Identification, Characterization and Expression Analysis Reveal the Potential Function of Annexin Genes in Response to Abiotic stress, Calcium and Hormones in Cassava (Manihot esculeta Crantz)

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Abstract

Background The calcium (Ca2+)-dependent phospholipid binding protein annexin gene family, which is known to be related to membrane lipid and cytoskeletal components, is involved in a diverse range of biological functions. However, in cassava (Manihot esculenta Crantz), no studies focusing on the roles of annexin genes in response to abiotic stresses, calcium, and hormones have been informed. Results 12 annexin genes were found and assigned to eight chromosomes in the cassava genome. All of the MeAnns contain a typical annexin domain with four 70-amino acid repeats. The MeAnns are classified into six groups in the phylogenetic tree. In their promoter regions, MeAnns possess at least 3 hormone response-related cis-elements and 1 abiotic stress response-related cis-acting element. MeAnn1, MeAnn2 and MeAnn5 exhibit very high levels of expression in each tested organs or tissues. By contrast, MeAnn12 exhibits very low levels in all the tested organs or tissues. qRT-PCR analysis indicates that both MeAnn5 and MeAnn9 have significantly high expression in leaves after cold, drought, and salt treatments and are highly responsive to CaCl2, GA and JA treatments. MeAnn2 and MeAnn10 are significantly downregulated in leaves by cold, drought and salt stress and negatively respond to CaCl2, GA and JA. The expression patterns of MeAnns under cold, drought, and salt stress are irregular in shoots. In roots, MeAnn1 and MeAnn9 are downregulated by cold, CaCl2 and JA treatments, while their other gene expression patterns are irregular. Conclutions In this study, we identified annexin genes in cassava and our expression profiling analysis demonstrated that cassava annexin genes responded to multiple stresses. Our results laid the foundation for further study of the function of cassava anesxin genes and provided an entry point for understanding the response mechanism of cassava to abiotic stress.

Background

As an evolutionarily conserved group, annexins are calcium (Ca^{2+})-dependent phospholipid binding proteins known to be related to membrane lipid and cytoskeletal components [1, 2]. The remarkable and special characteristics of these proteins function in membrane organization, vesicle trafficking and the Ca^{2+} signaling pathway and are involved in a diverse range of cellular functions [1]. Generally, annexins exist in almost all eukaryotes, among which plant annexins represent a monophyletic branch of homologs that evolved from green algae; however, some prokaryotes also have annexins [2]. Annexin multigene families have high diversity numbers across various organisms [3]. The primitive variety of annexin proteins date back to 1–1.5 billion years ago in *Giardia lambia* (unicellular protist) [4]. The annexin gene (*Ann*) was firstly detected in tomato over two decades ago [5], after which it has also been identified in *Giardia lamblia* [4], *Capsicum annuum* [6], *Nicotiana tabacum* [7], *Triticum aestivum* [8], *Arabidopsis thaliana* [9, 10], *Brassica juncea* [11] and *Oryza sativa* [12]. Among these, annexin genes in *A. thaliana*, *B. juncea* and *O. sativa* have been identified and well-characterized. So far, studies have shown that these plants have relatively large and diverse annexin families, whereas the original low single-cell plants, such as *Micromonas* and *Ostreococcus*,have small families [13, 14]. In vertebrates, low annexin A1 expression has been predicted to have a role in the induction of chemotherapy in oral cancer patients [15]. The annexin gene *Ann2* in vertebrates is a multipotent calcium and anionic phospholipid-binding

protein that is found to play a considerable role in endocytosis, exocytosis, ion channel conduction, membrane tissues and other processes [16]. During nodule formation in *Medicago truncatula*, annexins perform important functions in the generation and transmission of calcium signals [3]. A previous study has indicated that the *GbAnn6 interacted* with *Actin1* toregulating the fiber elongation in cotton [17]. *AtAnn1* from *A. thaliana* is significant for regulating H_2O_2-induced root calcium signaling [18].

Structurally, almost all annexins have four to eight repeats of a 60-70 amino acid motif [12]. It has been reported that annexins consist of four annexin motif repeats, with the conserved GxGT-(38 residues)-D/E calcium binding sequence exist in the first and fourth motif repeats [3]. Compared with vertebrates, annexins from plants have a short N-terminal region and lack calcium binding sites (CBS) in their second and third motif repeats [3]. Reports has also demonstrated the presence or absence of motifs that play key roles in calcium channels, peroxidases, and ATPase/GTPase activity [19]. From the phylogenetic point of view, plant annexins and animal annexins are independent populations [3, 19].

In recent studies, plant annexins have been reported to be expressed in many diverse tissues during plant development [9, 12]. Tissue-specific expression profiles of plant annexin families have been observed in plants such as *A. thaliana* [9], *triticum astivum* [20], and *O. sativa* [12]. *AtAnn1* is expressed in all examined tissues, but is richest in stems, while *AtAnn2* is most abundant in roots [9]. Additionally, many studies have reported the functions of annexins in response to abiotic stresses. Abiotic stresses start with stress perception and then lead to the activation of gene expression through signaling pathways, consequently influencing plant physiology, growth and development [21, 22]. It has been reported that some annexin genes related to membrane organization are involved in abiotic stress tolerance [20]. For instance, overexpression of *AtAnn1* from *A. thaliana* inplants enhanced drought tolerance, while *AtAnn1* knockout in plants reduced drought tolerance[10]. *AtAnn1* and *AtAnn4* from *Arabidopsis* can interact with one another and are adjusted to response to stresses such as aridity and salt [23]. Expression analysis also revealed differential annexin expression patterns in *O. sativa* seedling stages under diverse abiotic stresses such as salt, drought, cold and heat [12].

In the tropical and subtropical regions of the world, cassava (*Manihot esculenta* Crantz) is generally known as an essential food source for at least 500 million people [24, 25]. Recently, many studies have focused on increasing its productive yield and understanding the effects of abiotic stresses such drought and infertility [26]. In this present study, twelve *MeAnn* annexin genes from cassava have been identified. The conserved domains, motif distribution, evolutionary relationships, gene structures, and chromosomal localization of the *MeAnn* genes were inquired into. To identify the possible roles of *MeAnn*s in cassava, the cis-elements on the *MeAnn* promoters were predicted. The differential expression of *MeAnn*s under abiotic stresses, such as salt, cold and drought stresses, and during calcium ion and hormone signaling were examined by qRT-PCR in cassava. These results will help to ulteriorly reveal the important biological functions of *MeAnn* genes in cassava.

Results

Genome-wide identification and characterization of MeAnns

Using genome-wide retrieval techniques, in all, 12 supposed *Annexin* genes were found out in the cassava genome and named *MeAnn1-12*. ProtParam was used to characterize the MeAnn physiological and biochemical characteristics. The results showed that MeAnn lengths ranged from 309 (MeAnn12) to 382 (MeAnn6) AA (amino acids), with the predicted MW (molecular weights) ranging from 35.26 (MeAnn12) to 43.78 (MeAnn6) kDa. The GRAVY (grand average of hydropathicity) of the MeAnns ranged from -0.554 (MeAnn5) to -0.271 (MeAnn7), and the theoretical pI (isoelectric point) ranged from 5.80 (MeAnn6) to 9.46 (MeAnn9). The II (instability index) of MeAnn proteins ranged from 24.66 (MeAnn2) to 50.62 (MeAnn6), with six MeAnns (MeAnn2, 3, 7, 8, 11, and 12) denoted as stable proteins, while the other six MeAnns are unstable proteins. The AI (aliphatic index) of the MeAnns ranged from 80.14 (MeAnn10) to 100 (MeAnn7) (Table 1). Signal peptide (SP) analysis showed that only one of the twelve MeAnns (MeAnn6) has a signal peptide (Table 1). Subcellular localization prediction indicated that the MeAnns are located in one to three organelles including the cytoplasm (Cytop), mitochondria (Mito), nucleus (Nuc), chloroplast (Chlo) and plasma membrane (Plasm). Most MeAnns have two subcellular localization organelles. For example, MeAnn1 is located in the cytoplasm and mitochondria; MeAnns2, 3, 7 and 8 are located in the cytoplasm and nucleus; MeAnn4 is located in the mitochondria and chloroplast; MeAnn6 is located in the plasma membrane and nucleus; and MeAnn10 is located in the mitochondria and nucleus. MeAnn5 and MeAnn12 are located in three organelles, including the cytoplasm, mitochondria and nucleus, whereas MeAnn9 and MeAnn11 are only located in the mitochondria (Table 1).

Location and Distribution of MeAnns on Chromosomes

Using chromosomal localization analysis, the 12 *MeAnn* genes were assigned to eight chromosomes, including 4, 6, 9, 11, 12, 15, 17, and 18 (Fig. 1). The distribution of *MeAnn*s among the chromosomes is unequal. In particular, chromosomes 4 and 11 harbor 6 *MeAnn*s, with each one containing three genes. By contrast, chromosomes 6, 9, 12, 15, 17 and 18 each harbor only one *MeAnn* gene (Fig. 1). *MeAnn1, 2, 5* and *6* have the same orientation, whereas the other *MeAnn*sare in the opposite orientation.

Cassava MeAnn Sequence Alignment

To obtain detailed information on each cassava MeAnn protein, the 12 MeAnn proteins were subjected to multiple sequence alignment (Fig. 2.) All of the MeAnns contain typical annexin domains with four 70-amino acid repeats (Repeats □- □). The type □ Ca^{2+} binding sites appear in both Repeats □ and □. Only MeAnn7 and 8 have typical G-X-GTD-{38 residues}-E/D sites. The “G-X-GTD” is changed to “G-X-GTN” in MeAnn1-4; to “G-X-GVD” in MeAnn5 and 6; and to “G-X-GCD” in MeAnn9, 11 and 12. The typical G-X-GTD-

{38 residues}-E/D site is missing from MeAnn10 (Fig. 2). MeAnns 1-4 and 10 contain a phospholipid binding-related “W” residue in Repeat □ (Fig. 2). All the MeAnns except MeAnns 5-7 and 10 contain a peroxidase activity-related heme-binding site (“H” residue) (Fig. 2). MeAnn1, 2, 7 and 8 contain the typical GTP-binding site “DXXG” in Repeat □; however, the “D” residue is changed to “E” in MeAnns 3, 4, 9, 11 and 12; to “N” in MeAnns 5 and 6; and to “A” in MeAnn10 (the “DXXG” is changed to “AXXXG”) (Fig. 2). MeAnn1, 3-5 and 11 contain an F-actin binding site (IRI) in Repeat □; the “IRI” is changed to “IRV” in MeAnn2, to “VRI” in MeAnn6, to “VYI” in MeAnn7, to “VYV” in MeAnn8, to “IQI” in MeAnn9 and 12, and to “LEI” in MeAnn10 (Fig. 2). MeAnn10 has only one redox reaction-related S3 cluster in Repeat □, whereas the other 11 MeAnns have two S3 clusters, one each in Repeats □ and □ (Fig. 2).

Phylogenesis Analysis, Gene Structures and Conserved Motifs in cassava MeAnns

To make clear the relationships among theMeAnn members in cassava, a phylogenetic tree consisting of all the annexins from *M. esculeta*, *A. thaliana*, *O. sativa* and *S. lycopersicum* was framed using the Neighboring-Joining method. The results showed that the MeAnns in the phylogenetic tree are classified into six groups (Fig. 3). One MeAnn protein (MeAnn10) is clustered into Group □, three proteins (MeAnn9, 11 and 12) are clustered into Group □, MeAnn5 and 6 are clustered into Group □, MeAnn7 and 8 are clustered into Group □, MeAnn3 and 4 are clustered into Group □, and MeAnn1 and 2 are clustered into Group □. MeAnn10 is clustered together with two annexins from rice, whereas the other 11 MeAnns are clustered together with annexins from *Arabidopsis*, rice and tomato (Fig. 3).

The gene structures of the annexin members in cassava and *Arabidopsis* were determined using the GSDS online tool. The genomic DNA lengths of *MeAnn* genes vary from 1586 to 3162 bp, which are longer than their orthologs from *Arabidopsis*. However, their CDS lengths vary from 930 to 1149 (bp), which are similar to their orthologs from *Arabidopsis* (Table 1, Fig. 4B). The *MeAnn* gene structures have four (*MeAnn10*) to seven (*MeAnn4*) exons that are interrupted by three to six gene-specific introns with different lengths. Gene structure analysis revealed that the structures of *MeAnn9, 11* and *12* in Group □; *MeAnn5* and *6* in Group □; and *MeAnn7* and *8* in Group □ have six exons, which is similar to their orthologs from *Arabidopsis* (Fig. 4A, B). In Group □, the structure of *MeAnn3* is similar to its ortholog *AtAnn8*, which has six exons; however, *MeAnn4*, which is in the same group, has seven exons. In Group □, *MeAnn1* and *2* each have five exons, which is similar to *AtAnn2*, though it is different from *AtAnn16* and *7* in the same group. Only one gene (*MeAnn10*) in Group □ has four exons in its structure (Fig. 4A, B).

Ten different conserved motifs were identified from MeAnns and AtAnns (Fig. 3 C). Generally speaking, the members in the same groups share a similar motif structure, except for the members in Group □. All the MeAnns and AtAnns in Groups □-□ contain all ten motifs. All the MeAnns and AtAnns in Group □ contain nine motifs, only lacking motif10. In Group □, MeAnn5 consists of seven motifs, lacking motif3, 8 and 9, and MeAnn6 includes eight motifs, lacking motif3 and 8. By contrast, AtAnn4, which is also in

Group Ⅱ, contains six motifs, lacking motif3 and 8-10. In Group I, MeAnn10 includes seven motifs, lacking motif5, 6 and 8 (Fig. 3A, C).

Presence of hormone- and stress-related cis-acting elements in the MeAnn promoter

To better understand the feasible biological responses of *MeAnns* under hormone and abiotic stresses, the 2 kb sequence upstream of the translation start site of each MeAnn was analyzed by PlantCARE. Twelve hormone response *cis*-elements, including an ABRE, ABRE4, as-1, CCTCA motif, ERE, GARE motif, TATC box, TCA, TCA element, TGA box, TGA element and TGACG motif, were predicted (Fig. 4, Table S1). Fifteen stress response cis-elements, including an ARE, RDE core, LTR, MBS, Myb, MYB recognition site, MYB, Myc, MYC, STRE, TC-rich repeats, W box, WRE3, and WUN motif, were predicted (Fig. 4). MeAnns possess at least 3 hormone response-related cis-elements and 1 stress response-related cis-element, which indicates that *MeAnn* expression might be regulated by these hormone and abiotic stress factors. All twelve *MeAnns* contain one to seven EREs, suggesting potential responses to ethylene. One to four ABREs are present in nine *MeAnn*s (except *MeAnn9, 10* and *12*) and one to two ABRE4s are present in *MeAnn1*, *2*, *6*, *7* and *11*, which indicates that these genes might be regulated by ABA. One to three CGTCA motifs and TGACG motifs are found in three genes (*MeAnn1*, *4* and *9*), indicating that these genes might respond to MeJA. Four genes (*MeAnn 4*, *6*, *7* and *11*) contain a GARE motif and five genes (*MeAnn1*, *4*, *10-12*) contain a TATC box, figuring that these *MeAnns* might be adjusted by gibberellin (GA). Eight genes (except for *MeAnn1*, *2*, *4* and *12*) have TCA or TCA elements that might respond to salicylic acid (SA). The five genes (*MeAnn4*, *5*, *8*, *9* and *11*) with a TGA box or TGA element might respond to auxin. The genes (*MeAnn1*, *4* and *9*) with an as-1 cis-element may respond to auxin, salicylic acid and methyl jasmonate (JA). All the genes contain Myb, MYB, or MYB recognition sites, which indicates that all MeAnns might be regulated by MYB transcript factors. All the *MeAnns* except *MeAnn3* have Myc or MYC sites. Nine *MeAnns*, except for *MeAnn9*, *10* and *12*, show ARE motifs, which are cis-acting regulatory elements important for anaerobic induction. *MeAnn5*, *8* and *9* contain a DRE core, which is a cis-acting element related to dehydration, low temperature, and salt stress responses. *MeAnn1*, *8*, *9* and *11* contain an LTR motif, which is a cis-acting element involved in low temperature responsiveness. *MeAnn3*, *5*, *8*, *9* and *11* contain an MBS, which is a MYB binding site related to drought-inducibility. *MeAnn4-10* and *12* contain one to four stress-responsive elements (STREs). *MeAnn2*, *3* and *12* contain one to two cis-acting responsive elements related to defense and stress responsiveness (TC-rich repeats). Except for *MeAnn9*, all the other *MeAnn*s have at least one wound-responsive element (WRE3, W box or WUN motif). Among the hormone-associated cis-elements, ERE is the most abundant and ABRE is second. Among the stress-related elements, MYB- and MYC-associated cis-elements are the most abundant, followed by wound-responsive elements.

Analysis of Tissue-Specific Expression Patterns of the 12 MeAnns

To evaluate the tissue specific expression levels of the *MeAnn*s, RNA-seq data were downloaded from NCBI and analyzed. The gene expression levels of the 12 *MeAnn*s were analyzed in a variety of the organs or tissues in cassava, such as leaves, stems, fiber roots, storage roots, midveins, lateral buds, OES, FEC, petioles, RAM, and SAM. The results, which are shown in a heatmap, indicated that some *MeAnns* are expressed in all detected organs or tissues, suggesting that these genes may have essential biological roles in cassava growth and development. For instance, *MeAnn1*, *MeAnn2* and *MeAnn5* exhibited very high expression levels in all the tested organs or tissues. Their expression in young tissues, such as RAM and SAM, is higher than in mature organs, such as the leaves and roots. By contrast, *MeAnn12* exhibits very low levels in all of the tested organs or tissues. The other genes exhibit varying expression patterns (Fig. 6). The expression patterns of *MeAnn*s in leaves and midveins are similar; aside from *MeAnn4* and *MeAnn12*, the other *MeAnn*s are highly expressed.Moreover, *MeAnn* expression patterns in lateral buds and s hoot apical meristems are similar; aside from *MeAnn12*, the other eleven *MeAnn*s are highly expressed. The *MeAnn* expression patterns in fibrous roots are similar to that in root apical meristems, which show lower expression levels. The *MeAnn* expression patterns in stems and petioles are similar. The *MeAnn* expression patterns vary in other organs or tissues such as somatic organized embryogenic structures, friable embryogenic calli and storage roots (Fig. 6).

Examination of MeAnn gene expression under cold, drought and salt stresses

To uncover the changes in *MeAnn* expressionin response to cold, drought and salt stress, the relative expressions of the 12 *MeAnn*s were analyzed by qRT-PCR under 4℃, 20% PEG6000 and 300 mmol/L NaCl treatments, respectively, at different time points (from 0 to 48 h). The results indicated varied *MeAnn* expression profilesafter cold, drought, and salinity treatments (Fig. 7-9). Most obviously, *MeAnn5* and *9* were more sensitive to the three stresses (Fig. 7-9).

When cassava seedlings were under 4℃ cold stress, *MeAnn5*, *6* and *9* were significantly upregulated in leaves; *MeAnn1* and *10* were suppressed at all six cold stress stages; *MeAnn2* was downregulated at all stages except 9 h; *MeAnn3* was downregulated at 9, 12 and 48 h; *MeAnn4* was upregulated at 3, 6 and 24 h; *MeAnn7* was upregulated at 9 h and downregulated at 12 h; *MeAnn11* was only significantly upregulated at 9 h; and *MeAnn12* was downregulated at 9 and 12 h (Fig. 7). In shoots, *MeAnn1* was downregulated at 9 and 12 h; *MeAnn2* was upregulated at 9 and 12 h; *MeAnn4* was upregulated at 9 h; *MeAnn5* and *6* were upregulated at 9 h; *MeAnn7* was downregulated at 24 and 48 h; *MeAnn8* was downregulated at 12 h; *MeAnn9* was upregulated at 6 h and downregulated at 9, 12 and 24 h; *MeAnn10* was upregulated at 3, 9, 12, 24 and 48 h; *MeAnn11* was markedly upregulated at 3 and 12 h; and *MeAnn12* was upregulated at 6, 24 and 48 h. The differential expression of *MeAnn3* was weaker at all the

stages under cold stress (Fig. 7). In roots, *MeAnn1, 9* and *12* were significantly downregulated under cold stress at all six stages. Moreover, *MeAnn2* was upregulated at 12 h; *MeAnn7* was upregulated at 12 and 48 h; *MeAnn4* was downregulated at 9, 12, 24 and 48 h; *MeAnn10* was downregulated at 3, 6, 9 and 24 h; and *MeAnn11* was downregulated at 24 h cold stress. *MeAnn3*, *5*, *6* and *8* showed weaker differential expression under cold stress than under control conditions (Fig. 7).

