

Genome-wide identification of pepper (*Capsicum annuum* L.) AAT gene family and expression analysis under salt stress

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Abstract

Background

The AAT gene is a widely present and highly important amino transferase in organisms, connecting energy metabolism and nitrogen metabolism. Its role in the response of other plants to salt stress has been studied, but it is not yet clear in peppers.

Results

This study identified a total of 22 *CazAAT* genes from pepper varieties "Zunla-1". According to subcellular localization predictions, 18 *CaAAT* genes are distributed in cytoplasmic solutes, 2 in mitochondria, 1 in chloroplasts, and the remaining 1 gene potentially localizes to both mitochondria and chloroplasts. Phylogenetic analysis indicates that *CazAAT13* belongs to subfamily IV, alongside the salt stress-relieving AAT genes from *Aeluropus lagopoides* (Poaceae) and *Dendrobium officinale*. Under salt stress treatment, the expression levels of *CazAAT11* and *CazAAT13* were significantly upregulated. After exogenous Asp application, the expression of *CazAAT11* was further enhanced, whereas the upregulation of *CazAAT13* was reduced. These findings suggest that *CazAAT11* and *CazAAT13* may alleviate salt stress by regulating Asp metabolism. Furthermore, co-expression analysis suggests that regulating carbon and nitrogen metabolism could be another critical pathway through which *CazAAT11* and *CazAAT13* alleviate salt stress.

Conclusions

This study systematically analyzes the *CaAAT* gene family, providing preliminary insights for further research on pepper's response to salt stress.

Background

Capsicum annuum is one of the important vegetables and seasonings globally, rich in compounds such as capsaicin, vitamins, and carotenoids that benefit health [1]. In China, the total planting area of peppers reached 214,740 hectares in 2019, surpassing Chinese cabbage and tomatoes to become the largest vegetable in terms of cultivation area. Additionally, the total output value of peppers reached 250 billion yuan, accounting for 11.36% of China's total vegetable output value. Peppers have become the most widely planted vegetable and the most consumed spicy seasoning in China [2]. The current types of plantations in China are mainly divided into open-air planting and greenhouse cultivation. At present, greenhouse planting accounts for about 26% of the total area, but its proportion of planting land is constantly increasing. Although greenhouse cultivation greatly increases the yield of peppers per unit area, the extensive use of chemical fertilizers also exacerbates the degree of soil salinization [3].

Salt stress is a critical abiotic factor limiting the growth and yield of peppers. Peppers are moderately sensitive to salt, with yield loss of approximately 14% for every 1.0 dS/m increase in salinity [4]. The ion toxicity caused by salt stress is mainly achieved through Na^+ , and the excessive accumulation of Na^+ is a major cause of plant wilting and necrosis [5]. The high concentration of Na^+ in soil directly inhibits the absorption of water and nutrients by plant roots [6]. Lack of water and nutritional imbalance can further trigger primary pressures such as osmotic stress and ion stress [7]. The increase in reactive oxygen species levels induced by primary stresses exacerbates the damage caused by salt stress [8]. In order to adapt to salt stress, plants have also developed a series of coping strategies that combine endogenous signaling pathways with exogenous signals [9]. Previous studies have indicated that mitochondrial respiration processes, which provide reducing agents and ATP are essential for plant strategies to alleviate salt stress [10].

The tricarboxylic acid cycle (TCA), which links energy metabolism with carbon (C) and nitrogen (N) metabolism, makes mitochondrial respiration at the center of plant metabolic networks [11, 12]. Aspartic acid (Asp) and glutamic acid (Glu)

achieve the above characteristics of the TCA by linking amino acid metabolism with carbon metabolism of TCA-related organic acids and glycolysis pathways [13, 14]. When faced with salt stress, plants exhibit significant consumption of TCA-related organic acids and accumulation of related amino acids in tissues such as roots and leaves, with a significant increase in Asp content [13–16]. In salt-stressed plants, changes in Asp content are associated with alterations in protein metabolism [17].

Aspartate aminotransferase (AAT, also known as glutamate oxaloacetate transaminase (GOT)) mediates the N transfer of Asp and Glu, and its involvement in the transamination to form oxaloacetate is closely related to the glycolysis and oxidative phosphorylation pathways [18, 19]. In plants, AAT is mainly located in subcellular regions such as cytoplasm, mitochondria, and plastids, playing an important role in maintaining the dynamic balance of C and N and synthesizing other amino acids [20]. Meanwhile, existing evidence strongly suggests that AAT family genes are involved in abiotic stress tolerance [19, 20]. For instance, in cucumber, the accumulation of proline (Pro) is closely related to the increased AAT transcription, which enhances the plant's tolerance to salt stress [21]. In mulberry trees, AAT adjusts the plant's N metabolism ability to convert a large amount of free N into amino acids, thereby controlling ammonia toxicity in cells while regulating osmotic pressure to achieve plant protection under salt stress [22].

