Research Article

Characterization of Drought Responsive Genes of CIPK Families in Rice, Maize and Sorghum

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Abstract

The CIPK gene family plays a key role in plant development and in stress signal transduction. The orthologs for experimentally proven drought stress responsive CIPKs from rice was identified in maize and sorghum. A total of 49 genes from the three species were analyzed for their phylogenetic relationship, gene structure, expression level and tissue specificity upon drought stress. The drought stress tolerance specificity of intronless group of CIPKs and multi-stress responsive nature of intron rich CIPKs were identified. The group level characterization revealed the similarity in function among Group I and Group II (A) CIPKs as the number and distribution of motifs were similar. Functional similarity of the genes was analyzed by *in-silico* expression analysis using publicly available data and which confirmed the drought responsiveness of 31 genes as they had similar expression level and it also shows the conservation of functions between species.

Keywords

CIPK, Drought, Stress, Intron, Protein Kinase

Introduction

Climate change induced by emission of green house gas can cause severe drought [1,2]. The higher plants adopt numerous mechanisms to cope with drought. The role of protein kinase gene family in drought stress response was revealed by many genome-wide gene expression profiling studies and pointed that the drought stress response given by them are efficient, fast-acting and reversible [3-5] responses to many environmental stresses such as salinity, cold and drought [6]. The changes in concentration levels of Ca^{2+} are recognized by several Ca^{2+} binding proteins including calmodulin (CaM), calmodulin like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs) and results in downstream responses [7,8]. CDPKs are exceptional in this category as they have a kinase domain and other three Ca^{2+} sensors had no enzymatic domains. Except CDPKs, other Ca^{2+} sensors interact with their respective target proteins and modulate their activity [8]. Whereas, CDPKs serves as special sensor as they directly initiate the downstream phosphorylation events up on Ca^{2+} binding due to the presence of CaM like and protein kinase domains [9]. The target protein of CBLs is referred to as CBL-interaction protein kinases (CIPKs) [7] and is also known as SnRK3. CIPK proteins consist of a conserved N-terminal kinase domain followed by junction domain and C-terminal regulatory domain. The Ca^{2+} bound CBL proteins interact with target protein CIPK through a conserved NAF/FISL motif at the C terminal regulatory domain of CIPK and activate its catalytic activity [10]. Total of 33 CIPKs was iden-tified in rice through bioinformatics analysis [11,12], 43 CIPKs are identified in maize [13] and 32 CIPKs are identified in sorghum [14]. CIPKs are reported to be expressed in response to various stresses. Over expression of OsCIPK23 improved drought tolerance in rice [15]. In *Arabidopsis* AtCIPK24 and AtCIPK7 contribute to salt and cold stress [16,17]. A cotton CIPK gene GhCIPK6 was over expressed in *Arabidopsis* and found that the tolerance of the plant increased in drought stress [18].

The present study intended to expose the characteristics of potential drought responsive ortholog genes in maize and sorghum by comparative analysis of them with the experimentally proven drought responsive genes of rice. The drought responsive rice genes of CIPKs (CIPK1, CIPK2, CIPK5, CIPK9, CIPK11, CIPK12, CIPK15, CIPK17, CIPK20, CIPK21, CIPK22, CIPK23, CIPK24, CIPK29 and CIPK 30) [19] were selected for the study as they have experimental evidences.

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Materials and Methods

Datasets

Genomic, CDS and protein sequences of rice genes were retrieved from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu). Ensembl Plants (http://plants.ensembl.org) and NCBI (https://www.ncbi.nlm.nih.gov) were used for retrieving maize and sorghum gene sequences. The retrieved protein sequences of rice were subjected to a BLASTp (https://blast.ncbi.nlm.nih.gov) analysis to find the orthologous genes in maize and sorghum by means of reciprocal best hit approach. The protein sequences which showed identity ≥ 75% were considered as orthologous. The orthologous genes for CIPK 20, CIPK 22, CIPK 29 and CIPK 30 were not identified in sorghum and maize. Therefore a total of 49 protein sequences were analyzed further which included the homologues sequences of rice in sorghum and maize.

Phylogenetic analysis

The multiple sequence alignment of 49 full length protein sequences of all the three species were constructed using CLUSTALW [20]. Phylogenetic tree was constructed by using Neighbor-Joining method by considering 1,000 rapid bootstrap replicates with the help of MEGA X [21] and it was visualized using iTOL (http://itol.embl.de). The aligned sequence file was also used for finding out the discrete Gamma distribution to recognize the evolutionary rate difference. Number of discrete categories used for the analysis was 5. Substitution pattern and rates were estimated under the Jones-Taylor-Thornton [22] model (+G) [22]. The tree topology was automatically computed in MEGA X for estimating ML values.