When the cassava seedlings were under 20% PEG drought stress, in leaves, *MeAnn4-6* and *9* were markedly upregulated, *MeAnn2* and *10* were downregulated at all time points; *MeAnn8* and *11* were upregulated at 3 h; the expression of *MeAnn7* was markedly upregulated at 3, 6 and 12 h; *MeAnn12* was upregulated at 12 h; and *MeAnn1* was significantly downregulated at 9 and 48 h (Fig. 8). In shoots under drought stress, *MeAnn1*, *5* and *9* were downregulated at all time points; *MeAnn6*, *7* and *10* wereupregulated at 3 h; *MeAnn1* was downregulated and *MeAnn10* was upregulated at 6 h; and all the other genes had weaker differential expression at the six time points (Fig. 8). In roots, at 3 and 6 h, only *MeAnn2* was distinctly downregulated; at 9 h, only *MeAnn6* was distinctly upregulated; at 12 h, *MeAnn2*, *6*, *7*, *10* and *11* were upregulated and *MeAnn9* and *12* were downregulated; and at 24 and 48 h, only *MeAnn12* was distinctly downregulated (Fig. 8).

When the cassava seedlings were under 300 mmol/L NaCl stress, in leaves, *MeAnn5-7* and *9* were markedly upregulated, *MeAnn2* and *10* were downregulated at all time points; *MeAnn4* was upregulated from 3-24 h; *MeAnn11* expression was markedly upregulated at 24 and 48 h; and *MeAnn1* was significantly downregulated at 12 h (Fig. 9). In shoots, all 12 *MeAnns* had weak differential expression at 3 and 48 h; *MeAnn1*, *7* and *9* were downregulated at 6 h; *MeAnn4* and *12* were upregulated at 9 h; *MeAnn1*, *3-5*, *8* and *9* were significantly downregulated and *MeAnn7* and *MeAnn10* were upregulated at 12 h; and *MeAnn4* was upregulated at 24 h (Fig. 9). In roots, all the *MeAnn*s had slightly lower differential expression compared to the control at 3 h; only *MeAnn1* was upregulated at 6 h; *MeAnn4* and *7* were upregulated at 9 h; *MeAnn1*, *6*, *7*,and *9-11* were upregulated and *MeAnn2* and *8* were downregulated at 12 h; *MeAnn1*, *7*, *9* and *10* were upregulated at 24 h; and *MeAnn1* and *9* were upregulated at 48 h (Fig. 9).

Examination of MeAnn gene expression in response to Ca^{2+} signaling and hormones by qRT-PCR

To reveal the responses of the *MeAnns* to Ca^{2+} signaling, the cassava seedlings were treated with different concentrations of $CaCl_2$. The results indicated that *MeAnn1*, *2* and *10* were downregulated, while *MeAnn5* and *9* were upregulated in leaves under all the $CaCl_2$ treatments. *MeAnn6* and *7* had similar expression patterns in leaves under $CaCl_2$ treatments. *MeAnn1* and *9* had similar expression patterns and were downregulated under all treatments in shoots. *MeAnn3*, *4*, *8* and *12* were downregulated under 20 mmol/L $CaCl_2$ treatment in shoots. *MeAnn1* and *9* as well as *MeAnn4* and *12* had the same expression patterns in roots, which were downregulated under $CaCl_2$ treatments. *MeAnn2* was upregulated under 10, 20 and 50 mmol/L $CaCl_2$ treatments in roots. *MeAnn6* and *MeAnn7* were upregulated in roots under 50

mmol/L and 10, 40 and 50 mmol/L $CaCl_2$ treatments, respectively. *MeAnn10* and *11* were upregulated under the 40 mmol/L $CaCl_2$ treatment in roots. *MeAnn3* and *MeAnn8* were downregulated under 40 mmol/L and 20 and 30 mmol/L $CaCl_2$ treatment in roots, respectively (Fig. 10).

In leaves under ABA treatment, *MeAnn4* and *11* were upregulated and *MeAnn10* and *12* were downregulated. *MeAnn4-6* and *9* were upregulated and *MeAnn2* and *10* were downregulated by GA and JA treatments. *MeAnn4* and *6* were upregulated and *MeAnn9*, *10* and *12* were downregulated by SA treatments. In shoots, *MeAnn9* was downregulated by ABA treatment. *MeAnn7* and *8* were upregulated by GA treatment. *MeAnn9* and *12* were upregulated by JA treatment. All the *MeAnn*s in shoots were weakly responsive to SA treatment. In roots, *MeAnn2* and *9* were downregulated, while *MeAnn4*, *6* and *8* were upregulated by ABA treatment. *MeAnn9* was downregulated by the ABA, GA, JA and SA treatments. *MeAnn1* was downregulated by JA treatment, *MeAnn2* was downregulated by ABA treatment, *MeAnn3* was upregulated by SA treatment, *MeAnn4* was upregulated by ABA and SA treatments, *MeAnn6* and *8* were upregulated by ABA treatment, and *MeAnn11* was downregulated by SA treatment. *MeAnn5*, *7*, *10* and *12* showed slight responses to the four hormones (Fig. 11). Among all the *MeAnn*s, *MeAnn9* showed an obvious up- or down-regulated response to most of the hormones in different tissues.

Discussion

Annexin genes have been reported to play significant roles in plant growth, development, and overcoming abiotic adversity [27-37]. Numerous annexin family gene members have been identified in other plants. For instance, 8 *Annexins* from *Arabidopsis* [27], nine *Annexins* from tomato [33], 10 *Annexins* from rice [12, 38], and 12 *Annexins* from maize [39] have been reported. In this study, 12 *Annexin* genes from cassava have been identified and characterized (Table 1). MeAnns in cassava are classified into six well-delimited clusters (Fig. 3). The subcellular distribution profile of annexin proteins is one of the key factors associated with their functions. Previous studies indicated that plant annexin localization sites vary, including the cytoplasm, plasma membrane, nucleus, nucleolus and extracellular matrix [19, 34, 40, 41]. For instance, the *Apium graveolens* annexin gene *VCaB42* was detected to localize in vacuole membranes [41]. The AnnSp2from *Solanum pennellii* was localized in the nucleus [34], whereas GhFAnnxA in *Gosyypium hirsutum* was reported to be localized in the cytoplasmic-nuclear region [42]. Previous studies have also demonstrated that plant annexins show dynamic and reversible distribution profiles, with their distributions changing between the membrane and cytoplasm under different stress conditions [43-46]. In this study, the subcellular localization of MeAnns were predicted to have five sites including the cytoplasm, mitochondria, nucleus, chloroplast, and plasma membrane. Here, our results are in partial agreement with previous results.

Sequence analysis of the annexin amino acid sequences from cassava shows that the four repeats and some conserved motifs are present in MeAnns. There are two type II calcium-binding sites in the first and fourth annexin repeats, an IRI motif related to actin binding in the third repeat, and a DXXG motif related to GTPase activity in the fourth repeat. These results show the consistency of the annexin protein domains among cassava and other plants reported in other studies (Fig. 2) [12]. It has been reported that

all annexins have a core domain that is composed of four similar approximately 70-amino acid repeats [3]. The C-terminal region of the vertebrates annexin contains 4 motif repeats that have a greatly conserved sequence (GxGT-[38 residues]-D/E) for Ca^{2+} binding [3]. It has been demonstrated that cotton ANXD36 has higher GTPase activity than ATPase activity and is inhibited by calcium due to the presence of the GTPase activity site, such as the DXXG in the fourth repeat [47]. All plant annexins belong to soluble proteins and contain greatly conserved calcium-binding domains and diverse N-terminal regions and lack the long N-terminal regions compared to vertebrates [20]. Analysis of the full-length *MeAnn* genomic DNA sequences showed a variety of *MeAnn* gene structures with different numbers of exons and introns (Fig. 4B). Similar results are also found in other plants; for example, *AtAnn1* has two introns, *AtAnn6* and *7 have* three introns, *AtAnn2* has four introns and other *AtAnns* have five introns [5,6]. Previous studies have indicated that multiple annexin genes could be locate on one chromosome. For example, in *Arabidopsis*, 4 annexin genes are located on chromosome 5 [38]; in rice, two annexins are locate on chromosome 5 and three are located on chromosome 9 [38]; in poplar, three annexin genes are located on chromosome 1[38]; and in *Zea mays*, three annexin genes, *MeAnn7-9*, arelocated on chromosome 6 [39]. In cassava, both chromosomes 4 and 11 contain three annexin genes (Fig. 1). Thus, the conserved domain and the DNA structure reveal that *MeAnns* from *M. esculenta* belong to the annexin multifamily.

By analyzing the cis-acting elements present in the promoters, the possible biological functions of genes can be roughly inferred. In this study, the results indicate that *MeAnn* promoter regionspossess at least 3 hormone response-related cis-elements and 1 stress response-related cis-element [Fig. 5]. The stress- or hormone-related cis-acting elements also have been reported to exist in other annexin gene promoters. In maize annexin gene promoters, numerous of cis-elements, including ABRE, DRE, and LTRE, related to abiotic stress responses were found [39]. Hormone-related cis-elements, such as ABA, GA, or auxin recognition sites, and stress-related cis-acting elements, such as MYC or MYB recognition sites, were also found in the promoters of tomato annexins [33]. Hormone or stress treatments inducing the expression of annexin genes have been reported in *Indian mustard* [11], tomato (*Solanum pennellii*) [32], *Arabidopsis* [43, 48], and *alfalfa* [49]. The expression of annexin genes induced by auxin [50], SA [51], and methyl jasmonate (JA) [11] have also been reported. Here, at least one stress treatment was found to enhance the expression of several cassava annexin genes (Fig. 7-11). For instance, the expression of *MeAnn4*, *MeAnn6*, and *MeAnn8* in roots as well as *MeAnn4* and *MeAnn11* in leaves was highly enhanced by ABA treatment (Fig. 11). In cassava leaves, the expression of *MeAnn4-7* and *MeAnn9* was highly enhanced by GA and JA (Fig. 11), while the expression of *MeAnn4* and *MeAnn6* was enhanced by SA treatment (Fig. 11). However, it is hard to speculate the exact expression characteristics of a particular gene based only on cis-acting element analysis. For instance, nine *MeAnn* promoter regions contain putative ABA-responsive elements. However, in this study, only *MeAnn4*, *MeAnn6*, *MeAnn8* and *MeAnn11* were significantly enhanced by ABA (Fig. 5 and Fig. 11). JA-related cis-elements are found in the promoters of three *MeAnn*s, *MeAnn1*, *4 and 9.* However, the expression of only five *MeAnn*s, *MeAnn4-7* and *MeAnn9*, were highly enhanced by JA (Fig. 5 and Fig. 11). GA-related cis-elements are found in the promoters of seven *MeAnn*s, though the expression of only five *MeAnn*swere highly enhanced by GA (Fig. 5 and Fig.