Although the AAT gene plays a crucial role in plant resistance to salt stress, its related functions have not yet been studied in peppers. Based on the existing transcriptome data of our research group, we identified the AAT gene and conducted a comprehensive analysis of the AAT family genes (*CazAAT*) included in the entire genome of "*Zunla-1*". A total of 22 *CazAAT* family genes were identified, and their physicochemical properties, phylogenetic relationships, conserved domains, and homeotropic elements were analyzed. Our results provide valuable information for understanding the classification and putative function of the *CazAAT* gene.

Material and methods

Plant materials, Stress and Amino Acid Treatment

Soaked and disinfected the plump seeds in 15% NaClO for 10 minutes, followed by a 24-hour soak in distilled water. After that, they were germinated in a constant temperature incubator at 28°C, and subsequently transplanted them into seedling bowls containing substrate (vermiculite: nutrient soil = 3:1) for propagation, and the seedlings were grown in a light cultivation room with parameters set to 28°C/23°C (day/night), a light cycle of 16 h/8 h (bright/dark) and a light intensity of 20,000 lx [23]. When the seedlings developed 6–8 true leaves, we applied salt stress and amino acid treatment to seedlings that were free from pests and diseases and had similar growth. The control group seedlings were cultured with distilled water, while the experimental group seedlings were cultured with 200 mM NaCl solution. In addition, half of the seedlings in both the control and experimental groups were treated by spraying leaves with 0.4 mM aspartic acid solution. Treated 5 seedlings per group, with three replicates for each treatment. Collected the leaves of seedlings at 0, 3, 6, 12, and 24 hours after treatment. Samples were immediately frozen in liquid nitrogen, then stored them at -80°C for further processing.

Identification and Physicochemical Analysis of AAT Genes Family in Pepper

The whole genome and proteomic data of *Zunla-1* (v2.0) and *CM334* (v1.55) were obtained from Sol Genomics Network (https://solgenomics.net/organism/Capsicum_annuum/genome, accessed on 14 October 2024) [24]. The whole genome and proteomic data of *Capsicum chinense* (v1.2) were obtained from PEPPER GENOME (<http://peppergenome.snu.ac.kr/download.php>, accessed on 14 October 2024) [23]. The Hidden Markov Model (HMM) index (PF00155) of the AAT conserved domain was obtained from the Pfam database (<http://pfam.xfam.org/>, accessed on 16 October 2024) Query and obtain [25]. Subsequently, the Simple HMM Search tool of TBtools (v2.133) was used to screen for AAT genes in peppers, with an E-value threshold of less than 1e-5 [26]. Afterwards, we utilized SMART (<http://smart.embl-heidelberg.de/smart/batch.pl>, accessed on 16 October 2024) and Batch CD Search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 16 October 2024) [27] to identify the structural

domains of each AAT gene, and the AAT genes with the domain Aminotran_1_2, predicted by both methods, were selected as candidate genes. Finally, we utilized Euk-mPLOC 2.0 separately (<http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/>, accessed on October 17, 2024) [28] and TMHMM Server v2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>, accessed on October 17, 2024) [29] to predict the subcellular localization and number of transmembrane domains (TM) of AAT candidate genes. The physicochemical properties of candidate genes, including protein length, molecular weight, number of amino acids, and isoelectric point were analyzed using the ExPASy tool (<https://web.expasy.org/protparam/>, accessed on October 17, 2024) [30].