Characterization of phylogenetic groups

The gene structures of all the genes were predicted by aligning the coding sequence with its corresponding genomic sequence by using GSDS 2.0 server (http://gsds.cbi.pku.edu.cn). GSDS 2.0 is an improved version of GSDS and it supports two more widely used annotation formats, providing more comprehensive support for annotation files. To identify the conserved motifs in each group, the complete sequence of proteins at the groups were submitted to MEME suite (http://meme.sdsc.edu/meme/) [23]. This tool discovers the ungapped motifs in the sequence and splits the variable-length patterns in to more than two unique motifs. For the analysis we used the optimum width of motifs ranging from 12 to 60 by setting the search for 5 best motifs. The identified motifs were annotated by using Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) and InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search) [24]. The conserved domains were identified by Pfam [25]. Physiochemical properties of proteins were analyzed by using ProtParam tool available on ExPasy proteomics server (http://web.expasy.org/compute_pi) [26].

Gene expression analysis

To understand the function of the genes, expression pattern under drought stress was analyzed using GENEVESTIGATOR [27], which has a manually curated and well annotated database of expression data collected from variety of public repositories including Gene Expression Omnibus [28] and Array Express [29] (https://www.ebi.ac.uk/arrayexpress/). The gene expression at various development stages were observed for all the species. For rice, Affymetrix Rice Genome Array platform was used with water deficit microarray datasets GSE6901, GSE14275, GSE26280, E-MEXP-2401, GSE25176, GSE23211, GSE31077, GSE42683, GSE81253, GSE41647, GSE80246, GSE83378, and GSE57154. For maize, Affymetrix Maize Genome Array and mRNA- Seq Gene Level Zea Mays (ref: AGPV4) platforms were used with water deficit microarray datasets GSE16567, GSE43088 and GSE59533. In the case of sorghum the selected genes were analyzed for expression at various growing stage by using the Affymetrix Whole- transcriptome Sorghum Array platform with dataset GSE49879. The drought stress expression profile was analyzed using dataset GSE80699. The seven development stages in rice were considered for the analysis and they were seedling, tillering, stem elongation, booting, heading, flowering and milk stages. Three stages were considered in maize viz., seedling, stem elongation and anthesis. Five stages were included in sorghum analysis viz., seedling, stem elongation, booting, flowering and dough stages.

