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Research Article

In Silico Functional Prediction and Expression Analysis in Response to Drought Stress of the Natural Resistance-Associated Macrophage Protein (*NRAMP*) Gene Family in Maize

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ABSTRACT

Natural Resistance-Associated Macrophage Proteins (*NRAMPs*) function in plants as metal transition transporters to maintain metal homeostasis. The genome-wide identification and functional prediction of the maize *NRAMPs* have not yet been studied. The genome-wide analysis identified seven (*ZmNRAMP1, ZmNRAMP2, ZmNRAMP3, ZmNRAMP4, ZmNRAMP5, ZmNRAMP6, ZmNRAMP7*) proteins in maize with a molecular weight of 29.08 (*ZmNRAMP2*) to 63.20 (*ZmNRAMP5*) kDa. Except for *ZmNRAMP1* and *ZmNRAMP3*, all *ZmNRAMPs* are essential proteins (isoelectric points greater than 7). The number of introns in *ZmNRAMPs* ranges from 1 to 13, which is almost consistent among the phylogenetic groups (A and B) between maize and Arabidopsis. All proteins showed multiple motifs (ranging from 6 to 10) that were consistent among the groups between maize and Arabidopsis. We also conducted transmembrane domains, cis-regulatory elements, chromosomal mapping, and gene ontology annotation that revealed significant functional attributes. Significantly increased *ZmNRAMPs* (*ZmNRAMP1, ZmNRAMP2*, and *ZmNRAMP4*) expression in vegetative and reproductive tissues may play an essential role in heavy metal absorption, transport, and homeostasis. During drought stress, significant upregulation of *ZmNRAMPs* (*ZmNRAMP1, ZmNRAMP2, ZmNRAMP4, and ZmNRAMP7*) may play a role in drought tolerance or acclimatization in maize.

Keywords: *NRAMP*; Transporter; Drought; Gene expression; Maize

INTRODUCTION

Maize (*Zea mays*) is one of the world's major food crops and is of substantial value to food, feed, pharmaceutical, and various industries [1,2]. It is widely used as a raw material in food product development and different bioenergy industries [3]. Maize is the world's most productive and cultivated crop and a paradigm for genetics and genomics [4]. Among the top three crops in Asia, maize is one of them. In sub-Saharan Africa and Latin America, maize is considered the primary source of food security and economic development [5]. Various global environmental crises and stresses, including biotic and abiotic stresses, have posed a considerable threat to global maize production [6,7]. Therefore, it is necessary to clarify the molecular mechanism of maize in response to the unstable environmental situation [8]. Natural Resistance-Associated Macrophage Proteins (*NRAMP*) are known evolutionarily integral membrane proteins that play a crucial role in transporting a broad range of metal ions [9]. The *NRAMPs* are broadly distributed among various organisms, including bacteria, algae, yeast, plants, and animals [10]. The *NRAMP1* gene of the *NRAMP* family was first identified in mice and has been recognized as a divalent metal transporter in plants, fungi, bacteria, insects, and mammals [11–13].

The *NRAMPs* transport iron and manganese to protect against bacterial infection [14]. The availability of *NRAMPs* among diverse species indicates the significance of their functions. Previous research has shown that *NRAMP* family proteins play an important role in structural stability and functional divergence across species. The *NRAMP* family proteins work as proton-coupled metal ion transporters and help transport Iron (Fe^{2+}), Manganese (Mn^{2+}), Zinc (Zn^{2+}), Cadmium (Cd^{2+}), Copper (Cu^{2+}), Cobalt (Co^{2+}), Nickel (Ni^{2+}), and Aluminum (Al^{3+}) [10,15–19]. These *NRAMP* family proteins have been implicated in the intracellular transport,

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translocation, uptake, and detoxification of transition metals [10]. The *NRAMP* transporter transports proton-coupled metal ions in different plants and plays a crucial role in plant growth and development, nutritional support, heavy metal balancing, and signal transduction [9]. Previous studies showed that the importance of the *NRAMP* family proteins in different plants had been investigated, including *Arabidopsis thaliana* [20], *Populus alba* [21], *Oryza sativa* [22], *Theobroma cacao* [23], *Brassica napus* [24], and *Phaseolus vulgaris* [25,26].

In *Arabidopsis thaliana*, *AtNRAMP1* is found in the plasma membrane and works as an Iron (Fe^{2+}), Manganese (Mn^{2+}), and Cadmium (Cd^{2+}) transporter [27,28], whereas *AtNRAMP2* is found in the endomembrane and helps in the distribution of Manganese (Mn^{2+}) between intracellular organelles [29,30]. The *AtNARMP3* and *AtNARMP4* transport Manganese (Mn^{2+}) in plants that play a crucial role in photosynthesis and seed germination [31,32]. The *AtNARMP5* has been identified as an Iron (Fe^{2+}) and Cadmium (Cd^{2+}) transporter [33]. The intracellular transportation of Cadmium (Cd^{2+}) in plants is mediated by *AtNARMP6* [27]. In rice, the *NRAMP* family proteins transport Iron (Fe), Manganese (Mn), Cadmium (Cd), and Arsenic (As) for the growth and development of rice. *OsNARMP1* has been identified as a Fe, As, and Cd transporter [34–36], whereas Os*NRAMP2* accumulates a high amount of Cadmium (Cd) in shoots [37]. The *OsNRAMP3* helps transport Manganese (Mn) [38]. Furthermore, *OsNRAMP4* transports Aluminum (Al) [16], whereas *OsNRAMP5* transports Iron (Fe), Manganese (Mn), and Cadmium (Cd) [17,39]. *OsNRAMP6* is found in the plasma membrane that works as an Iron (Fe), and Manganese (Mn) transporter [40].

However, the significant functions of many *NRAMP* transporters have been intensively investigated in Arabidopsis and other plants, but the *NRAMP* transporters in maize have not been characterized to date. Therefore, the current research was conducted for genome-wide identification and functional characterization of *NRAMP* transporters in maize by intensive bioinformatics investigations. In this study, we identified *NRAMP* genes from the maize genome and performed a comprehensive analysis of phylogeny, exon-intron structure, conserved motif, three-dimensional protein model, transmembrane domain architecture, and responsive motif cis-elements in the promoters, and chromosomal mapping. Furthermore, for a deep understanding of the functional roles of *NRAMP* transporters, the expression of different maize tissues under developmental and drought stress conditions, and gene ontology were also analyzed.

MATERIALS AND METHODS

Identification of the *ZmNRAMP* gene family members in maize

The *NRAMP* proteins were identified following the Hidden Markov Model (HMM) profile of the conserved domains of *AtNRAMP* proteins based on the PFAM, CDD, and SMART databases. In brief, the complete maize genome (B73_RefGen_v4) sequence was downloaded from the maize database (http://www.maizesequence.org/index.html) and then TBtools software was used to construct a local database based on protein sequences. The known six *AtNRAMP* protein sequences were downloaded from TAIR (http://www.arabidopsis.org/) and used as queries to search against the maize genome *via* the local BLASTP program with an e-value of 1e-5 and where 50% identity was considered as the threshold. Further, all the *NRAMP* protein sequences of Arabidopsis have been aligned using ClustalX 2.1 (https://clustalx.software.informer.com/2.1/) for multiple sequence alignment, and the obtained alignments have been used to search the maize database using the online HMMER search tool. The results of HMMER search tools and BLASTP were compared and parsed by manual editing. For the confirmation of potential members of *ZmNRAMP* (PF01566) proteins, the PFAM database (http://pfam.xfam.org/), NCBI Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd/), and SMART database (http://smart.embl-heidelberg.de/) were used for removing redundant sequences. The remaining sequences were submitted to Inter-Pro-Scan (http://www.ebi.ac.uk/interpro/scan.html) to reconfirm the conserved domains. The maize genes were named according to the closeness of Arabidopsis genes. To analyze the physical and chemical parameters of the *ZmNRAMP* proteins, the ProtParam server (http://web.expasy.org/protparam/) was used [41–43].

Phylogenetic tree construction

The obtained protein sequence of *ZmNRAMPs* from the maize genome was aligned using ClustalX (version 2.1). Then, the phylogenetic tree of *ZmNRAMP* proteins was constructed by comparing *AtNRAMP* proteins following the Maximum Likelihood (ML) method and 1000 bootstrap replicates using MEGAX software [44,45].

Gene structures and conserved motifs analysis

The structure of *ZmNRAMP* proteins was investigated using the web-based GSDS server (http://gsds.cbi.pku.edu.cn/), and the conserved domains of the *ZmNRAMP* proteins were defined using Pfam (http://pfam.xfam.org/) and the TBtools software. The MEME suite (version 5.0.5) (http://meme-suite.org/tools/meme) was used to evaluate the conserved motifs and to identify the numbers of motifs based on the optimized E-values [45].

Prediction of three-dimensional modeling

The three-dimensional structures of representative *ZmNRAMP* proteins were constructed using the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2) to investigate structural differences and their effects on functions of the maize *ZmNRAMP* protein families [42].

Transmembrane domain prediction

For predicting transmembrane domains of the *ZmNRAMP* proteins, transmembrane topology crystal structures were constructed using the online tool HMMTOP (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py) and manually calculated the transmembrane helices [44].

Analysis of cis-regulatory elements in the promoters

The promoter sequences of *ZmNRAMP* genes were obtained from the maize whole-genome sequencing files. Subsequently, the upstream 2000 bp sequence relative to the translation initiation codon (ATG) of the promoter of each *ZmNRAMP* gene was selected as the promoter region, and the PlantCare online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict the cis-acting elements in the promoters of *ZmNRAMP* genes. Finally, the prediction map of the *ZmNRAMP* gene's promoters was visualized using TB tools [46].

Chromosomal mapping

The phenogram web server (http://visualization.ritchielab.org/phenograms/plot) was used to predict the chromosomal position of the *ZmNRAMP* genes. Phenogram creates ideograms with different colors according to the exact part of genes [47].

Expression analysis in different tissues and under drought stress conditions

To analyze the expression pattern of *ZmNRAMP* genes in different tissues [leaf, internodes, root, reproductive tissues (tassel, silk, and cob), embryo, endosperm, and seed], we downloaded the RNA-seq data of accession number SRP014652 (NCBI Bio-project 171684; PRJNA171684) from MaizeMine (http://maizemine.rnet.missouri.edu:8080/maizemine/begin.do), a freely accessible transcriptome database [48,49]. We also downloaded RNA-seq profiles of the accession number SRP347418 (NCBI Bio-project PRJNA782891: GEO accession GSE189392) from NCBI-SRA (https://www.ncbi.nlm.nih.gov/sra) to compare the expression patterns of respective transporter genes in control (drip irrigation) and under drought stress (negative pressure irrigation) [50]. An index of the maize genome sequence was built using bowtie2, and paired-end clean reads were mapped to the maize genome [51]. The cufflinks program was used to evaluate the expression level of the annotated genes in the reference genome [52]. The FPKM values were used to construct a heatmap using Tbtools [53].

Functional gene ontology analysis

To analyze the *ZmNRAMP* potential candidate genes and their corresponding IDs, they were subjected to the ShinyGO v0.61 database (http://bioinformatics.sdstate.edu/go/) to obtain Gene Ontology (GO) annotation against maize. GO enrichment was calculated by the p-value cut-off (FDR) at 0.05 for the genes [54].

RESULTS

Identification and characterization of the maize *NRAMP* gene family

After confirming the sequences, a total of seven *NRAMP* genes were identified in the maize genome and named *ZmNRAMP1*, *ZmNRAMP2*, *ZmNRAMP3*, *ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, *ZmNRAMP7*. Table 1 shows the biochemical and physiological features of all proteins. The *NRAMP* genes in maize ranged from 265 (*ZmNRAMP2*) to 586 (*ZmNRAMP5*) amino acids. The molecular weight of the *NRAMP* proteins varies from 29.08 kDa (*ZmNRAMP2*) to 63.20 (*ZmNRAMP5*) kDa. Except for *ZmNRAMP1* and *ZmNRAMP3*, all the *NRAMP* proteins have a pI value greater than 8, indicating that five of the *NRAMP* proteins are basic proteins. All proteins have positive GRAVY values, indicating that they are hydrophobic. In maize, four (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, and *ZmNRAMP7*) proteins are expected to be subcellular distributed in the plasma membrane, with three (*ZmNRAMP1*, *ZmNRAMP2*, and *ZmNRAMP3*) proteins in the vacuole.