11). Eight *MeAnn*s contained SA-related cis elements, but the expression of only three *MeAnn* genes were highly induced by SA (Fig. 5 and Fig. 11) . Similar results were also reported in tomato annexins [33]. Therefore, it is necessary to verify the functions of genes through detailed experiments.

The expression patterns of annexins in plant tissues or organs have been reported in a number of plant species. For instance, in *Arabidopsis*, *AtAnn1* was demonstrated to specifically express in mature pollen and germinated pollen [52, 53]. In *M. trancatula*, *MtAnn2* has been indicated to express in arbuscule-containing cells in mycorrhizal roots [54]. In *Solanum lycopersium*, *SlAnn4* is specifically expressed during fruit ripening, *SlAnn6* is specifically expressed in the stigma or ovary, and *SlAnn8* is specifically expressed in the stamen [33]. In *Tintorera* red grapes, *Annexin D1* was observed to express in fresh and raised grape fruits [55]. In *soybean*, annexin was differentially expressed in suspension-cultured soybean cells [56]. In our study, the expression patterns of *MeAnns* in 11 cassava tissues were detected [Fig. 7]. The heatmap showed that *MeAnn1*, *MeAnn2* and *MeAnn5* exhibit very high levels of expression in all the tested organs or tissues. By contrast, *MeAnn12* exhibits very low levels in whole of the detected organs or tissues. In leaves and midveins, all the *MeAnn*s except *MeAnn4* and *MeAnn12* are highly expressed. In lateral buds and s hoot apical meristems, all the *MeAnn*s except *MeAnn12* are highly expressed. The *MeAnn* expression patterns in fibrous roots are similar to those in root apical meristems, showing that their expression levels are lower than in other tissues. The *MeAnn* expression patterns in stems and petioles are also similar. By contrast, *MeAnn* expression patterns are differential in the remaining organs or tissues, such as OES (somatic organized embryogenics), FEC (friable embryogenic callus) and SR (storage roots) (Fig. 6).

Gene expression of annexin genes, in response to abiotic stresses such as drought, salt and cold, have been studied in some plants [27, 44, 48, 57]. In *M. sativa*, the annexin *MsAnn2* was observed to respond to salt and drought stresses [48]. *Arabidopsis* transcriptome analysis reported that annexins were upregulated under salt, cold and osmotic stresses [57]. This analysis showed that *AtAnn1i AtAnn6* and *AtAnn8* showed significantly increased expression in *Arabidopsis* under salt and dehydration stresses [27]. Subsequently, *AtAnn1* was intensively studied to reveal its major role in drought stress tolerance [10]. *AtAnn1* and *AtAnn4* were also revealed to interact with one another and in a light-dependent manner to regulate salt and drought stresses [23]. The role of *AtAnn8* in *Arabidopsis* salt and dehydration tolerance was also confirmed in a recent study [58]. In wild tomato, researchers have demonstrated that *SpUSP* functions in association with *SpAnn2* in drought stress signaling [59]. Overexpressing *SpAnn2* could enhance the salt and drought tolerance of transgenic tomato plants [34]. In rice, *OsAnn1* was reported to be associated with heat stress [44], while *OsAnn3* was reported to be associated with cold stress [36]. In our study, *MeAnn5* and *MeAnn9* in leaves were significantly upregulated by cold, drought and salt stresses (Fig. 7-9). Interestingly, the *MeAnn* expression patterns under cold stress were more similar to the expression patterns under $CaCl_2$ than under drought and salt stresses (Fig. 7-10). *MeAnn5*, *6* and *9* were significantly upregulated, while *MeAnn1* and *10* were downregulated at all six time points during cold stress to cassava leaves. In roots, *MeAnn1*, *9* and *12* were significantly downregulated under cold stress at all six time points (Fig. 7). *MeAnn4-6* and *9* were significantly upregulated, while *MeAnn2* and *10* were

downregulated under PEG-induced drought stress in leaves (Fig. 8). *MeAnn5-7* and *9* were significantly upregulated, while *MeAnn2* and *10* were downregulated under salt stress from 3-48 hours in cassava leaves (Fig. 9). Unlike the *MeAnn* gene responses to low temperature, no increase was observed in the transcription of any rice annexin genes in response to low temperature treatment, although decreased relative expression of *OsAnn2* and *OsAnn10* was observed in the untreated control [12]. In wheat, Breton et al. (2000) identified that there are four annexins that respond to low temperatures; they also showed their association with the membrane, but that this interaction was calcium-independent [8]. Gene expression of Annexin genes in response to drought has also been studied in *Arabidopsis*, which was also subject to oxidation-driven post-translational regulation [10]. Compared with the untreated control, the expression of *OsAnn6* and *OsAnn7* showed significant upregulation in response to NaCl treatment, while the expression of *OsAnn1* and *OsAnn10* showed a significant decrease under the same salt treatment [12]. The comparative discussion of expression patterns among cassava and other plants indicates that *MeAnn*s from cassava can respond to abiotic stresses, though with specific patterns for each gene, which are similar with other plants.

Conclusions

In this paper, annexin genes were searched in the cassava genome, systematically characterized and examined for their relationships and expression analysis. Twelve *MeAnn* genes were identified in the cassava genome. All MeAnns contain typical annexin domains. The *MeAnn*s were classified into six groups. All the MeAnns contain hormone or stress response-related cis-elements in their promoter regions. *MeAnn1*, *MeAnn2* and *MeAnn5* exhibit very high levels of expressions in all the detected organs or tissues. By contrast, *MeAnn12* exhibited very low levels in all the detected organs or tissues. Expression pattern analysis under abiotic stresses suggests that *MeAnn5* and *MeAnn9* have positive responses to abiotic stresses and calcium signaling and are induced by GA and JA in cassava leaves, while *MeAnn2* and *MeAnn10* have the opposite expression patterns under the same stresses. The expression patterns of *MeAnn*s under abiotic stresses are irregular in shoots. In roots, *MeAnn1* and *MeAnn9* have the similar expression patterns, as both are downregulated by cold, $CaCl_2$ and JA treatments, while the remaining genes display irregular expression patterns. These results will promote the subsequent functional investigations and the utilization of *MeAnns* in cassava and other plants.

Methods

Identification and Characterization of MeAnns from Cassava

To identify the annexin members in the cassava genome, the eight known annexins members *AtAnn1-8* from *A. thaliana* were used as probes for BLASTP of the *M. esculenta* V6.1 database. The MeAnn protein

sequences were further analyzed with the Pfam [60]and SMART[61] online tools. The physiological and biochemical characteristics of the MeAnn proteins, such as the molecular weight (MW), theoretical isoelectric point (pI), and hydrophilic mean (GRAVY), were analyzed with the ProtParam online tool [62]. The prediction of MeAnn protein subcellular location was performed with the CELLO2GO online tool[63].

Analysis of the Location and Distribution of MeAnns on Cassava Chromosomes

The chromosomal localization of each *MeAnn* gene and the chromosome sizes were obtained from the cassava open genome database (Bioproject: PRJNA234389). Then, the Mapinspect software (Mike Lischke, Berlin, Germany) was utilized to map the chromosomal localizations of the MeAnn genes. The Photoshop software CS (San Jose, CA, USA) was utilized to beautify and enrich the image.

MeAnn Phylogenetics, Structures and Motifs

The annexin protein sequences from cassava, *Arabidopsis*, rice and tomato were collected and multi-aligned with ClustalW. Then, a phylogenetic tree was produced using the MEGA7.0 software and based on the neighbor-joining method [64]. The *AtAnn* and *MeAnn* gene structureswere determined with the GSDS online software [65]. The MeAnn motifs were identified with the MEME Suite 4.12.0 online tool [66].

Analysis of the MeAnn Promoter Cis-Elements

To understand the cis-element distribution in the *MeAnn* promoters, 2000-bp sequences upstream of the initiation codon (ATG) of each *MeAnn* genewere downloaded from the public cassava genome database. The cis-elements in the MeAnn gene promoters were identified by searching the PlantCARE database website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The data were counted and processed with Microsoft Office Excel (One Microsoft Way, Redmond, WA98052-6399) to create tables and graphs.

MeAnn Expression Patterns in Cassava

To study the expression levels of the *MeAnn*s in different organs and tissues of cassava, the expression data for the 12 *MeAnn*s were downloaded from the public RNA-seq data of cassava (Bioproject ID PRJNA324539). The eleven different organs and tissues in cassava included the leaf, petioles, midveins, stems, shoot apical meristems (SAMs), lateral buds, fibrous roots, storage roots, root apical meristems (RAMs), somatic organized embryogenics (OES), and friable embryogenic calli (FEC). The heatmap was created with the OmicShare tools online (http://www.omicshare.com/tools).

Plant Materials and Treatments

The cassava cultivar SC8 was supplied by Cassava Research Center of the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Science. Thirty-day-cultivated SC8 cassava seedlings were individually placed in a 4℃ incubator for cold stress, in 300 mmol/L NaCl solution for salt stress, and in a 20% PEG-simulated dry environment for drought stress treatments. The samples were harvested at the treatment stages of 0, 3, 6, 9, 12 and 24 h, separately. The 30-day SC8 seedlings were individually placed in 50 μmol/L IAA solution, 2 mmol/L SA solution, 100 μmol/L GA_3 solution, 100 μmol/L JA solution, and 100 μmol/L ABA solution for 12 h for the hormone treatments. The 30-day SC8 seedlings were individually placed in 10, 20, 30, 40 and 50 mmol/L $CaCl_2$ for 12 h for the calcium treatments. The 30-day SC8 seedlings treated with H_2O were employed as blank controls. Each treatment was performed with three biological replicates. Collected cassava leaf, root and stem samples were outright frozen in liquid nitrogen and then saved at -80 ℃ until the follow-up experiments.