Results and Discussion

Analysis of protein properties

The nature of the proteins was analyzed by using physiochemical properties such as isoelectric point (pI), molecular weight, instability index and grand average of hydropathy (GRAVY). The pI value of the proteins ranged from 5.32 to 9.53 (Table 1). It shows that CIPK proteins have heterogeneous nature. Molecular weight of the proteins ranged from 40347.82 to 110358.1 Da. The variation in molecular weight among the members of the same group exists due to the variable number of domains contributing to protein size difference [30]. Except A0A1D6N844 all the other proteins exhibited hydrophilic nature since they had a negative GRAVY score [31]. Instability index showed that 26.5% proteins were unstable and 73.5% proteins were stable.
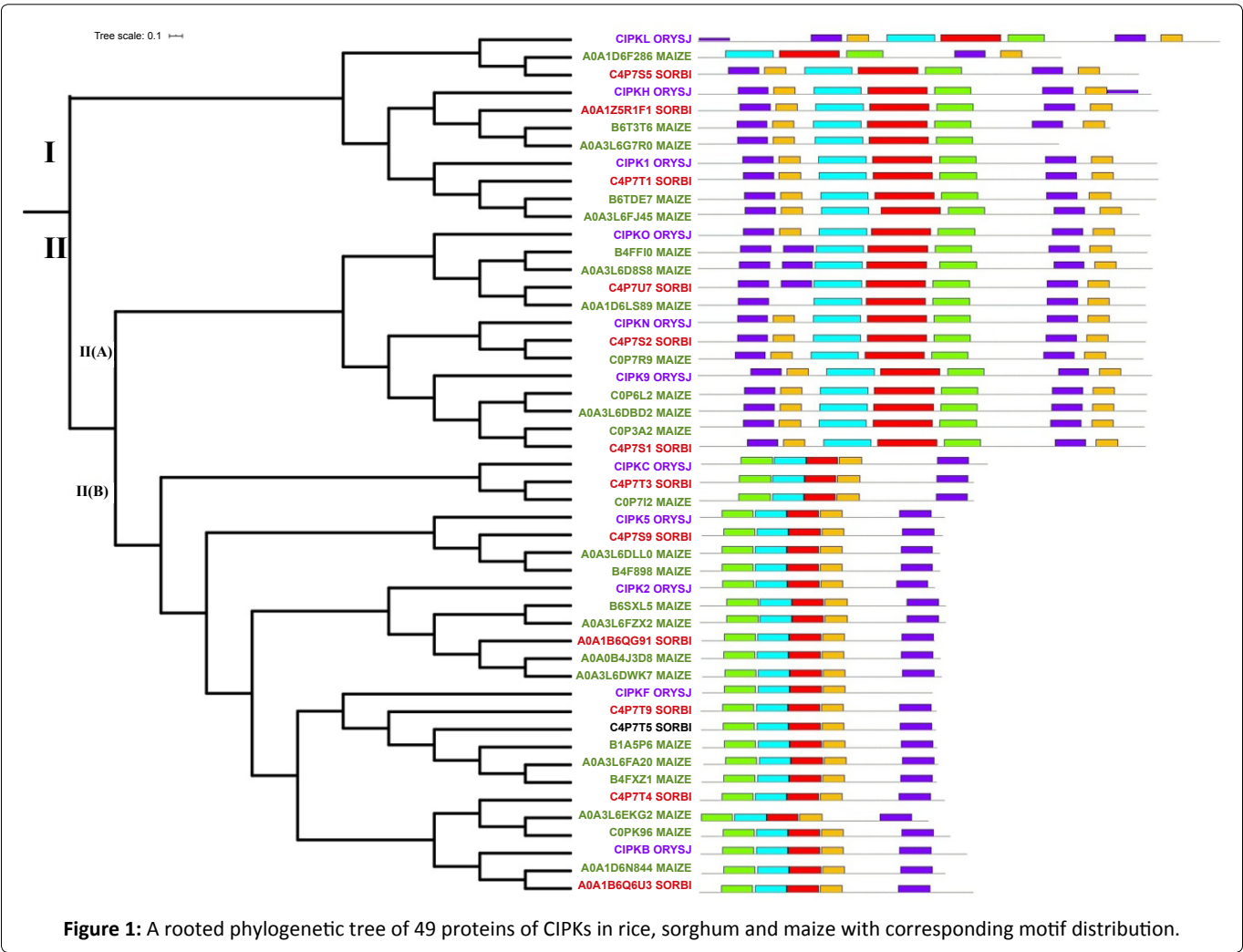
Phylogenetic analysis

To understand the evolutionary relationship of the drought responsive genes of CIPK gene family a rooted phylogenetic tree was constructed (Figure 1). Two major groups were identified from the analysis and the Group II had subdivision. Group I consisted of 3 members of rice, 5 members of maize and 3 members of sorghum. This group was found to be the smallest group. Group II (A) consisted of 3 members of rice, 7 members of maize and 3 members of sorghum. The largest group was Group II (B) which consisted total of 25 members; 5 from rice, 13 from maize and 7 from sorghum. The estimated value of the shape parameter for the discrete Gamma Distribution was 0.7910. Total of 5 categories were considered in the analysis of sites. Mean evolutionary rates in these categories were 0.07, 0.29, 0.63, 1.20, 2.81 substitutions per site. As the shape parameter is small most of the sites evolved very slowly in the evolutionary tree [32].

Table 1: Physiochemical properties of the selected proteins.