Phylogenetic analysis of the *NRAMP* gene family in maize

According to the findings of phylogenetic analysis, seven *ZmNRAMP* genes have been classified into two subclasses (Figure 1). The two subclasses are named groups A and B, respectively. The results revealed that group-A includes 3 (*ZmNRAMP1*, *ZmNRAMP2*, and *ZmNRAMP3*) genes. Group-B includes 4 (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, and *ZmNRAMP7*) genes. The subclass group-A and B of the *NRAMP* gene families of maize may be functionally similar to the *AtNRAMP2*, *AtNRAMP3*, *AtNRAMP4*, *AtNRAMP5* and *AtNRAMP1*, *AtNRAMP16* genes in Arabidopsis, respectively.

Analysis of the *NRAMP* gene family's gene structures and conserved motifs in maize

The number of introns in the *ZmNRAMP* gene family ranges from 1 (*ZmNRAMP2*) to 13 (*ZmNRAMP5*) in various cluster groups (Figure 2). The group-A *ZmNRAMP* proteins (*ZmNRAMP1*, *ZmNRAMP2*, and *ZmNRAMP3*) have 1 to 3 introns. The group-B *ZmNRAMP* proteins (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, and *ZmNRAMP7*) have 9 to 13 introns. According to exon-intron structures and phylogenetic analysis, members of the same group have similar gene structures.

Furthermore, a total of 10 motifs are identified and labeled as motifs 1 to 10 (Figure 3). The number of motifs in the *ZmNRAMP* family ranges from 6 (*ZmNRAMP2*) to 10 (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*). Among all *ZmNRAMP* proteins, only *ZmNRAMP4*, *ZmNRAMP5*, and *ZmNRAMP6* contain all 10 motifs. All the *ZmNRAMP* proteins have 6 (Motif 1, 3, 4, and 7, 8, 9) motifs. The group-A *ZmNRAMP* proteins (*ZmNRAMP1*, *ZmNRAMP2*, and *ZmNRAMP3*) have 6 to 9 motifs, and the group-B *ZmNRAMP* proteins (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, and *ZmNRAMP7*) have 9 to 10 motifs.

Analysis of three-dimensional modeling of *NRAMP* proteins in maize

The three-dimensional protein modeling reveals that all the *ZmNRAMP* proteins contain multiple alpha-helices, transmembrane helix, and coil structures (Figure 4). The percentage of the alpha-helix in the *ZmNRAMP* proteins varies from 69% (*ZmNRAMP3* and *ZmNRAMP7*) to 80% (*ZmNRAMP2*). The percentage of transmembrane helices in the *ZmNRAMP* proteins varies from 48% (*ZmNRAMP5*) to 53% (*ZmNRAMP3*). All the *ZmNRAMP* proteins have a 100% confidence level (Table S1).

Table 1: Identification and characterization of NRAMP gene family in maize

Transcript ID	Gene Name	Amino acid(aa)	Protein Molecular Weight(kDa)	pI	GRAVY	Subcellular Localization
Zm00001d030846	*ZmNRAMP1*	544	58.88	4.9	0.46	Vacuole
Zm00001d048129	*ZmNRAMP2*	265	29.08	9.05	0.52	Vacuole
Zm00001d033367	*ZmNRAMP3*	540	59.22	5.44	0.43	Vacuole
Zm00001d014391	*ZmNRAMP4*	550	59.52	8.26	0.57	Plasma membrane
Zm00001d005479	*ZmNRAMP5*	586	63.2	8.38	0.74	Plasma membrane
Zm00001d015133	*ZmNRAMP6*	551	58.94	8.01	0.58	Plasma membrane
Zm00001d019327	*ZmNRAMP7*	521	56.1	8.56	0.58	Plasma membrane

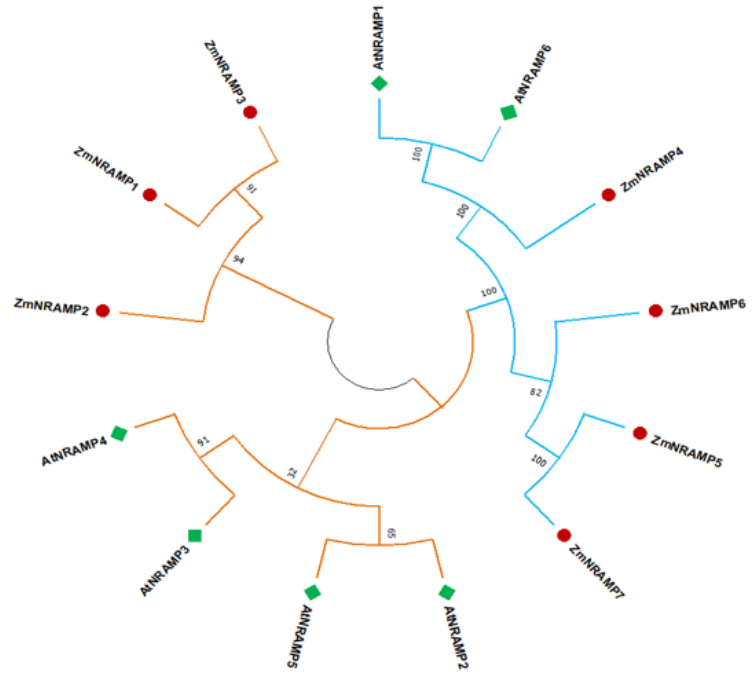

Figure 1: Analysis of phylogenetic tree of *NRAMP* gene family in maize. **Note:** (▭): Group A; (▭): Group B; (●): *ZmNRAMP*; (◆): *AtNRAMP*

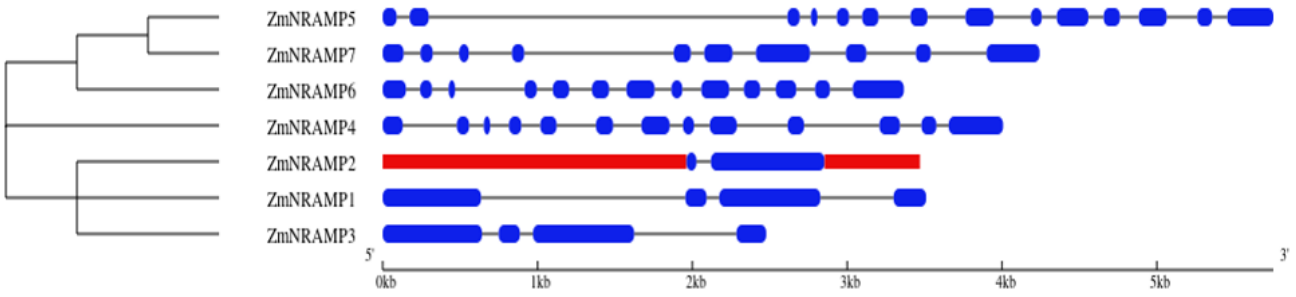

Figure 2: Analysis of exon-intron structure of NRAMP gene family in maize. **Note:** (▭): CDS; (▭): Upstream/downstream; (–): Intron