Multiple Sequence Alignment and Phylogenetic Analysis of AAT Genes

To investigate the phylogenetic relationship of AAT genes, sequences were collected from three pepper cultivars (*Zunla-1*, *Caz*; *CM334*, *Cam*; *Capsicum chinense*, *Cac*), tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), eggplant (*Solanum melongena*), *Arabidopsis thaliana*, *Zea mays* (vNAM-5.0), rice (*Oryza sativa*) (vIRGSP-1.0.59), and cotton (*Gossypium hirsutum*). The whole genome data of tomato (vTAG4.1), tobacco (v2017), and eggplant (v4.1) were obtained from Sol Genomics Network (https://solgenomics.net/organism/Capsicum_annuum/genome, accessed on 14 October 2024) [24]. The whole genome data of *Arabidopsis thaliana* (vTAIR10), *Zea mays* (vNAM-5.0), and Rice (vIRGSP-1.0.59) were downloaded from Ensembl Plants (<https://plants.ensembl.org/>, accessed on 14 October 2024) [31]. The complete genome data of Cotton (TM-1 ZJU v2.1) were obtained from CottonGen (<https://www.cottongen.org/>, accessed on 14 October 2024) [32]. Utilized the Two Sequence Files feature of TBtools for blastp analysis to obtain AAT homologous protein sequences of various plants. Finally, a phylogenetic tree was constructed using MEGA7's Neighbor-Joining method based on the Poisson model. Bootstrap analysis was performed with 1000 replicates to test the reliability of the tree, and missing data were corrected by partially deleting 95% of the data [33].

Conservative Domain, Gene Structure, and Conserved Protein Motif Analysis of AAT Family Genes

To identify the conserved domains of the *CaAAT* genes, we utilized SMART (<http://smart.embl-heidelberg.de/smart/batch.pl>, accessed on 16 October 2024) [34] and Batch CD Search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 16 October 2024) [27] to obtain information related to these domains. The conserved motif information of *CaAAT* genes was found in Multiple Em for Motif Elicitation (<https://meme-suite.org/meme/tools/meme>, accessed on 16 October 2024) [35]. Finally, the Gene Structure View (Advanced) tool of TBtools (v2.133) was used to integrate and visualize the phylogenetic trees, motif information, and conserved domain information of the three peppers varieties [26].

Collinearity Analysis and Cis-acting Element Analysis of AAT Family Genes

The chromosome length, gene density, and location information of the *CazAAT* genes were obtained based on the *Zunla-1* (v2.0) whole genome annotation file downloaded from Sol Genomics Network [24]. Used TBtools (v2.133) for One Step MC ScanX, Text Merge for MCScan, Text Transformat for MicroSyteny Viewer, and GXF Gene Position&info Extract tool to calculate and organize the collinearity of the *CazAAT* genes, Visualization was accomplished using the Advanced Circos tool [26].

To analyze the cis-acting elements, we extracted the upstream 2000bp sequence of the *CazAAT* genes as promoters using the Gtf/Gff3 Sequence Extract and Fasta Extract (Basic) tools of TBtools (v2.133). Cis-acting elements were predicted using the online website Plant Cis-Acting Regulatory Element (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 16 October 2024) [36], and finally the results were visualized with TBtools (v2.133) Simple Biosquare Viewer tool [26].

Gene Expression Analysis

Total RNA was extracted from the leaves of the pepper variety “Xiangla 107” (variety ID: 20914; seeds provided by Henan OULAND Seed Industry Co., Ltd., Zhengzhou, China) using the FG-Plant RNA Isolation Kit (NONAZYME, China) according to

the instructions. The extracted RNA was converted into cDNA to serve as the template for qRT-PCR. The specific primers and internal reference gene sequences are listed in Table S1.

qRT-PCR was carried out using ChamQ Universal SYBR Qpcr Master Mix (Vazyme, China) according to the manufacturer's protocol. All samples were independently repeated three times, and the relative expression levels of *CazAATs* were calculated using the $2^{-\Delta\Delta C_t}$ [37].

Transcriptome analysis

Expression data from 78 samples subjected to four abiotic stresses—cold, heat, salt, and osmotic stress—were retrieved from the SRP187794 project in the Sequence Read Archive (SRA) database of NCBI [38]. The HeatMap tool in TBtools (v2.133) was used to visualize the expression variations of *CazAAT* genes under these conditions.

Results

Identification and Physicochemical Properties Analysis of AAT Gene Family in Pepper

To investigate the *AAT* genes involved in the salt stress response, we analyzed existing transcriptome data. The results showed that *CazAAT11* and *CazAAT13* were significantly upregulated in roots and leaves, with *CazAAT13* showing greater upregulation (Fig. 1A, B). Additionally, *CazAAT13* was found to interact with genes whose expression levels were markedly altered under salt stress. Based on these findings, *CazAAT11* and *CazAAT13* were selected as candidate genes for further analysis (Fig. 1C).

A total of 68 homologous genes in *Zunla-1* (v2.0), *CM334* (v1.55), and *Capsicum chinense* (v1.2) based on the protein sequence of *CazAAT13*. