Article

Comprehensive analysis of calcium sensor families, CBL and CIPK, in *Aeluropus littoralis* and its expression profile in response to salinity

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Abstract: Plants have acquired sets of highly regulated and complex signaling pathways to respond to unfavorable environmental conditions during evolution. Calcium signaling, as a vital mechanism, enables plants to respond to external stimuli, including abiotic and biotic stresses, and coordinate the basic processes of the growth and development. In the present study, the calcium sensor families, including CBL and CIPK, were investigated in the halophyte plant, *Aeluropus littoralis*, with a comprehensive analysis. Here, six *AlCBL* genes, and twenty *AlCIPK* genes were presented. The analysis of the gene structure and conserved motifs, as well as physicochemical properties, showed that these genes are highly conserved during evolution. The expression levels of *AlCBL* genes and *AlCIPK* genes were evaluated under salt stress in leaf and root tissue. Based on the real-time RT-PCR results, *AlCIPK* gene family had a higher variation in mRNA abundance compared to the *AlCBL* gene family. The *AlCIPKs* were found to have a higher abundance in leaves than in roots. The results suggest that the interaction pattern of *AlCBL* genes with *AlCIPK* is tissue-specific, and different interactions can be expected in leaves and roots. Based on these patterns AlCIPK3.1 - AlCBL4.1 and AlCIPK1.2 - AlCBL4.4 can interact in root tissue, while the AlCBL10 has the potential to interact with the AlCIPK5, AlCIPK26 and AlCIPK12.3 in the leaf tissue. These findings provide valuable information on the structure and function of calcium sensor families in *Aeluropus littoralis* a halophyte plant, for future research on the biological function of CBLs and CIPKs on salt stress resistance.

Keywords: Calcium sensors; CBL; CIPK; Salt stress; Kinases; Cell signaling

1. Introduction

In sessile organisms such as plants, perception and signaling of environmental stimuli is necessary for survival and growth regulation. Calcium (Ca^{2+}) is one of the signal transduction components that acts as a second messenger in all eukaryotes [1–3]. Ca^{2+} is stored in organelles such as vacuoles, mitochondria and endoplasmic reticulum, where abiotic stresses such as salt, cold and drought cause rapid increase of Ca^{2+} concentration in the cytosol [3–6]. However, biotic stresses, pH dynamics, and phytohormones also can affect the Ca^{2+} concentration [7–10]. In addition, pollen tube development and guard cell regulation are also associated with changes in Ca^{2+} concentration [8]. Calcium sensors or calcium-binding proteins recognize the modification in Ca2+ concentrations in plant cell,

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and downstream pathways are induced by affecting the phosphorylation status of calcium sensors and activating protein kinases [11,12]. Calmodulin (CaM), calcium-dependent protein kinases (CDPKs), and calcineurin B-like (CBLs) are part of the known calcium sensors in plants [13]. CBLs are plant-specific sensors that, after sensing a specific calcium signature, can physically interact with a protein kinases, CBL-interacting protein kinases (CIPKs), to activate downstream signaling components [14–16]. CBL proteins share a common helix-loop-helix structural motif (the EF-hand), where acts as Ca2+ binding region [17]. Besides, it seems that the EF-hand composition could affect the affinity rate of calcium ions [17].

In the plant model system Arabidopsis diverse roles were reported for CBLs: The *cbl1* mutant was very sensitive to the abiotic stresses such as drought, extreme salinity, and hyperosmotic stress. Likewise, *CBL9* gene is involved in ABA signal transduction and stress-induced ABA biosynthesis pathways [18]. In addition, it was reported that CBL9 and CBL1 are participated in pollen germination and flower fertilization [19]. Furthermore, it was stated that CBL1 is involved in response to aluminum stress [20], cold stress [21,22]. Moreover, CBL7 is associated with *Arabidopsis* responses to alkaline stress [23]. Interestingly, it was reported that CLBs, such as CBL3 and CBL4, could modulate the potassium channel and affect the potassium homeostasis [21,24]. It has also been found that the expression patterns of the *CBL* genes are dependent on the tissues and developmental stages and the type of stress. For example, *CBL1* expression is not affected by external application of abscisic acid (ABA), but is induced in response to environmental stresses such as salt, cold, drought, and wounding [25]. While *CBL2* and *CBL3* do not respond to abiotic stress stimuli, they are transcriptionally induced by light stress [26]. *CIPK* genes also have differential expression patterns. For example, *CIPK9* transcriptional regulation is more induced in response to ABA treatment, and is mainly activated in shoot tissues [27]. In addition, *CIPK* genes in *Medicago truncatula*, including *MtCIPK2*, *MtCIPK17*, and *MtCIPK18* were found to be upregulated in response to salinity, PEG and ABA treatments [28]. Recently, it has been reported that a *CIPK* gene from chrysanthemum, *CmCIPK8*, could affect the expression patterns of ion transport-related genes, and may enhance tolerance to salinity [29]. Moreover, *CIPK10* in potato (*StCIPK10*) could increase tolerance to osmotic and drought stress by affecting the content of osmoregulation substances [30]. Also, it was reported that StCIPK10 can interact with several StCBLs, including StCBL4, StCBL8, StCBL1, StCBL6, StCBL12, and StCBL11 [30]. In *Beta vulgaris* it was described that, *BvCIPKs*, are upregulated in response to NaCl treatment [31]. In *Saccharum spontaneum*, *CIPK* genes were shown to respond to abiotic stresses as cold and water stress, and ABA treatment [32]. Overall, it seems that cell signaling networks linked with CBL-CIPK play critical roles in response to abiotic stresses [33–35].

Aeluropus littoralis as a halophyte model can grow under high salt concentrations [36,37]. Identifying genes related to resistance in plants such as *A. littoralis*, as a valuable germplasm, and determining their function can provide a better understanding of resistance mechanisms in plants [38]. According to the mentioned materials above, the genes of the CBL and CIPK family play a key role in responding to environmental stresses and regulating downstream signaling pathways, but these gene families have not been identified and investigated in *A. littoralis*. Here, we identified the members of CBL and CIPK families and analyzed the structure and evolution as well as their regulatory systems. In addition, the expression profile of *AlCBL* and *AlCIPK* genes were evaluated under salinity in root and leaf tissues of *A. littoralis*.

2. Materials and Methods

2.1. Identification of CBL and CIPK family genes in A. littoralis

In this study, the putative protein sequences of CBL and CIPK in rice were retrieved from the RGAP database (http://rice.plantbiology.msu.edu/) and for *Arabidopsis thaliana* from TAIR database (https://www.arabidopsis.org/). Sequences were used as query in

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blastp and tblastn tool, E-value< $1e^{-10}$ to identify members of the *CBL* and *CIPK* gene families from the transcriptome platform e!DAL of *A. littoralis* [39].The presence of PKinase- and NAF-domains in CIPK proteins, as well as EF-hand domains in CBL proteins was tested and confirmed using the CDD database [40], SMART [41], and InterPro Scan [42]. The confirmed protein sequences were renamed based on their orthologs in *Arabidopsis*. Further AlCBL and AlCIP proteins were analyzed by ExPASy online database ProtParam tool [43] to predict the physiochemical properties including molecular weight (MW), GRAVY, and isoelectric point (pI).

2.2. Phylogenetic analysis and classification of AlCBL and AlCIPK gene family

To investigate the evolutionary relationships in the calcium sensor gene families, the protein sequences of AlCBL and AlCIPK families along with their orthologues in *Arabidopsis* and rice were analyzed. First, the sequences were aligned by the ClustalW tool [44] and then a phylogenetic tree was drawn by IQ tree software [45] using the maximum likelihood (ML) method with 1000 bootstrap replications. Finally, the tree file was restored and upgraded in the iTOL database [46].

2.3. Motif analysis and gene structure of AlCBLs and AlCIPKs

Ten conserved motifs into AlCBL and AlCIPK protein sequences were predicted using MEME motif finder [47] based on the default setting. Besides, the gene structure of *AlCBL* and *AlCIPK* genes was illustrated based on exon and intron distribution using Tbtools [48].

2.4. Plant materials growth conditions and salt treatments

Cultivation of the *A. littoralis* seeds was carried out at a temperature of 25 ± 3, a photoperiod of 16 hours of light and 8 hours of darkness. Then the cloned samples were transferred to Hoagland's solution and after two months, salt stress treatment was started. In order to apply salinity stress, sodium chloride was gradually added; 100 mM salt every 3 days was added to solution until the final concentration reached 600 mM. Sampling of leaf and root tissues was done in the time series of 0 (as a control sample), 3, 12, 24 hours after the application of salt stress. The collected samples were kept in a freezer at -80 for the next steps. All experiments were performed in three biological replications.

2.5. RNA extraction and cDNA synthesis

Extraction of total RNA from leaf and root tissues was done using the Trizol kit (Threezol, Riragene). To remove genomic DNA from RNA, DNase I treatment (DNase I RNase-free, Thermo Scientific) was applied. Finally, cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) based on the company's instructions, and diluted four times.

2.6. Real-time PCR

In the present study, the levels of mRNA abundance from six *AlCBL* and twelve *AlCIPK* genes were investigated in two tissues, roots and leaves, under salinity and normal conditions. Genes were selected based on phylogenetic analysis. Primers of candidate genes were designed using AlleleID [49] (Table S1 and S2). The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used to evaluate the relative expression based on manufacturer's instructionswith a Bio-Rad CFX96 machine. The temperature cycle was performed in two stages according to the manufacturer's instructions: 10 minutes activation stage at 95 ºC, 40 cycles at 95 ºC for 15 seconds, and 60 ºC for 1 minute. In the current study, three reference genes, including *AlUBQ*, *AlRPS3*, and *AlRPS3*, for each tissue were used. The geometric mean of these genes was used to normalize the data. Finally, the relative expression levels of each target gene was calculated using the $2^{-\Delta\Delta CT}$ method [50].

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3. Results

3.1. Physicochemical properties of AlCBLs and AlCIPKs

In the present study, six *AlCBL* genes and twenty *AlCIPK* genes are identified in the genome of *A. littoralis*. The evaluation of the physicochemical characteristics of CBL proteins revealed variable molecular weight in the range of 18.70 (AlCBL 4.4) to 34.67 kDa (AlCBL 10), and all AlCBLs were predicted as acidophilic proteins, pI less than 5.5 (Table 1). Furthermore, all AlCBLs (except for AlCBL 10 protein) had negative GRAVY value, revealing that most AlCBLs have hydrophilic properties. In general, based on the physicochemical characteristics, AlCBL 10 protein was different from other members of the AlCBL gene family, which can be more considered in molecular functional research. According to the physicochemical characteristics, AlCIPK family members showed more diversity than AlCBLs. Molecular weight in AlCIPKs ranged of 42.04 (AlCIPK 10.6) to 58.9 kDa (AlCIPK 10.1), and pI varied from 6.21 (AlCIPK 21) to 9.28 (AlCIPK 10.2).

Table 1. Physicochemical properties of identified AlCBLs and AlCIPKs encoded proteins from *A. littoralis.*

Family	Gene ID	Gene Name	Length (aa)	Intron number	MW (kDa)	pI	GRAVY
CBL	Alg14121	*AlCBL2*	226	7	25.87	4.98	-0.219
	Alg15558	*AlCBL4.1*	214	7	24.35	4.71	-0.196
	Alg11525	*AlCBL4.2*	213	7	24.33	4.94	-0.259
	Alg8494	*AlCBL4.3*	217	7	24.88	5.19	-0.299
	Alg13204	*AlCBL4. 4*	166	5	18.70	4.78	-0.341
	Alg5886	*AlCBL10*	303	8	34.67	5.28	0.133
CIPK	Alg4127	*AlCIPK1.1*	473	12	53.48	6.52	0.372
	Alg7902	*AlCIPK1.2*	454	11	50.69	6.62	-0.320
	Alg7566	*AlCIPK3.1*	442	13	50.76	7.64	-0.460
	Alg12052	*ALCIPK3.2*	448	13	50.63	8.23	-0.407
	Alg15044	*AlCIPK4*	427	0	46.34	8.59	-0.115
	Alg5583	*AlCIPK5*	450	0	48.19	-	0.054
	Alg12300	*AlCIPK10.1*	523	0	58.97	9.03	0.401
	Alg9524	*ALCIPK10.2*	438	0	49.72	9.28	-0.260
	Alg4701	*AlCIPK10.3*	421	0	47.98	9.03	0.400
	Alg3308	*AlCIPK10.4*	478	0	54.66	9.13	0.514
	Alg13906	*AlCIPK10.5*	410	1	45.99	8.93	-0.307
	ALg9805	*AlCIPK10.6*	383	1	42.04	8.99	0.480
	Alg2698	*AlCIPK11*	433	0	47.40	8.95	-0.151
	Alg8115	*AlCIPK12.1*	516	0	57.36	8.64	-0.341
	Alg10559	*AlCIPK12.2*	515	0	57.47	8.06	-0.374
	Alg11449	*AlCIPK12.3*	490	0	54.06	8.84	-0.254
	Alg11347	*AlCIPK20*	456	0	51.64	9.08	-0.422
	Alg8711	*AlCIPK21*	430	13	48.54	6.21	-0.303
	Alg1003	*AlCIPK23*	449	13	50.51	9.16	-0.371
	Alg7179	*AllCIPK26*	448	13	50.44	8.41	-0.395

3.2. Phylogenetic analysis of AlCBLs

AlCBL proteins along with their orthologues in rice and *Arabidopsis* were subjected to a phylogenetic analysis. Results disclosed that CBL proteins could be classified in four

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main groups (Figure 1). None of AlCBLs could be identified in group I. AlLAC 4.1, AlLAC 4.2, AlLAC 4.3, and AlLAC 4.4 were located in group II, AlLAC 10 in group III, and AlLAC 2 in group IV. In addition, AlCBLs and rice CBLs showed more similarity to each other that *Arabidopsis* CBLs. Overall, our results propose that the diversity in CBL family has been occurred after the splitting of monocots and dicots.

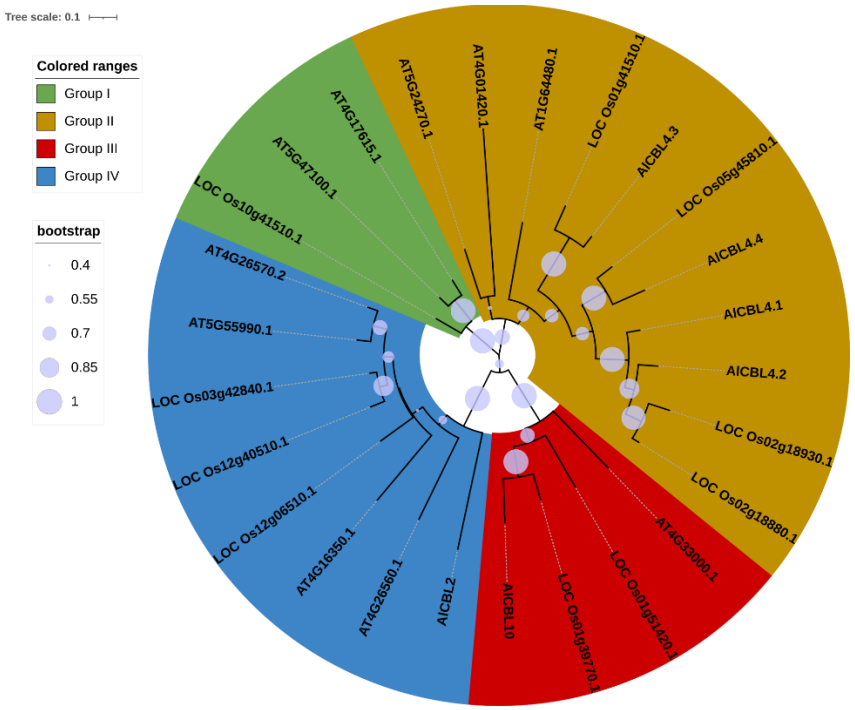

Figure 1- Phylogeny tree of CBL family proteins in *A. littoralis* (AlCBL), *O. sativa* (started with LOC Os), and *A. thalianas* (started with AT).

3.3. Phylogenetic analysis of AlCIPKs

To determine the evolutionary origin of AlCIPKs, the phylogenetic tree of AlCIPKs with their orthologues in *Arabidopsis* (26 CIPK proteins) and rice (33 CIPK proteins) was made based on protein sequences (Figure 2). Results illustrated that CIPKs could be separated in four main groups. The highest number of CIPKs were found in group III, and the lowest number were observed in group IV. Similar to AlCBLs, AlCIPKs also showed a close relationship with rice CIPKs. In addition, it can be concluded that probably the expansion in the CIPK family has been occurred after the derivation of monocots and dicots.

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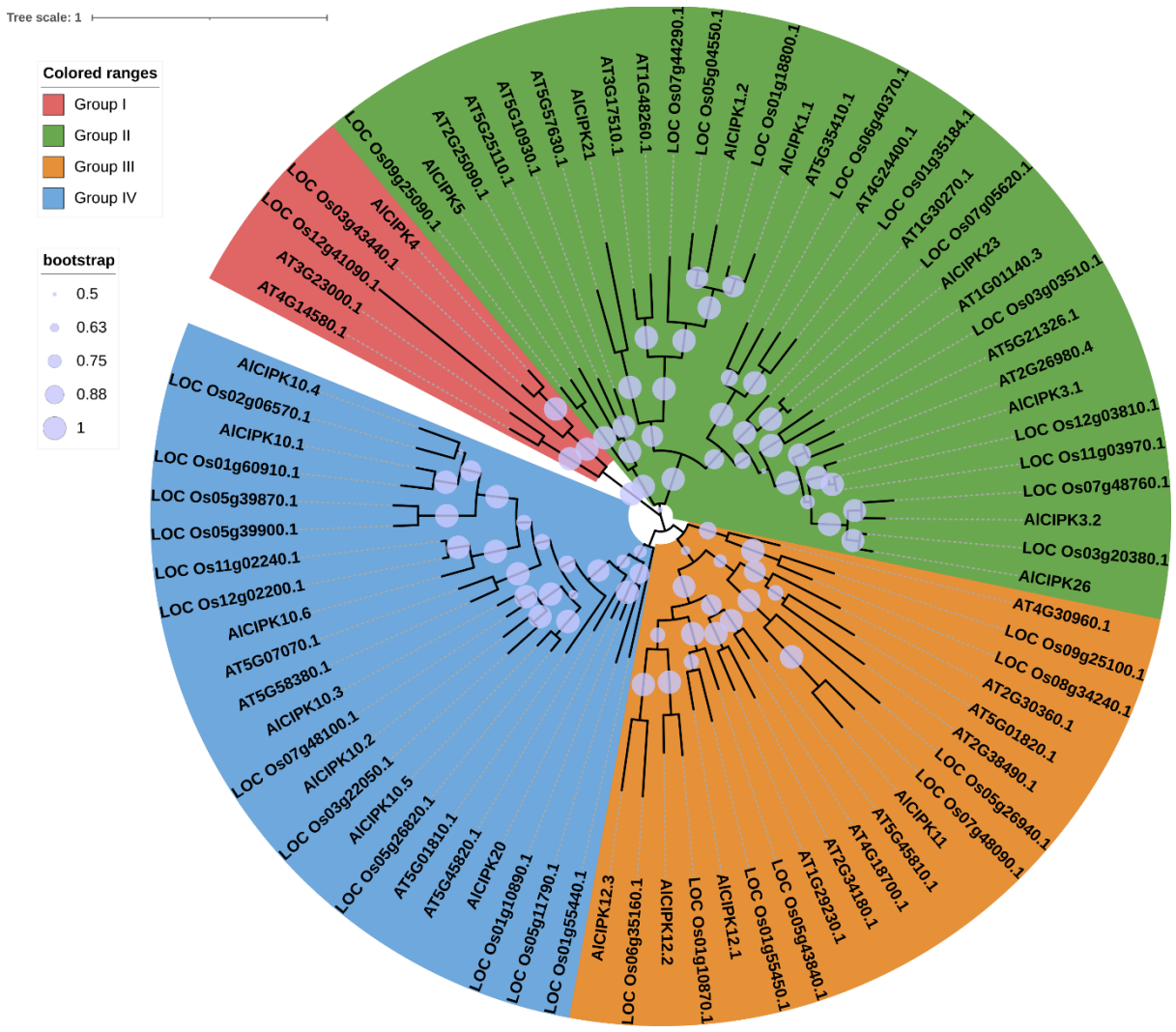

Figure 2. Phylogeny tree of CIPK family proteins in *A. littoralis* (AlCBL), rice (started with LOC), and Arabidopsis (started with AT).

3.4. Gene structure and conserved motifs of AlCBLs

AlCBLs with their orthologous in rice (OsCBLs) were analyzed based on the conserved motifs and domains, and gene structure (Figure 3). Ten conserved motifs were recognized in AlCBLs and OsCBLs that motifs 6 and 9 were not detected in CBLs from group II and motif 9 was only observed in OsCBLs from group III (Figure 3a). Calcium binding superfamily, EF-hand 7, EF-hand 5 and EF-hand 1 domains were observed in AlCBLs and OsCBLs, although they differed based on the location and number of domains (Figure 3b). In addition, two copies of EF-hand 7 and EF-hand 1 domains were found into AlCBL 10 and its orthologue, OsCBL 9, suggesting that AlCBL 10 probably has more potential to interact with downstream elements of involved pathways. Besides, *AlCBLs* were different based on gene structure and all *AlCBLs* had a high number of exons/introns (Figure 3c).

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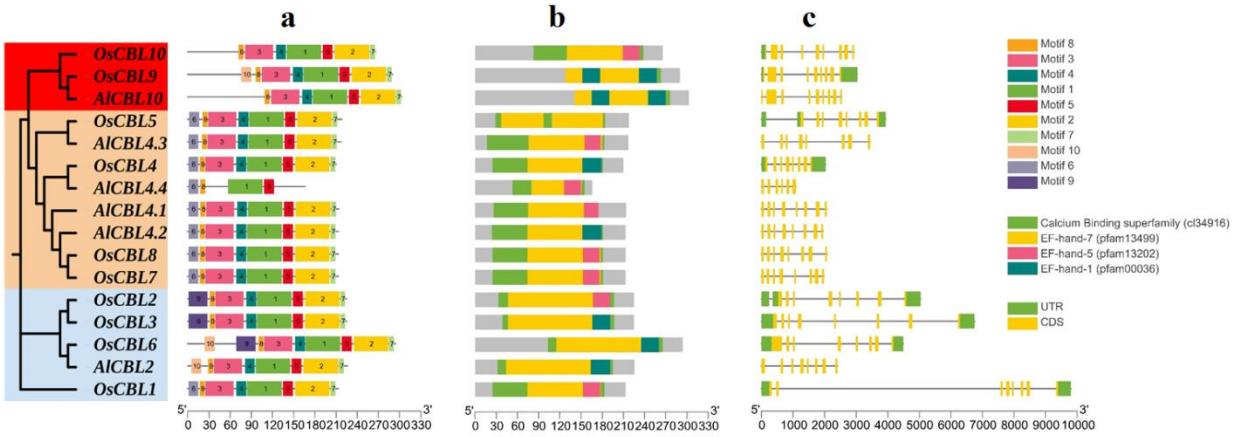

Figure 3. Distribution of conserved motifs (a), functional domains (b), and exon/intron (c) in AlCBL and OsCBL family members.

3.5. Gene structure and conserved motifs of AlCIPKs

To identify conserved motifs and determine the position of these motifs in KINAS and NAF domains, AlCIPK proteins with their orthologues in rice (OsCIPKs) were analyzed using MEME tool (Figure 4a). The results show that the spatial distribution of the motifs in the investigated proteins is strongly conserved. All ten identified motifs were observed in AlCIPK3.1, AlCIPK3.2, AlCIPK10.1, AlCIPK10.2, AlCIPK10.4, AlCIPK20, AlCIPK23 and AlCIPK26 proteins, while motif 10 was not detectable in AlCIPK1.1, AlCIPK12.1, AlCIPK12.2 and AlCIPK12.3. Motif 5 was not detected in AlCIPK4, motif 4 in AlCIPK5, motif 1 in AlCIPK10.3 and motif 2 in AlCIPK10.5. In AlCIPK1.2 protein, motifs 10 and 3, in AlCIPK11 protein, motifs 10 and 6, in AlCIPK10.6 protein, motifs 3 and 8, and in AlCIPK21 protein, motifs 10 and 4 are not present. This result indicates that the main (conserved) motifs play an important role in the function of CIPK proteins. Besides, two KINAS and NAF domains were identified in AlCIPKs and OsCIPKs (Figure 4b), that all studied proteins showed one copy of KINAS and NAF domain. Based on gene structure analysis, 60% of *AlCIPK* genes have one exon and no intron, about 20% of genes have 14 exons and 13 introns, and about 10% of genes have 2 exons and 1 intron, about 5% of genes have 13 exons and 12 introns, and about 5% of genes have 12 exonsand 11 are introns (Figure 4c).

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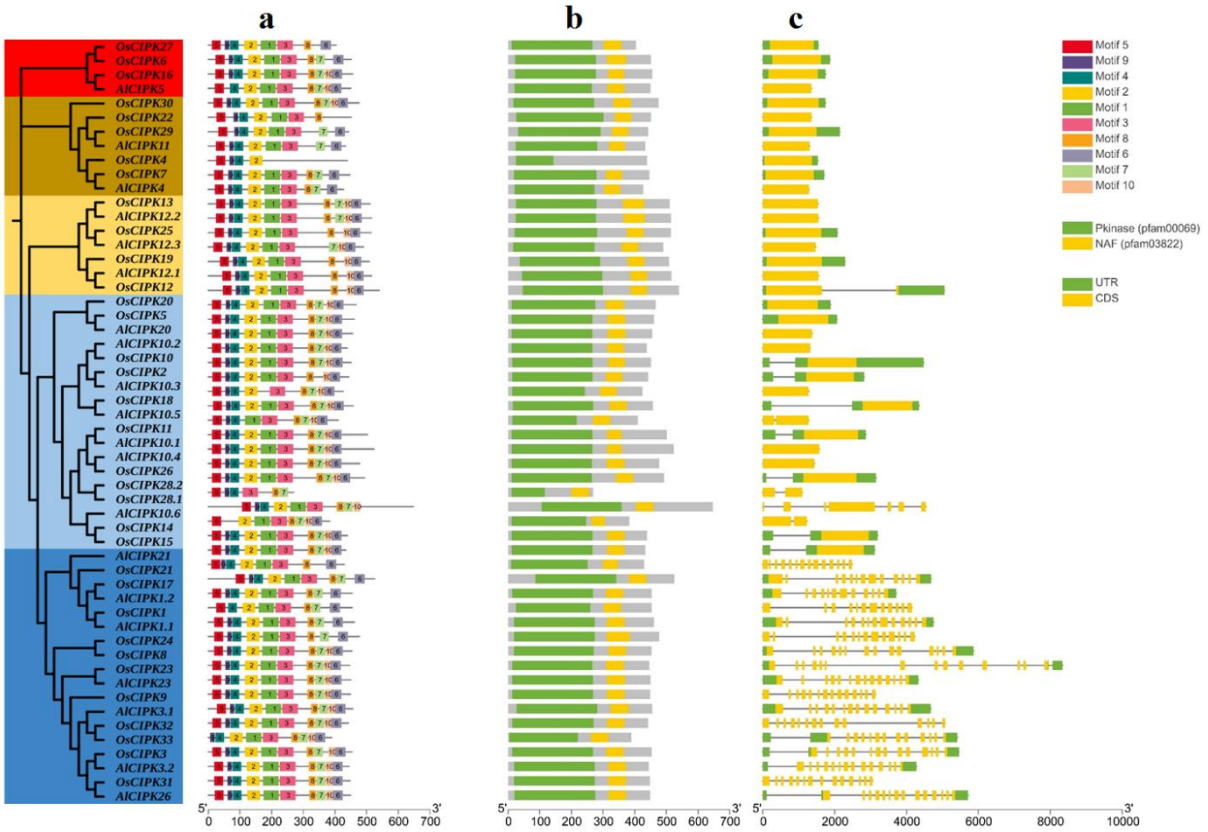

Figure 4. Distribution of conserved motifs (a), functional domains (b), and exon/intron (c) in AlCIPK and OsCIPK family members.

3.6. Promoter analysis

In the present study, the upstream of *AlCIPKs* and *AlCBLs*was screened to identify the cis-regulatory elements related to stress, hormone and growth and development (Figure 5). Most recognized elements were related to common cis-regulatory and elements with unknown function (Figure 5a). Besides, putative cis-regulatoryelements related to transcription factors binding site, and response to phytohormones, and stresses were observed in upstream site of *AlCIPKs* and *AlCBLs*. Cis-regulatory elements involved in response to ABA hormone were frequently identified in promoter site of *AlCIPKs* and *AlCBLs* (Figure 5b). Besides, the putative *cis*-regulatory elements related to GA, auxin, SA and MeJA hormones were recognized in upstream site of *AlCIPKs*, while in *AlCBLs*, regulatory elements responding to GA and MeJA hormones were observed. Cis-regulatory elements involved in responsive to abiotic stresses, including low-temperature, MBS, DRE, and STRE, and biotic stresses including wound, elicitor and defense were identified in promoter region of *AlCBLs* and *ALCIPKs* (Figure 5c). In addition, the binding site of several TFs such as MYB, MYS, and WRKY were observed in upstream site of *AlCIPKs* and *AlCBLs* (Figure 5d). In general, *AlCIPKs* were richer than *AlCBLs* based on the number of stress-related *cis* elements.

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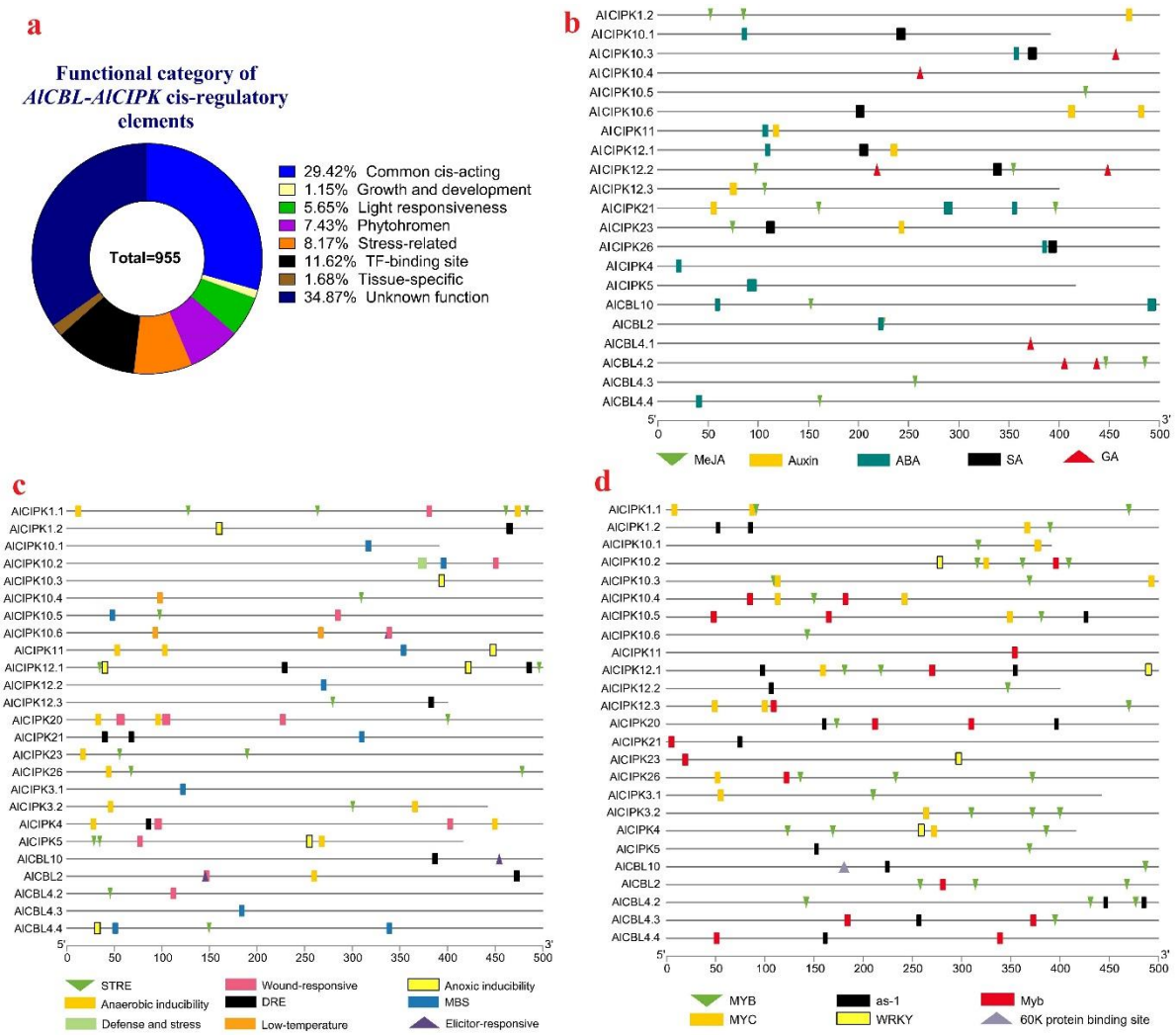

Figure 5. Distribution of cis-regulatory elements in upstream of *AlCBLs* and *ALCIPKs*. Grouping of cis-regulatory elements based on their functions (a). Distribution of cis-regulatory elements involved in response to phytohormones (b), and stress (c), TF binding site (d).

3.7. Expression profile of AlCBL genes in response to salinity

Expression levels of *AlCBL* genes were investigated under salinity in root and leaf tissues. According to our results, *AlCBL2* was not expressed under the tested conditions. After 3h of salinity treatment, *AlCBL4.1*, *AlCBL4.2*, and *AlCBL4.4* showed an upregulation in root tissues (Figure 6). Three *AlCBL* genes, including *AlCBL4.3*, *AlCBL4.4*, and *AlCBL10*, were highly induced after 24h that all three genes were upregulated in root while they were down-regulated in leaf.

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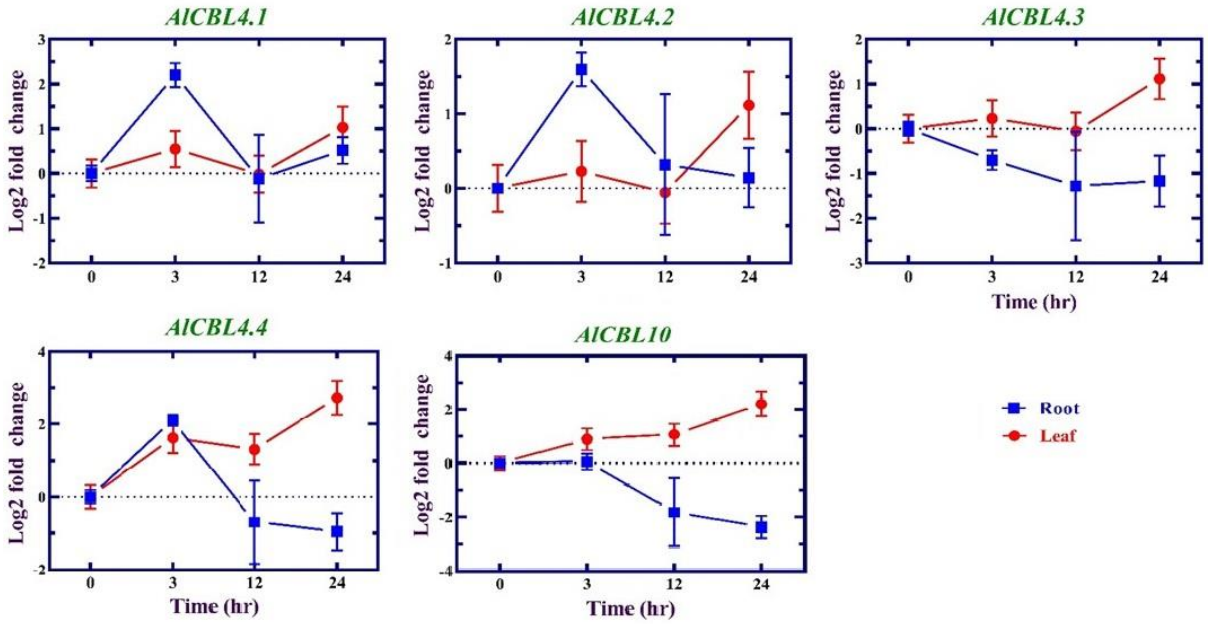

Figure 6- Expression patterns of *AlCBL* genes in response to salinity in two tissues root and leaf. Expression levels are presented based on log2 fold change stress/normal condition.

3.7. Expression profile of AlCIPK genes in response to salinity

In the root tissue, the expression levels of *AlCIPK1.2* (1.96 time), *AlCIPK3.1* (4.90 times), *AlCIPK5* (2.32 times), *AlCIPK11* (4.21 times), *AlCIPK12.1* (2.62 times) and *AlCIPK26* (4.63 times) were increased after three hours (hr) applying salt stress (Figure 7). In the leaf tissue at 3 hours after applying salt stress, *AlCIPK11* (4.10 times), *AlCIPK1.2* (2.89 times), *AlCIPK4* (1.82 times), *AlCIPK12.3* (1.77 times), *AlCIPK5* (1.69 times), *AlCIPK10.2* (-2.50 times), *AlCIPK10.6* (-1.91 times) were more induced. In the root tissue, after 12 hr of salinity, *AlCIPK10.2* gene (-4.46 times) just showed a sharp downregulation, while in leaf tissues, *AlCIPK4* (3.43 times), *AlCIPK10.2* (-6.39 times), *AlCIPK11* (3.72 times), *AlCIPK1.2* (2.00 times), *AlCIPK3.1* (-1.82 times) and *AlCIPK5* (2.13 times), *AlCIPK10.6* (-1.34 times), *AlCIPK12.1* (1.58 times); *AlCIPK26* (2.00 times) and *AlCIPK12.3* (2.61 times) showed a significant modification in their expression levels after 12 hr. Interestingly, *AlCIPK10.2* was notably downregulated in both root and leaf tissues. In addition, the expression levels of *AlCIPK4* (3.47 times) and *AlCIPK12.3* (3.78 times) were increased in leaf tissue after 24 hours. In total, *CIPKs* were more expressed in leaf tissue, while *AlCIPK12.3* was expressed only in leaf tissue and *AlCIPK1.1* gene expression was observed only in root tissue. *AlCIPK4*, *AlCIPK5*, *AlCIPK10.2*, *AlCIPK10.6*, *AlCIPK11*, and *AlCIPK12.3* genes were significantly expressed in leaf tissue at all times of stress.

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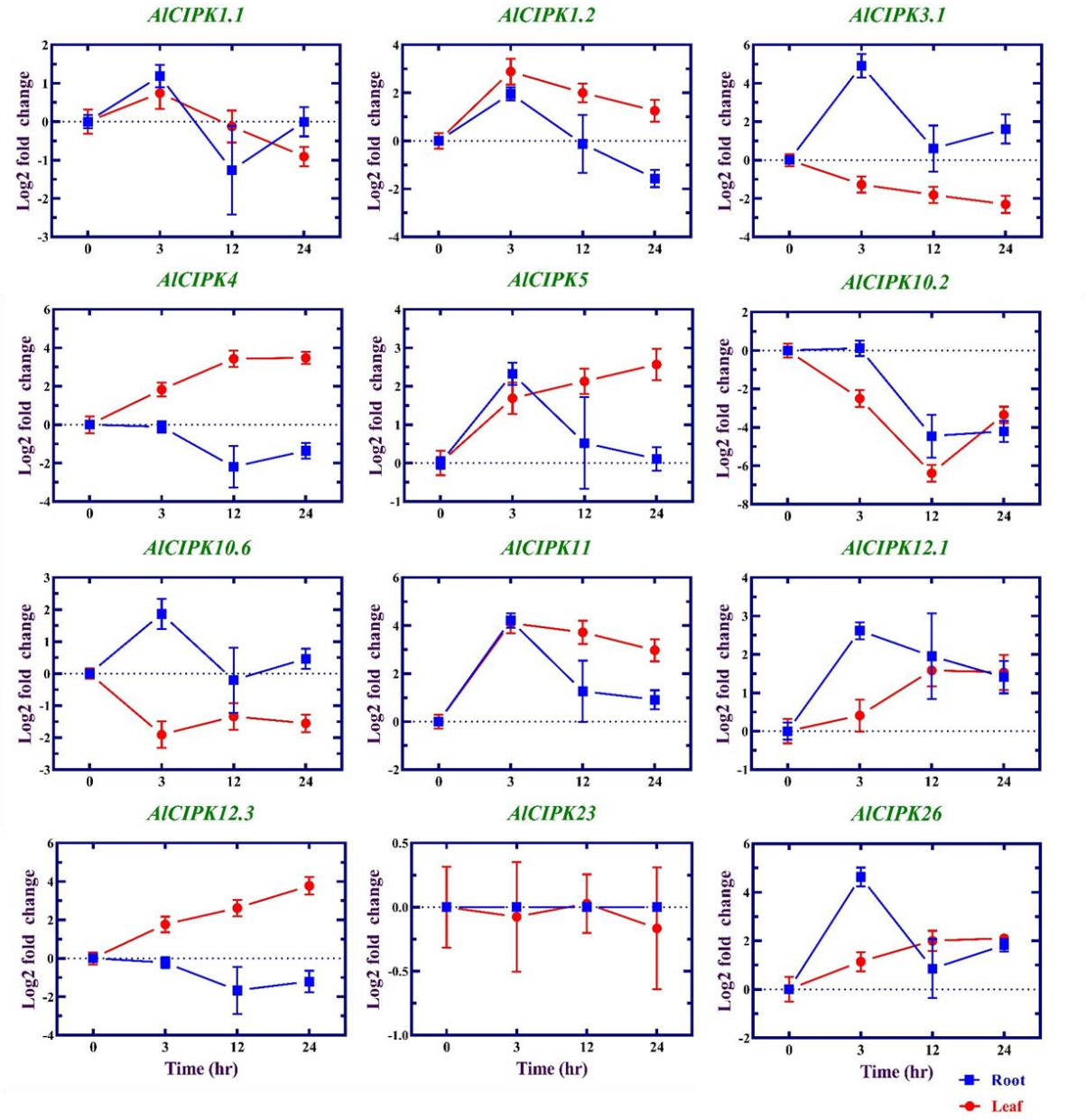

Figure 7. Expression patterns of *AlCIPK* genes in response to salinity in two tissues root and leaf. Expression levels are presented based on log2 fold change stress/normal condition.

4. Discussion

Calcium sensors such as calcineurin B-like proteins (CBL) and CBL-interacting protein kinases (CIPKs) not only participate in the processes of plant growth and development, but they are also involved in stress responses [30]. In the present study, the available genome of *A. littoralis* was used as resource [38] and screened for the respective gene families. Six *AlCBL* genes and twenty *AlCIPK* genes were identified. Due to the importance of calcium-dependent signaling pathways, these gene families, CBL and CIPK, have been studied in various plants. 23 *CBLs* and 58 *CIPK* genes were identified from the genome of *Medicago sativa* [28], 27 *CIPK* genes from potato [30], 9 *CBLs* and 30 *CIPK* genes from the pecan genome [51], 10 *CBLs* and 26 *CIPKs* from *Arabidopsis* [17], 7 *CBLs* and 20 *CIPK* genes from bread wheat [52], 7 *CBLs* and 23 *CIPK* genes from canola [53], 16 *CBLs* and 41 *CIPK* genes from quinoa [35], and 20 *CIPK* genes from sugar beet [31]. The different number of members of this gene family suggests that they may have been subjected to evolutionary pressures differently in each plant [54,55]. Based on the physicochemical properties, AlCBL proteins have similar properties, except for AlCBL10 protein. The proteins of the

 doi:10.20944/preprints202302.0407.v1

AlCIPK family show more diversity. This result support the hypothesis that AlCIPKs are highly diverse due to their involvement in different pathways [56,57]. While *AlCIPKs* showed a high variation in term of gene structure the *AlCIPKs* can be separated into two groups. This grouping is also reflected by their low intron number (less than three introns) and high intron number (more than ten introns). Moreover, this feature has also been reported in previous studies where *CIPKs* have been classified into two groups based on their intron structure [35,58]. It was stated that partial duplication probably has affected the intron number of gene family members [59]. Besides, it was reported that expression levels of genes can be affected by intron number and genes with low intron number could be faster induced [60]. According to phylogenetic analysis, both AlCBL family and AlCIPK family are closer related to their rice orthologs ., This finding suggest that the diversity in these gene families occurred after derivation of monocots and dicots species [61,62].

Halophyte plants have a high potential to grow in substrates of high salinity. Therefore these species are of great interest to investigating the mechanisms of tolerance to salinity. This aims at mechanisms such as absorption, transport and homeostasis of ions, osmotic regulation and salt removal from leaves [37,38]. Although the cultivation of these plants is not an easy task, while the germplasm of halophyte plants is considered as a valuable source for providing genes resistant to environmental conditions, for the implementation of plant breeding programs [63]. In the current study, the expression profiles of *AlCBLs* and *AlCIPKs* were investigated under salt stresses in roots and leaves of *A. littoralis*. *AlCBLs* and *AlCIPKs* showed tissue specific expression patterns. For instance, *AlCIPKs* mRNAs were higher abundant in leaves than in roots, while *AlCBL4.3, AlCBL4.4*, and *AlCBL10* showed upregulation in root and downregulation in shoot. This pattern might be related to the presence of as-1 specific motifs in the promoter region of *AlCBL* genes. Each of *AlCBL4.3*, *AlCBL4.1*, and *AlCBL2* genes had two as-1 motifs, while six as-1 motifs in *AlCBL4.2*, and three as-1 motifs in *AlCBL4.4* and an as-1 motif 3 motifs were observed in promoter region.

The results revealed that the interaction pattern of AlCBL proteins with AlCIPK was tissue-specific and different interactions were observed in two tissues of leaves and roots. Based on the expression pattern, *AlCIPK3.1-AlCBL4.1* and *AlCIPK1.2-AlCBL4.4* genes can potentially interact in root tissue, while in leaf tissue *AlCBL10* gene can interact with *AlCIPK5, AlCIPK12.3* and *AlCIPK26* genes. Positive correlation was reported between CBLs and CIPKs in response to stresses, such as salinity [64], drought [65], and disease [58]. In Arabidopsis, the interaction between CBL4 (called SOS3) and CIPK24 (called SOS2) could active the kinases and +/H+ antiporters called SOS1 and vacuolar H+-ATPase to increase stress tolerance [53,66,67]. Subsequent research in Arabidopsis showed that the AtCBL10 gene also interacts with AtCIPK24. Thus, the CBL10-CIPK24 complex interacts with vacuoles to protect the shoot from damage caused by salt stress [67]. This result suggests the fact that calcium sensors may exhibit very different functions despite high sequence similarity or close evolutionary relationship.

5. Conclusions

This review is the first comprehensive study of the family of calcium sensors with the aim of clarifying the evolution, expression patterns and possible functions of the genes of this superfamily in *A. littoralis* in response to abiotic stresses. These findings provide information to predict the function of calcium sensor genes in plant tolerance to salt stress. Additional studies on the expression of AlCBL and AlCIPK family genes under different abiotic stresses in future research can be useful in understanding the mechanism of gene expression adjustments related to the SOS pathway. The *AlCIPK* genes reported in this research, while providing preliminary information, provide a basis for identifying the functions and mechanisms of the stress response, especially the responses related to the CBL / CIPK pathway in the *A. littoralis* plant.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: List of primers of *AlCBL* genes used in qPCR analysis; Table S2: List of primers of *AlCIPK* genes used in qPCR analysis.

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Author Contributions: Conceptualization, M.A., S.H.H., H.N.Z. and G.N.; methodology, M.A., S.H.H. and P.H.; software, M.A. and S.H.H.; validation, S.H.H., H.N.Z. and G.N.; formal analysis, S.H.H.; investigation, P.H. and M.K.; writing—original draft preparation, P.H.; writing—review and editing, P.H. and M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable

Acknowledgments: Costs for open access publishing were partially funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, grant 491250510)

Conflicts of Interest: The authors declare no conflict of interest.

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