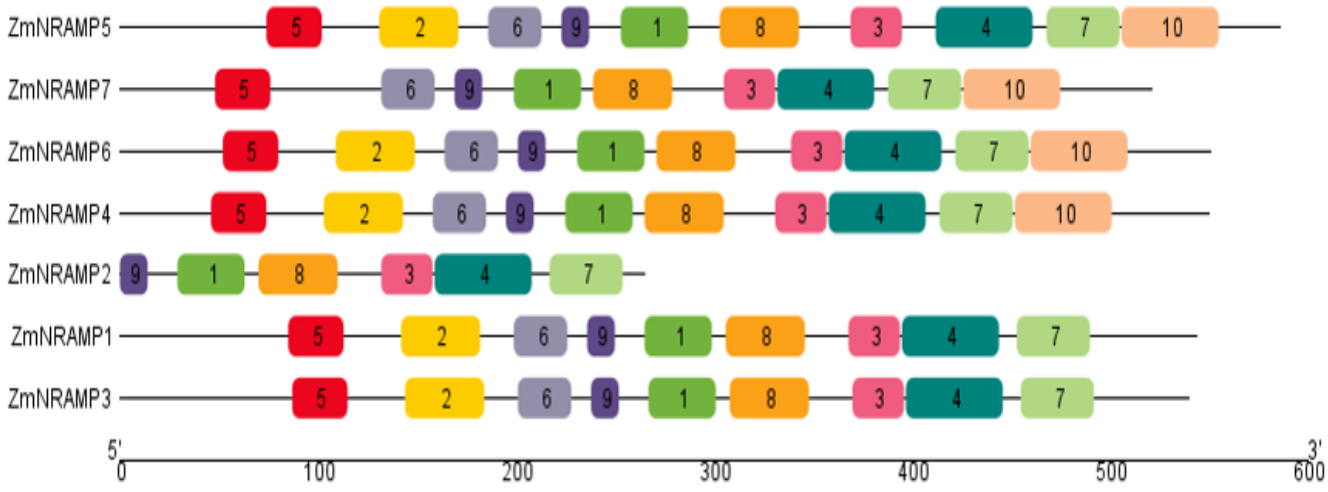

Figure 3: Analysis of conserved motif of *NRAMP* gene family in maize. **Note:** (▭): Motif 1; (▭): Motif 2; (▭): Motif 3; (▭): Motif 4; (▭): Motif 5; (▭): Motif 6; (▭): Motif 7; (▭): Motif 8; (▭): Motif 9; (▭): Motif 10

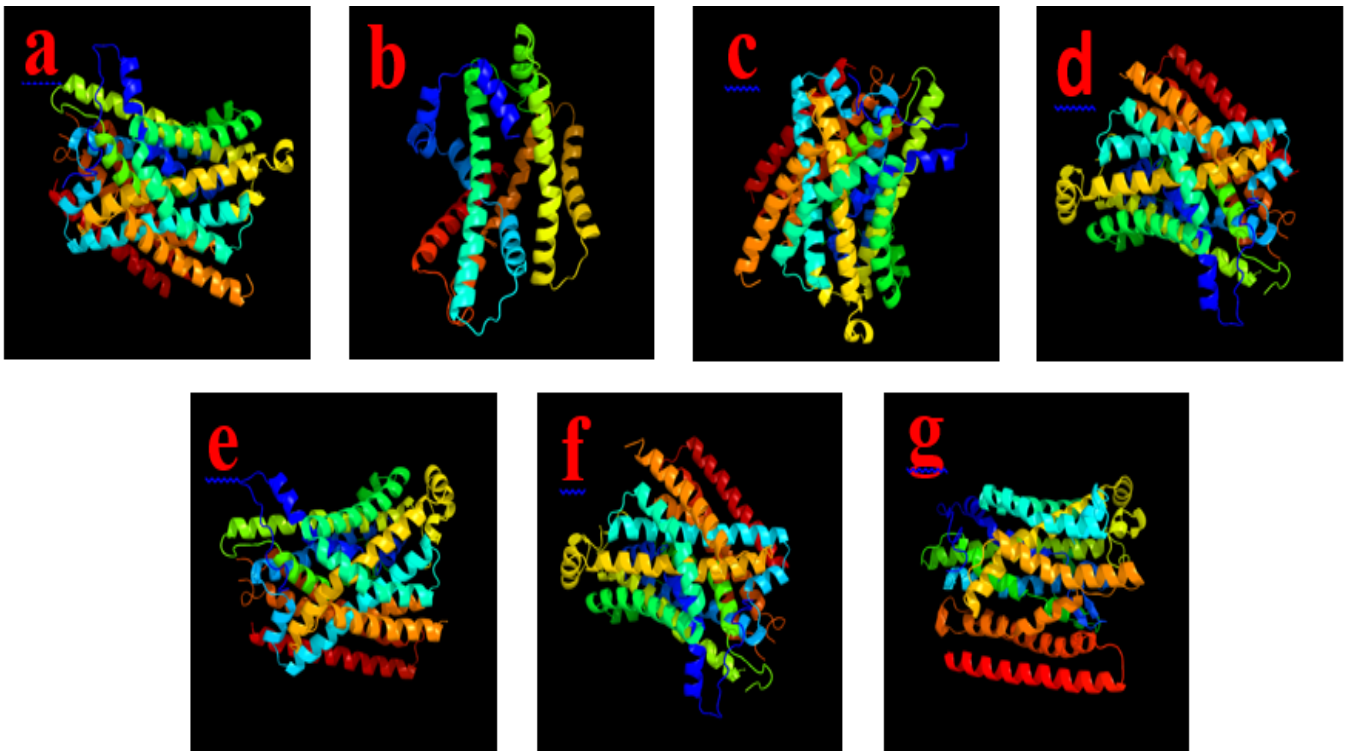

Figure 4: Analysis of three-dimensional protein structure of *NRAMP* gene family in maize. **Note:** a) *ZmNRAMP1*; b) *ZmNRAMP2*; c) *ZmNRAMP3*; d) *ZmNRAMP4*; e) *ZmNRAMP5*; f) *NRAMP6*; g) *NRAMP7*

