Table S1) *Zea mays* germin-like protein gene (ZmGLP) sequences (PDF)

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M.I., I.A., D.N.B., and S.U. collected the data, did the analysis, and wrote the original draft; T.M., B.A., S.M.E., R.I., I.A., and S.H.A.B. helped with interpretation, manuscript writing, and editing.

Notes

The authors declare no competing financial interest.
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