Sl. No	Protein ID	Protein name	Gene	pI	MW	GRAVY	I-I
1	Q0D4B2	CBL-interacting protein kinase 21	LOC_Os07g44290	9.26	59116.06	-0.427	43.15
2	A0A1D6F286	Non-specific serine/threonine protein kinase	Zm00001d006944	5.32	41113.77	-0.343	39.58
3	C4P7S5	Non-specific serine/threonine protein kinase	SORBI_3002G390100	8.79	50265.89	-0.351	31.8
4	Q75L42	CBL-interacting protein kinase 17	LOC_Os05g04550	6.93	50915.03	-0.395	34.14
5	A0A1Z5R1F1	Non-specific serine/threonine protein kinase	SORBI_3009G034200	6.33	51772.04	-0.351	39.14
6	B6T3T6	Non-specific serine/threonine protein kinase	Zm00001d024762	6.61	46241.68	-0.328	45.77
7	A0A3L6G7R0	Non-specific serine/threonine protein kinase	Zm00014a023444	5.84	40347.82	-0.328	41.12
8	Q9LGV5	CBL-interacting protein kinase 1	LOC_Os01g18800	6.26	52201.57	-0.403	38.1
9	C4P7T1	Non-specific serine/threonine protein kinase	SORBI_3003G139500	6.55	51915.56	-0.306	40.48
10	B6TDE7	Non-specific serine/threonine protein kinase	Zm00001d040567	7.2	51328.93	-0.298	39.79
11	A0A3L6FJ45	Non-specific serine/threonine protein kinase	Zm00014a022070	8.43	49785.37	-0.303	40.46
12	Q69Q47	CBL-interacting protein kinase 24	LOC_Os06g40370	8.52	50935.69	-0.202	35.67
13	B4FFI0	Non-specific serine/threonine protein kinase	Zm00001d000407	8.61	50809.36	-0.219	29.19
14	A0A3L6D8S8	Non-specific serine/threonine protein kinase	Zm00014a001662	8.64	51381.96	-0.219	29.78
15	C4P7U7	Non-specific serine/threonine protein kinase	SORBI_3010G186300	8.79	50415.05	-0.188	32.15
16	A0A1D6LS89	Non-specific serine/threonine protein kinase	Zm00001d036879	8.95	50798.5	-0.247	34.36
17	Q6ZLP5	CBL-interacting protein kinase 23	LOC_Os07g05620	9.23	50708.19	-0.408	38.92
18	C4P7S2	Non-specific serine/threonine protein kinase	SORBI_3002G034700	9.16	50702.31	-0.387	35.97
19	C0P7R9	Non-specific serine/threonine protein kinase	Zm00001d018799	9.16	50649.16	-0.402	35.73
20	Q10SC8	CBL-interacting protein kinase 9	LOC_Os03g03510	7.17	51033.5	-0.378	36.82
21	C0P6L2	Non-specific serine/threonine protein kinase	Zm00001d048460	7.18	50501.78	-0.414	32.05
22	A0A3L6DBD2	Non-specific serine/threonine protein kinase	Zm00014a016888	7.18	50529.79	-0.415	32.48
23	C0P3A2	Non-specific serine/threonine protein kinase	Zm00001d027455	7.26	50088.4	-0.388	36.62
24	C4P7S1	Non-specific serine/threonine protein kinase	SORBI_3001G523200	7.64	50280.61	-0.402	32.71
25	Q5JLS2	CBL-interacting protein kinase 12	LOC_Os01g55450	8.3	59807.67	-0.285	48.23
26	C4P7T3	Non-specific serine/threonine protein kinase	SORBI_3003G302800	8.93	56989.73	-0.26	43.4
27	C0P7I2	Non-specific serine/threonine protein kinase	Zm00001d043389	8.84	57268.12	-0.296	45.2
28	Q9LWM4	CBL-interacting protein kinase 5	LOC_Os01g10890	9.28	51965.79	-0.402	33.26
29	C4P7S9	Non-specific serine/threonine protein kinase	SORBI_3003G024400	9.26	50830.45	-0.338	36.61
30	A0A3L6DLL0	Non-specific serine/threonine protein kinase	Zm00014a022960	9.17	50985.68	-0.366	36.16
31	B4F898	Non-specific serine/threonine protein kinase	Zm00001d008901	9.17	50985.68	-0.366	36.16
32	Q7X996	CBL-interacting protein kinase 2	LOC_Os07g48100	9.18	50272.36	-0.421	28.7
33	B6SXL5	Non-specific serine/threonine protein kinase	Zm00001d007167	9.13	52709.9	-0.422	39.29
34	A0A3L6FZX2	Non-specific serine/threonine protein kinase	Zm00014a043100	9.13	52709.9	-0.422	39.29
35	A0A1B6QG91	Non-specific serine/threonine protein kinase	SORBI_3002G417300	9.22	49984.95	-0.401	29.67
36	A0A0B4J3D8	Non-specific serine/threonine protein kinase	Zm00001d022450	9.12	51349.25	-0.475	37.33
37	A0A3L6DWK7	Non-specific serine/threonine protein kinase	Zm00014a022344	9.12	51321.14	-0.488	35.5
38	Q2RBF0	CBL-interacting protein kinase 15	LOC_Os11g02240	9.53	49697.65	-0.398	36.91
39	C4P7T9	Non-specific serine/threonine protein kinase	SORBI_3008G032000	9.28	50721.66	-0.461	31.64
40	C4P7T5	Non-specific serine/threonine protein kinase	SORBI_3005G012000	9.33	50375.25	-0.423	34.81
41	B1A5P6	Non-specific serine/threonine protein kinase	Zm00001d052340	9.22	50000.8	-0.392	29.29
42	A0A3L6FA20	Non-specific serine/threonine protein kinase	Zm00014a039214	9.17	50087.83	-0.416	30.26
43	B4FXZ1	Non-specific serine/threonine protein kinase	Zm00001d052340	9.17	50087.83	-0.416	30.26

44	C4P7T4	Non-specific serine/threonine protein kinase	SORBI_3004G049500	9.19	52737.53	-0.484	39.47
45	A0A3L6EKG2	Non-specific serine/threonine protein kinase	Zm00014a013270	8.83	48888.8	-0.5	43.83
46	C0PK96	Non-specific serine/threonine protein kinase	Zm00001d015325	8.99	53633.23	-0.506	43.71
47	Q0JI49	CBL-interacting protein kinase 11	LOC_Os01g60910	8.48	56558.9	-0.448	43.73
48	A0A1D6N844	Non-specific serine/threonine protein kinase	Zm00001d043038	6.65	110358.1	0.068	40.82
49	A0A1B6Q6U3	Non-specific serine/threonine protein kinase	SORBI_3003G339700	7.59	58316.97	-0.474	44.18

Figure 1: A rooted phylogenetic tree of 49 proteins of CIPKs in rice, sorghum and maize with corresponding motif distribution.

Characterization of the Groups by gene structure analysis, motif analysis and protein properties analysis

The characterization of the phylogenetic groups was carried out by gene structure analysis which showed the divergence among the groups. Above 60% of the genes in Group I had ≥ 4 introns. All the sorghum genes belonged to this category (Figure 2). In rice and sorghum 40% and 20% genes were intronless respectively. All the genes in Group II (A) showed intron richness (≥ 4 introns). The Group II (B) dominated in intronless feature. In this category all the sorghum genes were intronless. In the case of rice, 80% of genes were intronless and 20% of genes belonged to the category of genes with single intron. Similarly, 84% of genes in maize were intronless and an equal distribution of genes (8% in each category) was present in ≥ 4 introns and 3 introns category. The loss or gain of introns could have played in grouping of CIPK genes. The variation in the number of introns among the three Groups points to the genome evolution by means of selection pressure and population size [33,34]. Moreover the intron rich behavior of the genes will add to the functional diversity through alternate splicing and exon shuffling [35]. Therefore the intron rich Group I and II (A) genes might be involved in multiple pathways in response to various abiotic stress signals. The intronless feature of Group II (B) CIPK genes strongly indicates that they are single stress inducible genes and possibly the stress is drought [36]. The significance of intron poor genes in drought tolerance was analyzed by Zhu, et al. [36], and proved its role in soybean CIPK-intron poor clade genes.

The conserved motifs and their organization were identified in each group (Figure 1). It was found that all the motifs
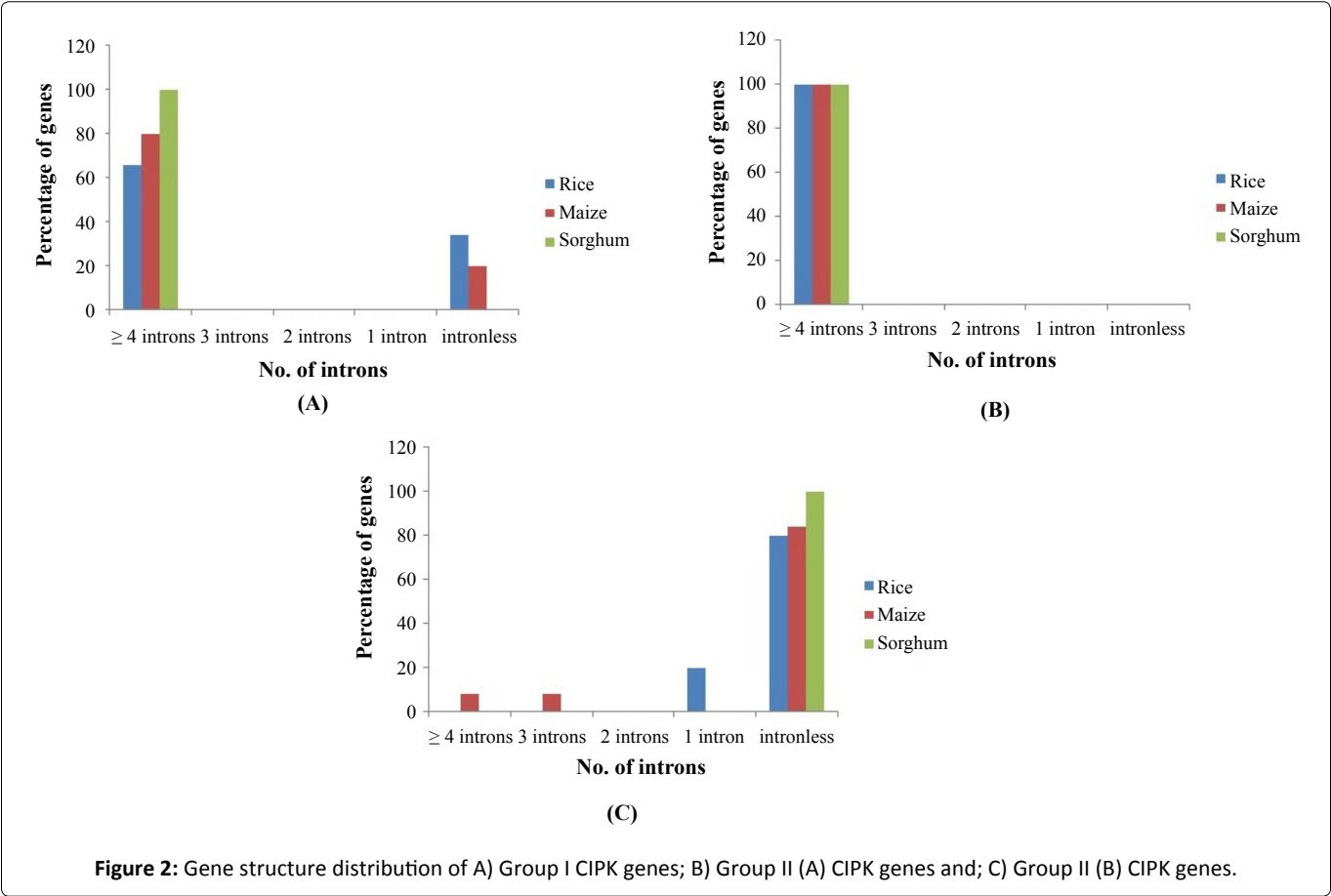
Figure 2: Gene structure distribution of A) Group I CIPK genes; B) Group II (A) CIPK genes and; C) Group II (B) CIPK genes.
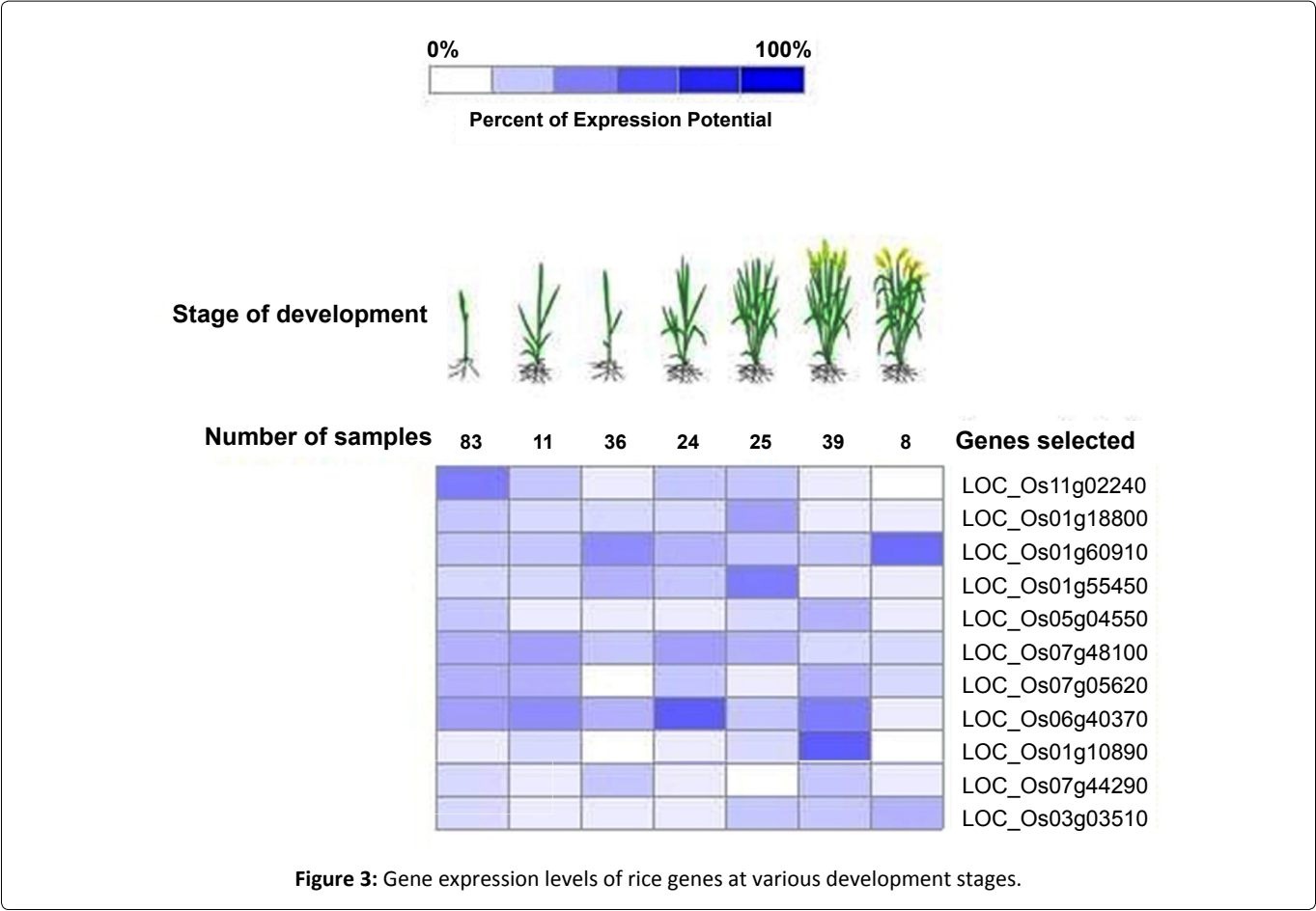

Figure 3: Gene expression levels of rice genes at various development stages.

were part of protein kinase domain. The Protein kinase domain which is conserved at the N-terminus included a protein kinase ATP binding site followed by serine/threonine protein kinase active site. Group I and Group II (A) had similarity in distribution of motifs which points to their similarity in function. In Group I number of motifs varied from 5 to 8 and in Group II (A) it was varied from 6-7. Meanwhile, in Group II (B) 5 motifs were reported and all of them were highly conserved among the members. Motif 1 was either present nearby N-terminus or in the N-terminus and it represented a protein kinase ATP binding site domain. These domains are glycine rich with lysine residue in vicinity and are located at N terminus [37].

Functional analysis by gene expression level

The CIPK genes play an important role in the growth and development of the plants [12,38]. Hence the expression levels at various development stages were analyzed. In rice, CIPK genes showed up regulated expression at all the stages except 4 genes, LOC_Os11g02240, LOC_Os07g05620, LOC_Os01g10890 and LOC_Os07g44290 at milk, stem elongation, milk and stem elongation and heading stages respectively as seen in Figure 3. It is also noted that they possessed high expression potential at other stages. The expression level in tissues of leaf, seedling, sheath, panicle, roots, pistil, caryopsis, flag leaf and anther were analyzed for all the genes and it was observed up regulated expression for 4 genes viz., LOC-Os11g02240, LOC-Os01g18800, LOC-Os07g48100 and LOC-Os06g40370 respectively in all the selected tissues as depicted in Table 2. Medium level of expression in all the tissues were reported for CIPK12 (LOC-Os01g55450).

Table 2: Tissue level expression of genes in rice, maize and sorghum.

Gene	Source	Expression level at tissues		
		High	Medium	Low
LOC_Os11g02240	Os	Leaf, seedling, sheath, panicle, roots, pistil, caryopsis, flag leaf, anther	-	-
LOC_Os01g18800	Os	Leaf, seedling, sheath, panicle, roots, pistil, caryopsis, flag leaf, anther	-	-
LOC_Os01g60910	Os	Caryopsis, panicle, leaf	Seedling, sheath, roots, pistil, flag leaf, anther	-
LOC_Os01g55450	Os	Flag leaf	Leaf, seedling, sheath, panicle, roots, pistil, caryopsis, anther	-
LOC_Os05g04550	Os	Pistil, leaf, seedling, flag leaf	Anther, root, sheath, caryopsis, panicle	-
LOC_Os07g48100	Os	Leaf, seedling, sheath, panicle, roots, pistil, caryopsis, flag leaf, anther	-	-
LOC_Os07g05620	Os	Seedling, sheath, roots, pistil, caryopsis, flag leaf, anther	Panicle	-
LOC_Os06g40370	Os	Leaf, seedling, sheath, panicle, roots, pistil, caryopsis, flag leaf, anther	-	-
LOC_Os01g10890	Os	Anther, pistil, leaf, flag leaf, seedling	Sheath, panicle, roots, caryopsis	-
LOC_Os07g44290	Os	Pistil, seedling, panicle, roots	Sheath, caryopsis, leaf, anther, flag leaf	-
LOC_Os03g03510	Os	Leaf, seedling, panicle, roots, pistil, caryopsis, flag leaf, anther	Sheath	-
Zm00001d040567	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d022450	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d008901	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d048460	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d015325	Zm	-	Foliar leaf, shoot, roots	-
Zm00001d043389	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d052340	Zm	Foliar leaf, shoot, roots	-	-
Zm00001d036879	Zm	-	Foliar leaf, shoot, roots	-
Zm00001d000407	Zm	-	Foliar leaf, shoot	-
SORBI_3002G034700	Sb	Rind, internode, shoot, pith, roots	Leaf	-
SORBI_3003G139500	Sb	Rind, internode, shoot, pith, leaf, roots		

SORBI_3003G024400	Sb	Leaf	Shoot	Rind, internode, pith, roots
SORBI_3001G523200	Sb	Rind, internode, shoot, pith, leaf, roots	-	-
SORBI_3003G339700	Sb	-	Rind, internode, shoot, pith, leaf, roots	-
SORBI_3004G049500	Sb	-	Rind, internode, shoot, pith, leaf, roots	-
SORBI_3003G302800	Sb	Rind, internode, shoot, pith, leaf	Roots	-
SORBI_3005G012000	Sb	Rind, leaf	Internode, shoot, pith, roots	-
SORBI_3008G032000	Sb	-	Rind, internode, shoot, pith, leaf	Roots
SORBI_3002G390100	Sb	Rind, internode, shoot, pith, leaf, roots	-	-
SORBI_3010G186300	Sb	Internode, shoot	Rind, pith, leaf, roots	-

*Os: Oryza sativa; Zm: Zea mays; Sb: Sorghum bicolor.

In maize, the gene expression analysis was carried out by observing the expression level at three development stages with respect to drought stress. All the 9 selected genes had up regulated expression at three stages of development. The tissues analyzed for expression level were foliar leaf, shoot and root. It was observed that genes Zm00001d015325 and Zm00001d036879 had medium level of expression in all the three tissues. The gene Zm00001d000407 had low level expression in roots and medium expression in foliar leaf and shoot.

The expression of 11 genes in sorghum was analyzed at various tissues and it was observed that the gene SORBI_3003G024400 was not expressed at dough stage. All the other genes exhibited potential for expression at all development stages. The tissues analyzed for expression were rind, internode, shoot, pith, leaf and roots. The genes SORBI_3003G139500, SORBI_3001G523200 and SORBI_3002G390100 showed high level of expression in all the selected tissues. Drought response of the 11 sorghum genes were analyzed manually by using the dataset GSE80699 and it was observed that 6 genes viz., SORBI_3003G139500, SORBI_3003G024400, SORBI_3001G523200, SORBI_3004G049500, SORBI_3003G302800 and SORBI_3010G186300 were up regulated and 5 genes viz., SORBI_3002G034700, SORBI_3003G339700, SORBI_3005G012000, SORBI_3008G032000 and SORBI_3002G390100 were down regulated during the treatment.

It was identified that above 80% of the genes in respective groups have shown medium or high level expression up on drought stress. It was also noted that all the genes had high or medium level expression at leaf and shoot part. This expression analysis indicates that these orthologous genes posses similar expression with respect to drought stress. The similar expression of orthologous genes are already reported by Kong, et al. [39].

Conclusion

The present study screened 49 genes and scrutinized 31 genes in rice, maize and sorghum for potential drought stress response based on functional analysis. The experimentally proved drought tolerant genes of CIPKs from rice and their orthologous genes in maize and sorghum were grouped by phy-

logenetic analysis. Maximum number of rice orthologs was found in maize. Comparative analysis of the gene structure showed that Group II (B) CIPK genes dominated intronless feature whereas, Group II (A) CDPK genes and Group I CIPK genes dominated intron rich feature. The intron richness indicates that the genes might have included in multiple stress signal transduction other than drought. The genes in Group II (B) are specifically induced in drought due to their intronless feature [36]. Alternate splicing and exon shuffling could be the reason for functional diversity among CIPK groups [35]. This also points to the adaptation of plants with respect to environmental changes during evolution which in turn altered their phenotypes significantly by transforming the form and function of genes [40]. The most common motifs seen among the groups were parts of protein kinase domain. The similar distribution of motifs in Group I and Group II (A) indicates the functional similarity of the groups. Gene expression analysis showed that above 80% of the genes in respective groups have shown medium or high level expression up on drought stress. The similarity in the expression pattern also shows their functional similarity [39] and conservation of functions between species.

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