Transmembrane domains of the *NRAMP* gene family in maize

As a consequence of the transmembrane domain analysis, multiple transmembrane helices were found in the *ZmNRAMP* gene family in maize. The *ZmNRAMP* proteins generally contain 1 to 12 transmembrane helices, and the intracellular sequences are extended from the N-terminus to the C-terminus (Figure 5). The four *ZmNRAMPs* (*ZmNRAMP3*, *ZmNRAMP4*, *ZmNRAMP5*, and *ZmNRAMP6*) contain all (12) the number of transmembrane helices. In the *ZmNRAMP* gene family, transmembrane domain length ranges from 265 (*ZmNRAMP2*) to 586 (*ZmNRAMP5*) and the number of transmembrane domains ranges from 6 to 12 (Table S2).

Analysis of cis-regulatory elements in the promoters of the *NRAMP* gene family in maize

The identification of putative cis-elements in promoter sequences is necessary for understanding gene regulation and function. To investigate the cis-elements of the *ZmNRAMP* genes, 2000-bp of the upstream sequence from the start codon was analyzed. The cis-elements of the *ZmNRAMP* genes were classified into three categories: plant growth and development, phytohormone responsiveness, and stress responses. The promoters of *ZmNRAMP* genes comprise three types of cis-elements. In the first category (related to growth and development), the elements included meristem expression (CAT-box), endosperm expression (GCN4_motif), seed-specific regulation (RY-element), and zein metabolism regulation (O_2-site). In the second category, five types of phytohormone-responsive cis-elements were detected, auxin-responsive (TGA-element and AuxRE), Abscisic Acid-Responsive (ABRE), methyl jasmonate-responsive (CGTCA-motif and TGACG-motif), Gibberellin-Responsiveness (P-box, TATC-box, and GRE), and salicylic acid-responsive (TCA-element). In the third category (stress-responsive), the elements included MYB-binding sites involved in drought inducibility (MBS), Low-Temperature-Responsiveness (LTR), as well as defense and stress responsiveness (TC-rich repeats). All of the *ZmNRAMP* gene promoter sequences contained members of the three cis-element categories, with numbers ranging from 10 (*ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, and *ZmNRAMP7*) to 16 (*ZmNRAMP1* and *ZmNRAMP3*), and the proportions of hormone-responsive elements were significantly greater. For example, *ZmNRAMP1* and *ZmNRAMP3* contained a total of 16 cis-elements. Among them, *ZmNRAMP1* had 11 hormone-responsive cis-elements, and *ZmNRAMP3* had 12 hormone-responsive cis-elements, (Figure 6, Table S3).

Chromosome mapping of maize *NRAMP* genes

The chromosomal mapping phenogram of seven *ZmNRAMP* genes revealed that all the genes were widely distributed on different chromosomes of maize. The phenogram detected the gene's position from the chromosomal annotations. According to the combination of phylogenetic analysis and chromosomal mapping, chromosomes 1 and 9 contained the group A genes, and chromosomes 2, 5, and 7 included all the genes from group B. Chromosome 1 carried the *ZmNRAMP1* and *ZmNRAMP3* genes, chromosome 2 carried the *ZmNRAMP5* gene, chromosome 5 carried the *ZmNRAMP4* and *ZmNRAMP6* genes, and chromosome 7 and 9 carried the *ZmNRAMP7* and *ZmNRAMP2* genes, respectively (Figure 7).

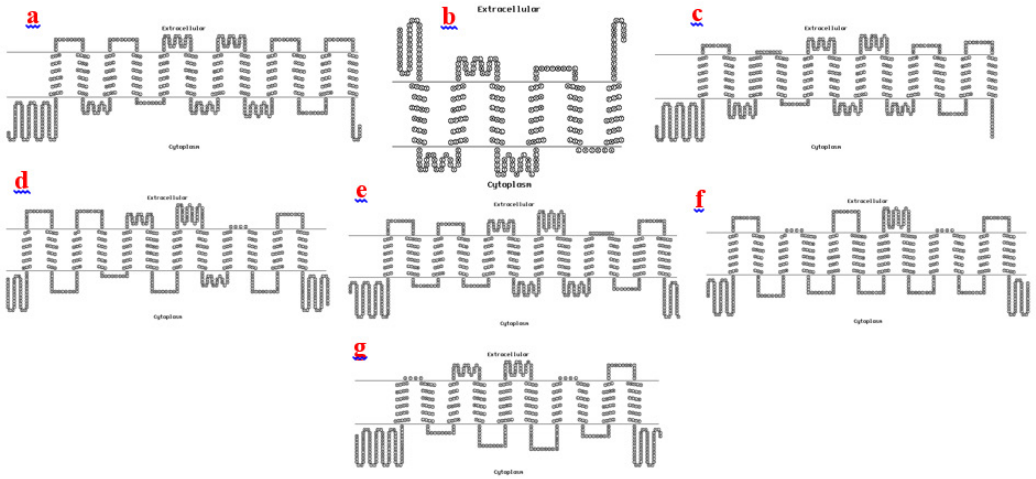

Figure 5: Analysis of transmembrane topology of *NRAMP* gene family in maize. **Note:** a) *ZmNRAMP1*; b) *ZmNRAMP2*; c) *ZmNRAMP3*; d) *ZmNRAMP4*; e) *ZmNRAMP5*; f) *NRAMP6*; g) *NRAMP7*

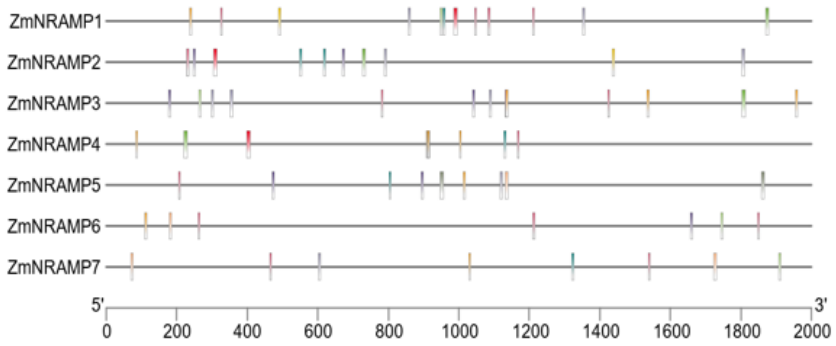

Figure 6: The cis-elements of *NRAMP* genes in maize. **Note:** ( ) Salicylic acid responsive; ( ) Endosperm specific; ( ) MeJa responsive; ( ) Low temperature responsive; ( ) Zein metabolism; ( ) Auxin responsive; ( ) Drough inducibility; ( ) Abscisic acid responsive; ( ) Meristem expression; ( ) Gibberellin-responsive; ( ) Defense and stress responsive

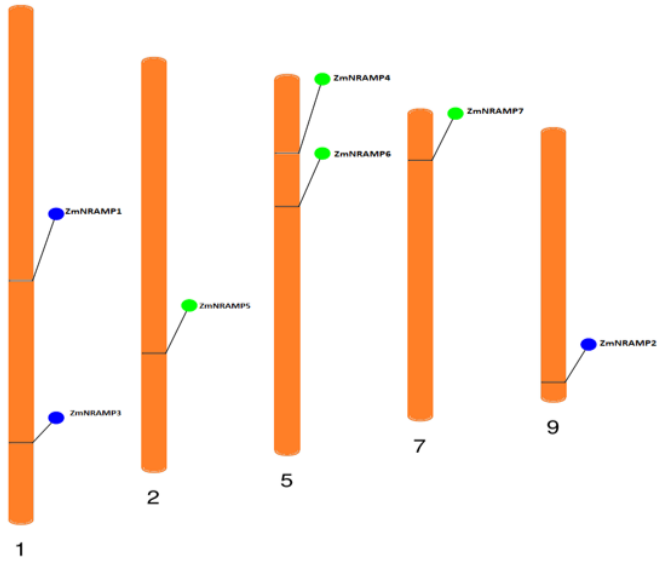

Figure 7: Chromosomal mapping of *NRAMP* genes. **Note:** (●) Group 1; (●): Group 2

Expression analysis of maize *NRAMP* genes in different tissues and under drought stress conditions

The expression patterns of *ZmNRAMPs* were studied utilizing RNA-seq data acquired from specific tissues at various developmental stages from an online database. The normalized log2 (FPKM) value was displayed on the heat map (Figure 8). The *ZmNRAMPs* were classified into two categories based on their expression patterns. In the first cluster, three genes (*ZmNRAMP1, ZmNRAMP2,* and *ZmNRAMP4*) had a reasonably high expression level and were stable in practically all tissues at various developmental stages. In the second cluster, two genes, *ZmNRAMP3* and *ZmNRAMP5*, were expressed at low levels in most tissues, except the leaf and anthers. The remaining two genes in the second cluster (*ZmNRAMP6* and *ZmNRAMP7*) showed a wide range of expression patterns. Furthermore, some genes were highly expressed in multiple tissues, such as leaves, and internodes, roots, reproductive tissues, and seeds. For example, *ZmNRAMP2* and *ZmNRAMP4* were highly expressed in leaves, internodes, and roots and moderately expressed in seeds and reproductive tissues. On the other hand, the *ZmNRAMP7* showed relatively high expression in leaves and roots (Figure 8A).

During drought stress, the *ZmNRAMP1, ZmNRAMP2, ZmNRAMP4,* and *ZmNRAMP7* genes were significantly upregulated in the leaves of maize. Among the *ZmNRAMP* family members, the transcript of the *ZmNRAMP4* gene was highly upregulated, and *ZmNRAMP1, ZmNRAMP2* and *ZmNRAMP7* were moderately upregulated in the leaves of maize under drought stress, which may be due to their significant role in drought tolerance or acclimatization during drought stress in maize (Figure 8B).

Functional gene ontology analysis of maize *NRAMP* genes

The functional ontology of *ZmNRAMP* genes was predicted by the ShinyGO database, which showed the involvement of these genes in various molecular functions and biological processes. These genes showed significant molecular functions and biological processes, including cadmium, manganese, and aluminum transmembrane transporter activity; metal and inorganic cation transporter activity (Figure 9).

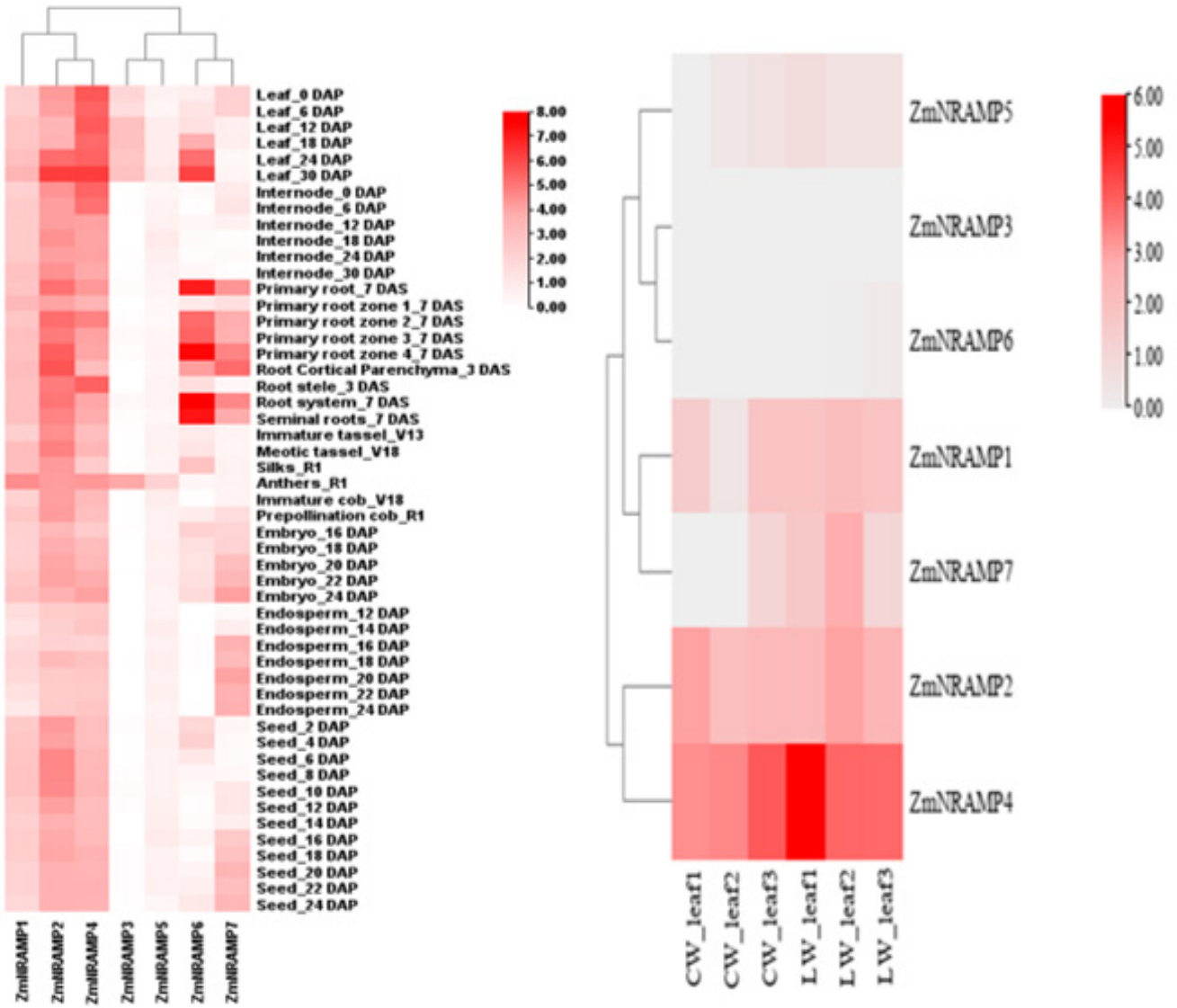

Figure 8: Analysis of transcript expression in different tissue specific region. **Note:** (A) and under drought stress condition (B) in maize *NRAMP* genes.

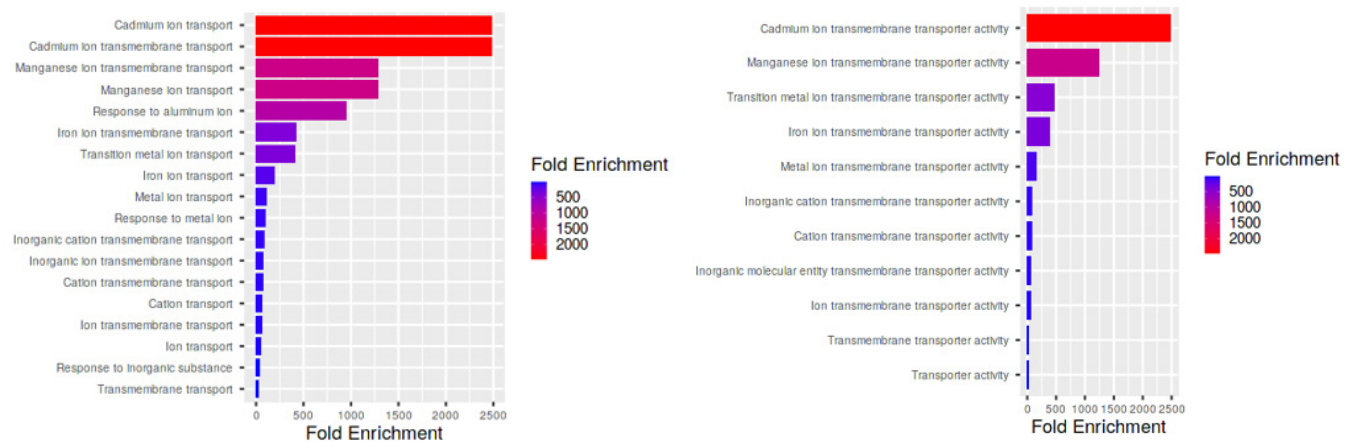

Figure 9: Gene ontology analysis of *NRAMP* gene family in maize. **Note:** (A) The biological process of *Zm*NRAMP genes. (B) The molecular function *Zm*NRAMP genes.

DISCUSSION

Natural Resistance-Associated Macrophage Proteins (*NRAMPs*) are a prominent gene family in plants that play a significant role in the absorption and transport of heavy metals such as Iron (Fe), Copper (Cu), Zinc (Zn), Lead (Pb), Cadmium (Cd), and Manganese (Mn). The *NRAMPs* also show a crucial impact in response to heavy metal stress. There is an abundance of research that has been carried out on the *NRAMP* gene family and identified 48 members from 6 species, including *Solanum tuberosum*, *Arabidopsis thaliana*, *Solanum Lycopersicum*, *Capsicum annuum*, *Oryza sativa*, and *Nicotiana attenuate* [55]. In the current study, we identified *ZmNRAMP* family proteins from the maize genome database based on the conserved domain of identified *AtNRAMP* family proteins and assessed their functional and structural properties. The phylogenetic analysis of the current study indicated that the *NRAMP* gene families in maize and Arabidopsis are classified into two groups based on their identified protein sequences. They are named groups A and B. The exon-intron structure analysis of a specific plant genome indicates a close evolutionary similarity compared to another plant genome [56]. Exons and introns are integral structural components and play a significant impact on regulatory gene function [57]. Indeed, the structural variations of exon-introns, transmembrane domains, and motif compositions are significantly conserved within the same subfamilies [58]. However, functional diversity is the consequence of the structural changes of exons and introns [59]. The exon-intron structure analysis of our study revealed that *ZmNRAMPs* have 1 (*ZmNRAMP2*) to 13 (*ZmNRAMP5*) introns. Therefore, *ZmNRAMP5* has the highest possibility of functional diversity, whereas *ZmNRAMP2* has the least during maize genome evolution. The results of the conserved motif study, on the other hand, revealed that the number of motifs within sub-groups is nearly identical, and some amounts of motifs may have changed due to early evolution. The frequent mutations and deletions of conserved motifs are the result of functional alteration in plants [43]. In this current study, the gene structures and motif patterns of *ZmNRAMPs* are almost similar when compared to *AtNRAMPs* except for some variations. The consistent gene structures and motif patterns in maize and Arabidopsis genomes may share the same regulatory characteristics. However, some variation in gene structures and motif patterns among the members of *ZmNRAMPs* may be due to gene duplication and different evolutionary origins during the evolutionary process of maize genomes. The three-dimensional protein structures that consist of multiple alpha-helices reveal a protein's evolutionary history and functional efficiency. The consistent transmembrane helices indicate that the relevant proteins are functionally active [43,48]. The analyzed three-dimensional protein structures of *ZmNRAMP* proteins contain multiple alphas and transmembrane helices. Consequently, the protein structures of *ZmNRAMP* and *AtNRAMPs* from different groups (A and B) are nearly identical, and this is the identification of functional activation.

The previous study suggested that the *NRAMP* proteins generally contained around 500 amino acid residues and 10–12 transmembrane domains in different species. In rice, the *OsNRAMP* proteins contain 10–12 transmembrane domains and 518–550 amino acid residues [60]. The *PvNRAMP* proteins in beans consist of 12 transmembrane domains and the amino acid length ranges from 507 to 554 [25]. Similarly, the *CsNRAMP* proteins in tea plants contain 3–12 transmembrane domains, and the length of amino acids ranged from 279 to 1373 [26]. However, in this current study, the identified *ZmNRAMP* proteins contain 6–12 transmembrane domains and the length of amino acids ranges from 265 to 586. The transmembrane domain structure analysis assesses the core membrane protein structure and overall topological characteristics. The impact of gene expressions and structural variations in plants was also evaluated through a transmembrane domain study [61]. According to the position of the N/C-terminal and the number of predicted transmembrane domains, the transmembrane domain structure could be divided into cytoplasmic, extracellular, and both cytoplasmic and extracellular N/C terminals. Among all the *ZmNRAMPs*, cytoplasmic N/C terminals were attributed to 10–12 TMs, which included *ZmNRAMP1*, *ZmNRAMP3*, *ZmNRAMP4*, *ZmNRAMP5*, *ZmNRAMP6*, *ZmNRAMP7*; only *ZmNRAMP2* proteins were predicted in extracellular N/C terminals and showed 6 TMs; there were no both cytoplasmic and extracellular N/C terminal TMs. According to the phylogenetic tree analysis of maize and Arabidopsis *NRAMP* family members, they are phylogenetically related and may share common functional properties. Similarly, the comprehensive investigation into subcellular localization, exon-intron structure, conserved motifs, 3-D protein structure, a transmembrane domain, and cis-regulatory elements of promoter

characteristics of maize and Arabidopsis showed similar structural properties. As a result, this current study estimated that the *ZmNRAMP* family proteins may have the same functional properties as Arabidopsis.

Our research has identified many growth and development, phytohormone, and stress-responsive cis-acting elements in the promoter region of *ZmNRAMP* genes. In addition, cis-acting components regulate the expression of stress-inducible genes, which helps them control significant biological processes [62]. The promoters of *ZmNRAMP* genes contain endosperm, seed, and meristem-specific cis-acting elements that lead to growth and developmental processes and control multiple stress signals. This is consistent with the essential functions of *NRAMPs* [63].

The expression patterns were performed to evaluate the probable functions of the *ZmNRAMPs* in maize growth and development. In contrast, *ZmNRAMP* genes were expressed at different levels in various tissues. Three genes (*ZmNRAMP1, ZmNRAMP2,* and *ZmNRAMP4*) in the *ZmNRAMP* gene family showed relatively high expression in leaves, internodes, and root zone. Indeed, *ZmNRAMP2* from the first cluster was highly expressed in maize leaves and roots, and *ZmNRAMP4* was highly expressed in maize leaves. Additionally, we have discovered the greater relative expression of *ZmNRAMPs* in leaves, internodes, and roots than in other maize tissues. Therefore, the greater expression patterns of *ZmNRAMPs* may play a significant role in the absorption, transport, and homeostasis of heavy metals. Interestingly, four *ZmNRAMP* genes (*ZmNRAMP1, ZmNRAMP2, ZmNRAMP4,* and *ZmNRAMP7*) were significantly upregulated in the leaves of maize during drought stress conditions. In particular, the *ZmNRAMP4* gene was highly upregulated in maize leaves. Significant upregulation of transcripts of the above genes may be a consequence of their essential role in drought tolerance or acclimatization in maize. In general, the expression pattern of *ZmNRAMPs* in distinct tissues and drought stress showed their critical role in other organ development and controlling drought stress conditions in maize. This current study also identified the functional annotation of gene ontology, including molecular functions and biological processes. Gene ontology annotation revealed that *ZmNRAMP* proteins play a crucial role in cadmium, manganese, and aluminum transmembrane transporter activity. The *ZmNRAMPs* also play a role in metal and inorganic cation transporter activity.

CONCLUSION

In summary, this is the first compr`ehensive and systematic investigation of the *NRAMP* gene family in maize. We identified seven *NRAMP* genes from the maize genome and systematically investigated the genome-wide identification, chromosomal distribution, gene structure, conserved motifs, three-dimensional protein modeling, and transmembrane domain of *NRAMP* gene family members in maize. We subsequently constructed a phylogenetic tree. We also analyzed the cis-regulatory elements in the promoter region, chromosomal mapping, and gene ontology annotation of maize *NRAMP* genes. We further evaluated the expression patterns of *NRAMP* gene family members to determine their possible roles in the absorption and transport of heavy metals like Cd, Zn, Fe, and Mn in maize. Our research work will provide an inclusive framework for studying the maize *NRAMP* gene family. It will also contribute to the *ZmNRAMP's* functional analysis and its applications.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Characterization of three-dimensional modeling of the *ZmNRAMP* gene family in maize; Supplementary Table S2. Prediction of the transmembrane domain region of the *ZmNRAMP* gene family in maize; Supplementary Table S3. The numbers of different cis-elements in the *ZmNRAMP* genes.

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AUTHOR'S CONTRIBUTIONS

Md. Numan Islam and Md. Golam Rabby designed this study. Md. Golam Rabby and Md. Sakib Hasan performed comprehensive analyses. Md. Numan Islam, Md. Mostafa Kamal and Md. Mossabbir Hossain wrote and revised the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The readers interested in using the data may contact the corresponding author.

DECLARATIONS

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

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