Total RNA isolation and qRT-PCR analysis

A RNAplant plus (TIANGEN, China) was used to extract total RNA from cassava. A PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan) was used to synthesize the first cDNA chain. qPCR SYBR Premix Ex Taq II (Takara, Japan) was used to conduct qRT-PCR analysis in a ABI 7900HT Fast Real-Time PCR System (ABI, USA).The relative expression levels of the *MeAnn* genes were evaluated by the $2^{-\triangle\triangle Ct}$ method and normalized by log2 values. The normalized relative expression data were used to construct a heatmap. Three technical replicates were settled. The qRT-PCR primers are listed in Table S2.

Abbreviations

GA: gibberellin ; JA: jasmonic acid; *Ann:*annexin gene; CBS: calcium binding sites; AA : amino acids; MW: molecular weights; GRAVY: grand average of hydropathicity; PL: Protein length; MW: Molecular weight of the amino acid sequence; GRAVY: grand average of hydropathicity; pI: theoretical isoelectric point; II: Instability index; AI: Aliphatic index; S: stable; U: unstable; Cytop: cytoplasm; Mito: mitochondria; Nuc: nucleus; Chlo: chloroplast; and Plasm: plasma membrane; OES: the somatic organized embryogenics; FEC: the friable embryogenic calli; FR: the fibrous roots; SR: storage roots; RAM: the root apical meristems; and SAM: shoot apical meristems.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

All of the authors consent to publish this article.

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

RL and YH were responsible for all aspects of the research, including experimental design, data acquisition and analysis, and manuscript preparation. YW, YZ, and TQ worked on the preparation of the studied materials, and qRT-PCR. YS, YY, JL and SF worked on primer design, and technical and informatics analyses of these genes. XH and JG were responsible for the programs and all experiments, critically revised the manuscript, and provided the final approval of the article.

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Tables

Table 1. A list of the twelve MeAnns identified in this study.

GenBank ID	Locus name	Chromosomal location	Genomic position (5'-3')	gDNA (bp)	CDS (bp)	P L (aa)	pI	MW (kDa)	GRAVY	II	AI	SP	Subcellular Location
MeAnn1	Manes.12G135200	Chr12	28973589-28976387	2799	951	316	6.78	35.93	-0.431	45.24U	91.77	-	Cytop, Mito
MeAnn2	Manes.18G068000	Chr18	5847458-5849722	2265	951	316	6.34	36.03	-0.517	24.66S	89.56	-	Cytop, Nuc
MeAnn3	Manes.04G165300	Chr04	28577361-28579255	1895	945	314	8.28	36.24	-0.454	38.97S	96.88	-	Cytop, Nuc
MeAnn4	Manes.11G003900	Chr11	452875-456136	3162	1065	354	9.29	40.36	-0.376	43.73U	97.32	-	Mito, Chlo
MeAnn5	Manes.11G003800	Chr11	450617-452202	1586	954	317	6.18	36.36	-0.554	49.70U	88.96	-	Cytop, Mito, Nuc
MeAnn6	Manes.04G165200	Chr04	28574893-28577069	2177	1149	382	5.8	43.78	-0.352	50.62U	92.43	+	Plasm, Nuc
MeAnn7	Manes.04G165100	Chr04	28571325-28573513	2189	957	318	6.67	35.89	-0.271	34.21S	100	-	Cytop, Nuc
MeAnn8	Manes.11G003700	Chr11	445585-448437	2853	957	318	5.94	35.88	-0.333	38.25S	96.01	-	Cytop, Nuc
MeAnn9	Manes.15G138400	Chr15	10627745-10630221	2477	948	315	9.46	35.79	-0.444	42.65U	91.14	-	Mito
MeAnn10	Manes.09G138900	Chr09	25831927-25833788	1862	1038	345	8.5	38.98	-0.379	45.06U	80.14	-	Mito, Nuc
MeAnn11	Manes.06G001900	Chr06	527782-530910	3129	951	316	9.26	35.61	-0.327	34.27S	90.51	-	Mito
MeAnn12	Manes.17G089200	Chr17	23235068-23236817	1750	930	309	9.17	35.26	-0.375	34.57S	93.46	-	Cytop, Mito, Nuc

Notes: PL, Protein length; MW, Molecular weight of the amino acid sequence; GRAVY, grand average of hydropathicity; pI, theoretical isoelectric point; II, Instability index; AI, Aliphatic index; S, stable; U, unstable; Cytop, cytoplasm; Mito, mitochondria; Nuc, nucleus; Chlo, chloroplast; and Plasm, plasma membrane.

Figures

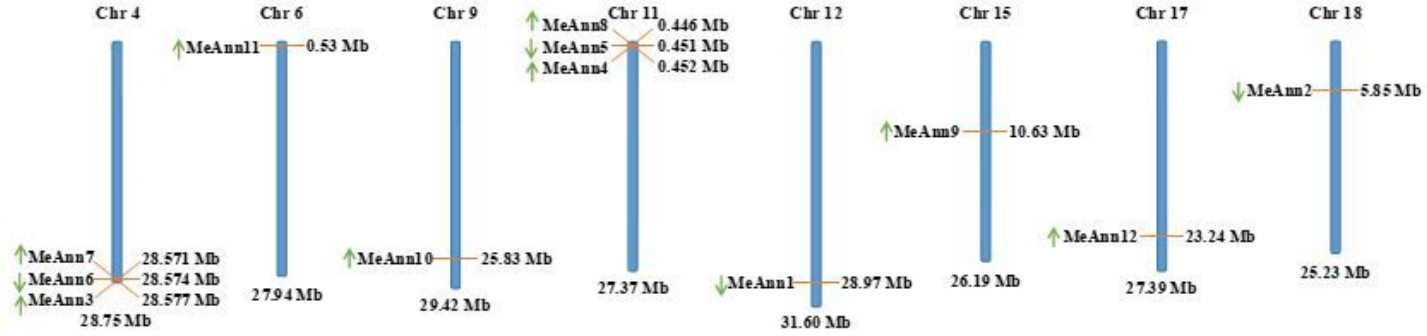

Figure 1

Location and distribution of the 12 MeAnns on the chromosomes. The chromosome number is labeled at the top of each chromosome. The gene orientation on the chromosomes is indicated by green arrows. The chromosome size is labeled at the bottom of each chromosome. The position of gene on the chromosome is labeled to the right of each gene.

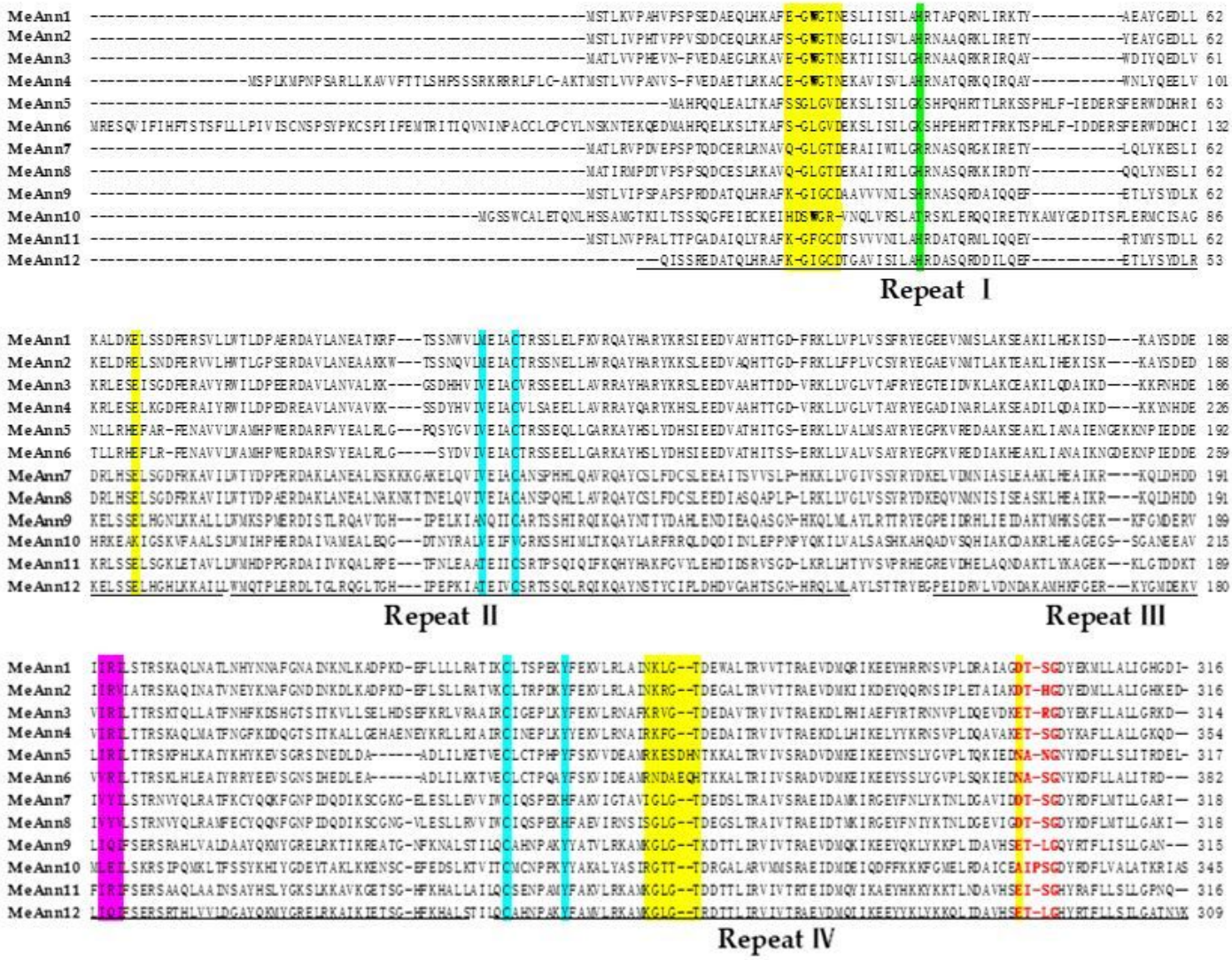

Figure 2

Multiple sequence alignment of the cassava MeAnns. The putative MeAnn repeats Ⅰ-Ⅳ are underlined; shaded yellow sequences denote the type Ⅱ calcium binding sites; shaded green sequences are the conserved heme-binding sites; shaded purple red sequences represent the actin binding sites; shaded light blue sequences are the putative S3 clusters related to redox reactions; red bold amino acids within Repeat IV are the putative GTP-binding motifs; and black bold amino acids within Repeat I indicate the putative phospholipid-binding sites.

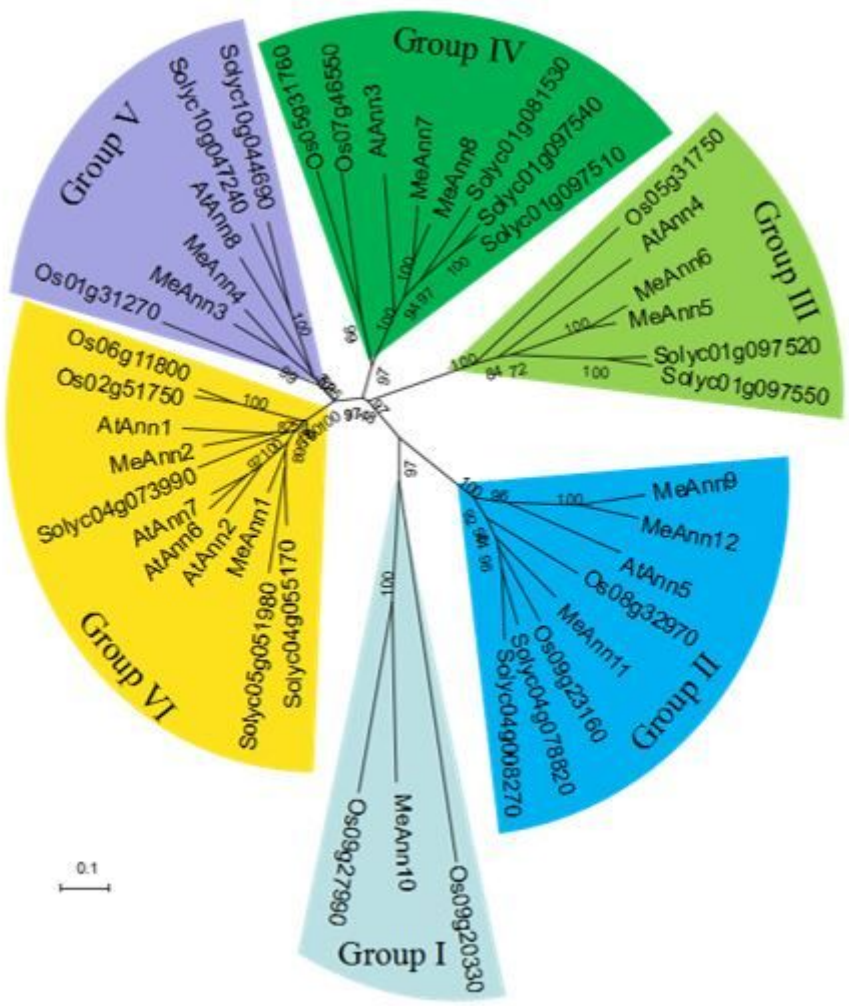

Figure 3

Phylogenetic relationships among MeAnns and other plant annexins. The prefixes At, Os, Solyc and MeAnn indicate proteins from O. sativa, S. lycopersicum, A. thaliana and M. esculenta, respectively.

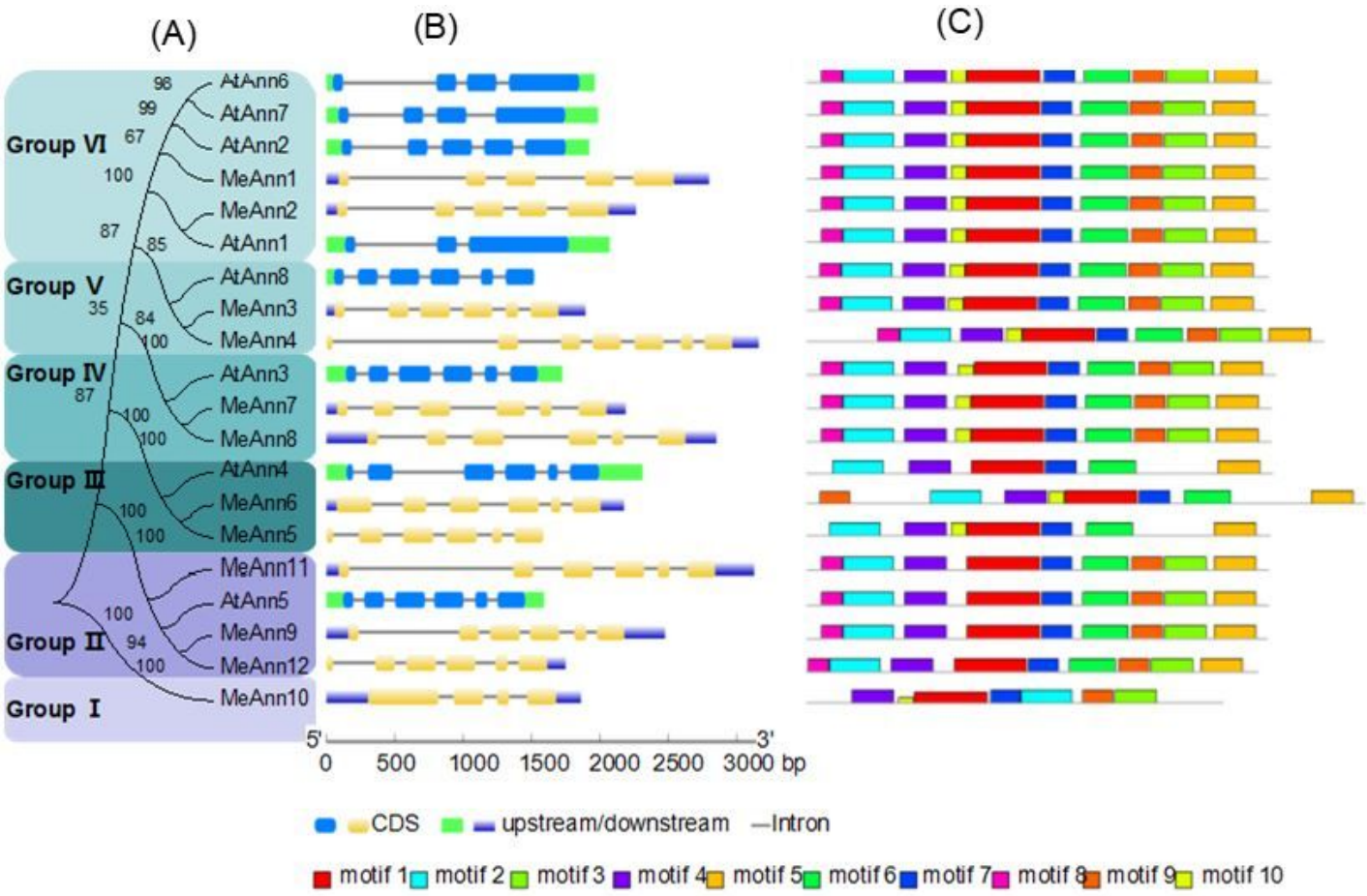

Figure 4

Comparative analyses of the phylogenetics, structures, and motifs of MeAnns and AtAnns. (A) The phylogenetic relationship of MeAnns and AtAnns. (B) The gene structures of MeAnns and AtAnns. (C) The motifs in the MeAnn and AtAnn proteins. The genes in Figures A, B and C are ordered in the same.

Type	Hormone-relative elements												Stress-relative elements												
cis-element	ABRE	ABRE4	as-1	CGTCA-motif	TGACG-motif	ERE	GARE-motif	TATC-box	TCA	TCA-element	TGA-box	TGA-element	ARE	DRE core	LTR	MBS	Myb	MYB recognition site	MYB	Myc	MYC	STRE	TC-rich repeats	W box	WRE3	WUN-motif
MeAnn1	4	1	3	3	3	6		1					1		1		3			2	2			1		2
MeAnn2	2	2				7							1						4	1			1	1	1	1
MeAnn3	2					7				1			1			1	1						1			1
MeAnn4	1		1	1	1	6	1	1				1	2				2		6		4	1				2
MeAnn5	2					1				1		1	2	1		1	2	2	1	1	3	1				2
MeAnn6	3	1				2	2		1	1			1						3		4	2			1	1
MeAnn7	3	1				2	2		1	1			1						4		4	1			1	1
MeAnn8	2					1				2		1	3	1	1	1	2	2	1	1	3	2		1		3
MeAnn9			2	2	2	5			1	2	1			1	3				6		2	4				
MeAnn10						6		1		1							2		4		7	2				5
MeAnn11	1	1				1	1	2		1		1	1		2	3	5		2	2	3			1	2	
MeAnn12						6		2								2	3				1	1	2	1		

Figure 5

The stress- and hormone-related cis-acting elements in the cassava MeAnn gene promoters.

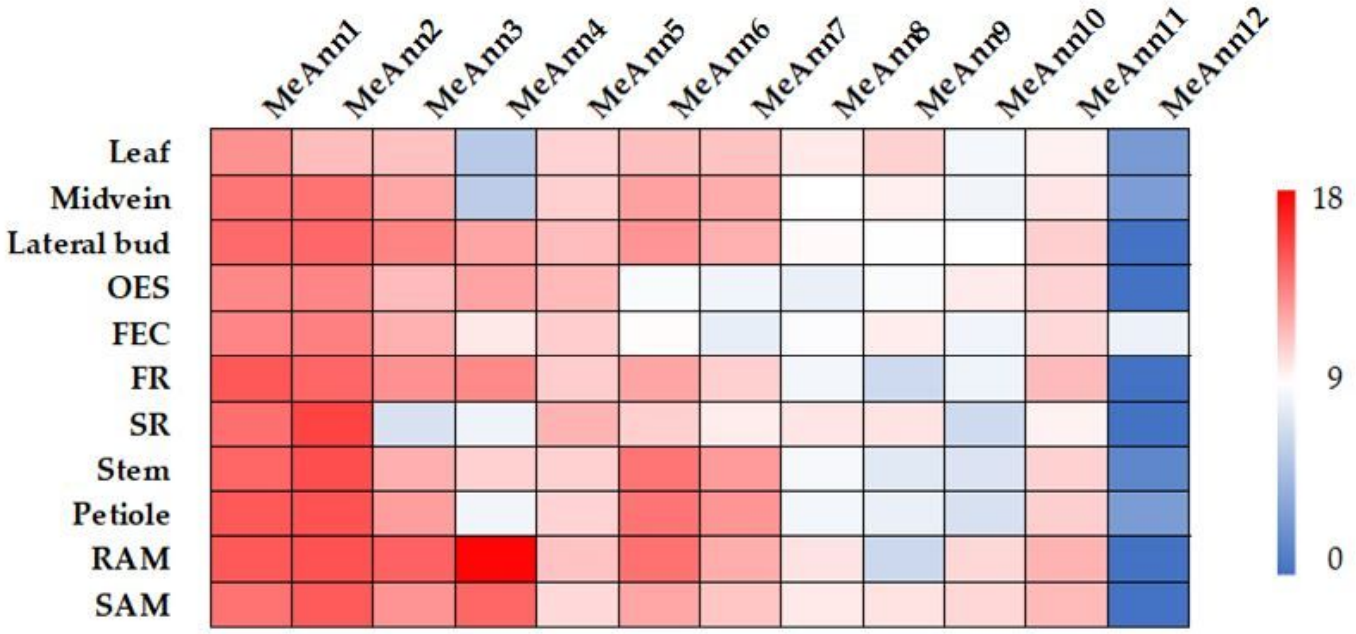

Figure 6

Gene expression levels of MeAnns in multiple cassava organs or tissues. OES, the somatic organized embryogenics; FEC, the friable embryogenic calli; FR, the fibrous roots; SR, storage roots; RAM, the root apical meristems; and SAM, shoot apical meristems. The gradient bar on the right indicates the Log2-corrected FPKM values (fragments per kilobase of exons per million reads mapped).

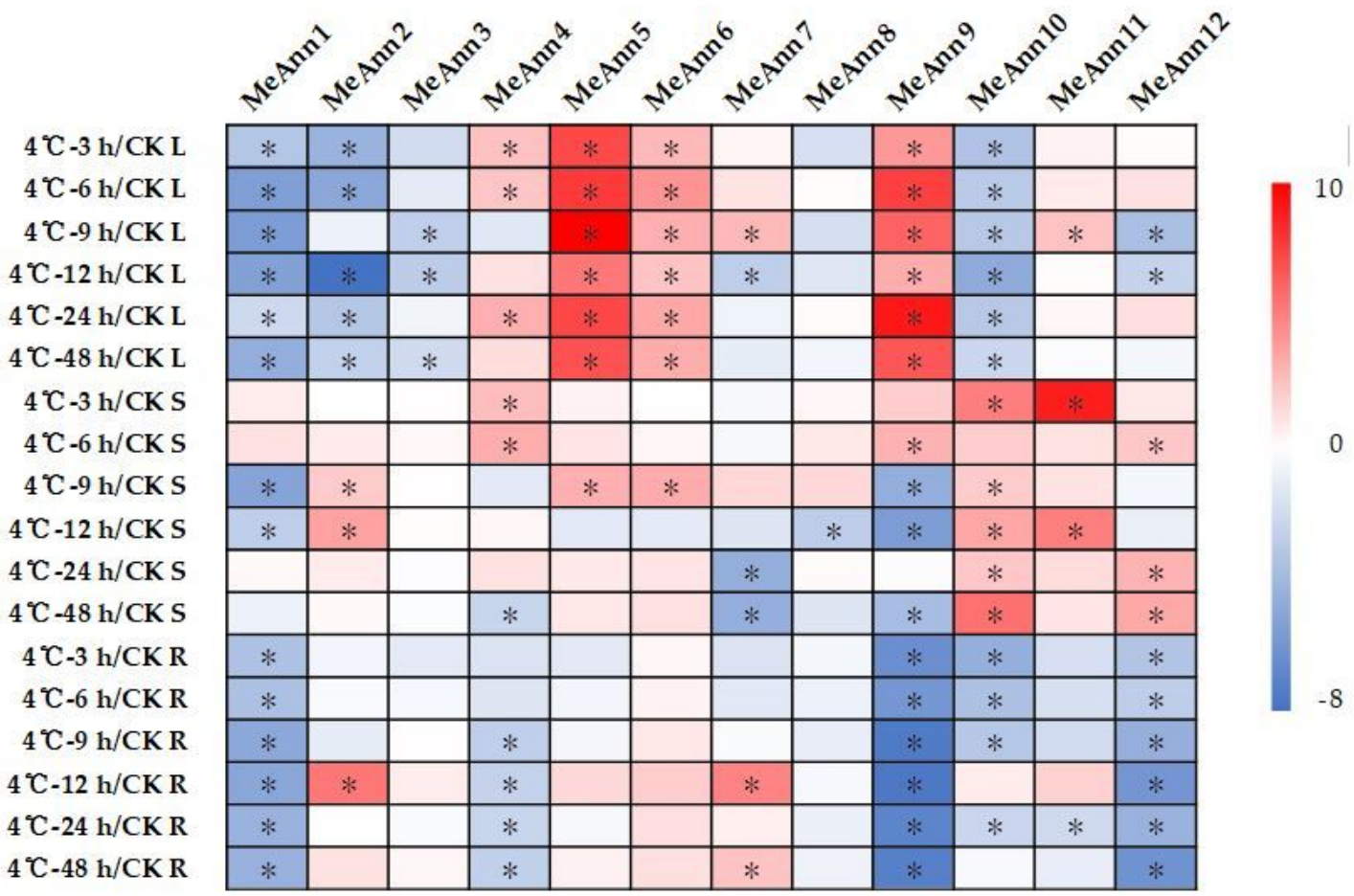

Figure 7

Expression patterns of MeAnns in leaves, shoots and roots under 4°C cold stress. The gradient bar on the right indicates the Log2-normalized relative gene expression levels. L, leaves; S, shoots; and R, roots. The stars indicate values greater than 2-fold.

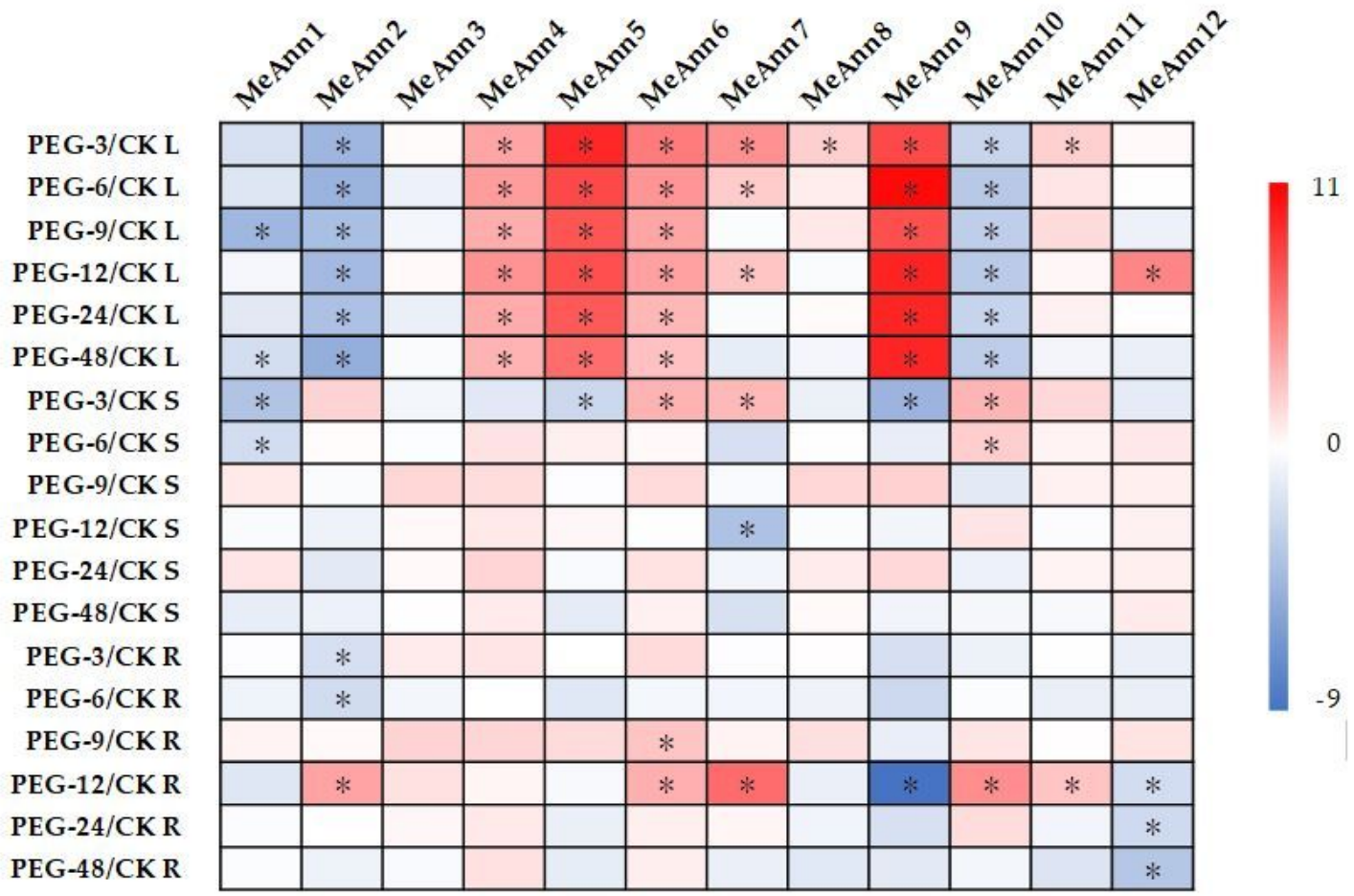

Figure 8

Expression patterns of MeAnns in leaves, shoots and roots under 20% PEG6000-induced drought stress. The gradient bar on the right indicates the Log2-normalized relative gene expression levels. L, leaves; S, shoots; and R, roost. The stars indicate values greater than 2-fold.

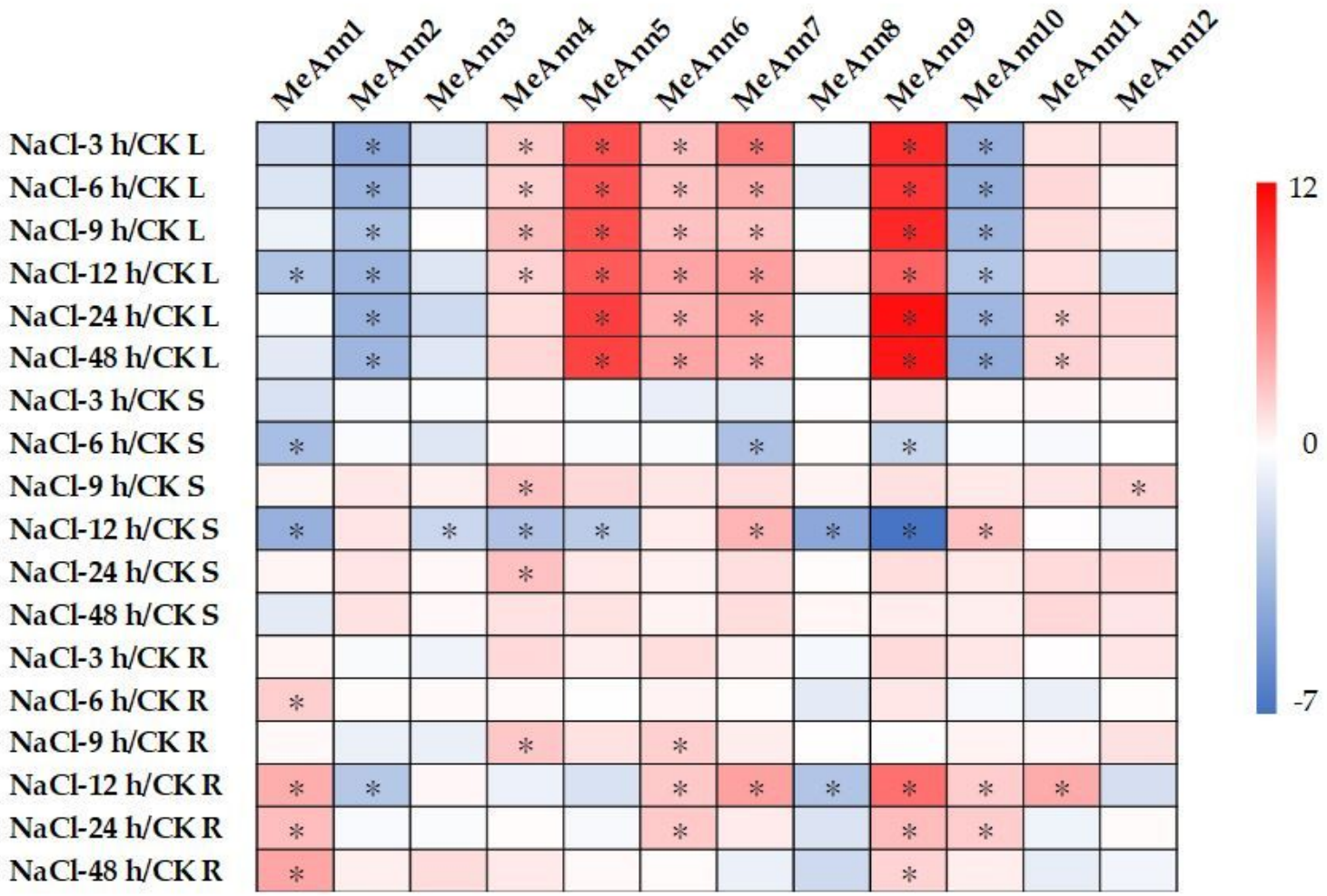

Figure 9

Expression patterns of MeAnns in leaves, shoots and roots under 300 mmol/L NaCl-induced salt stress. The gradient bar on the right indicates the Log2-normalized relative gene expression levels. L, leaves; S, shoots; and R, roost. The stars indicate values greater than 2-fold.

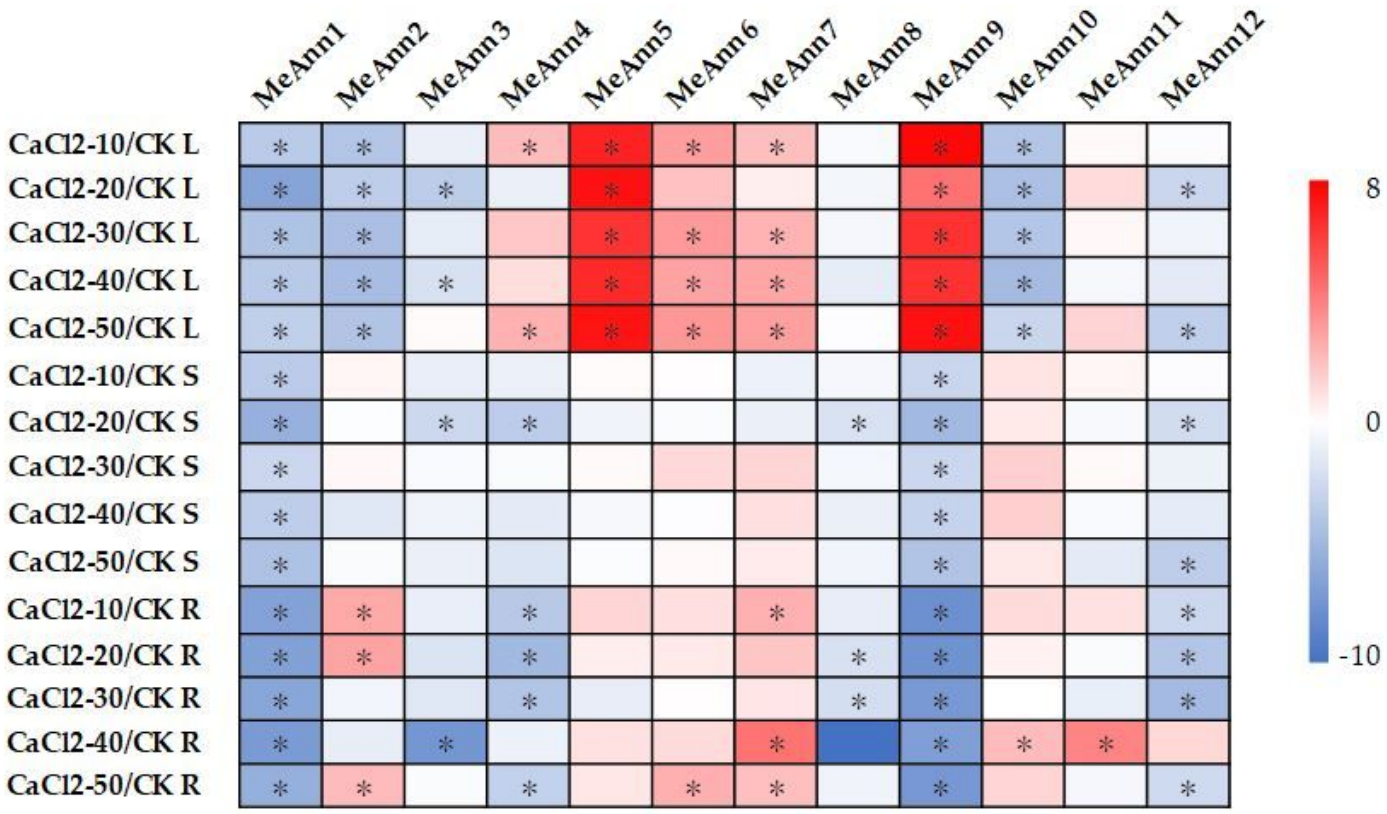

Figure 10

Expression patterns of MeAnns in leaves, shoots, and roots under CaCl2-induced Ca2+ signaling. The gradient bar on the right indicates the Log2-normalized relative gene expression levels. L, leaves; S, shoots; and R, roost. The stars indicate values greater than 2-fold.

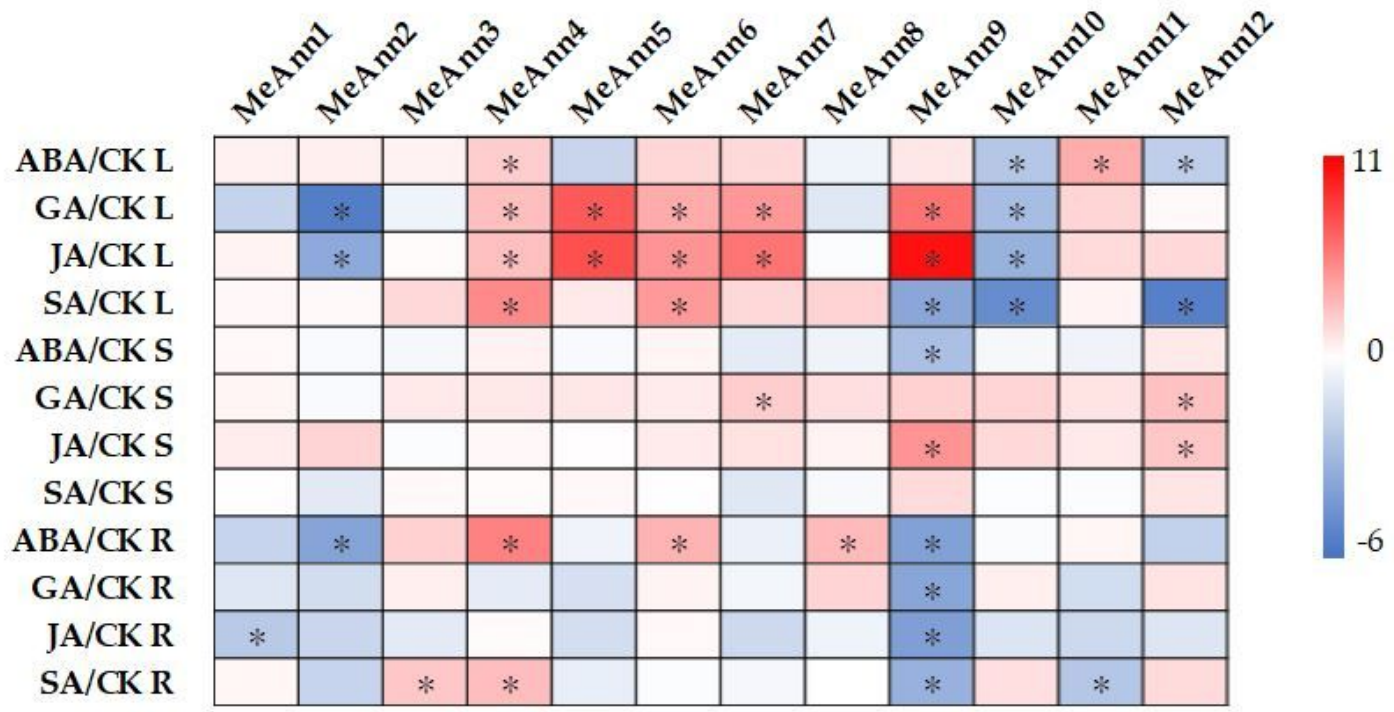

Figure 11

Expression patterns of MeAnns in leaves, shoots, and roots under different hormone treatments. The gradient bar on the right indicates the Log2-normalized relative gene expression levels. L, leaves; S, shoots; and R, roost. The stars indicate values greater than 2-fold.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS.xls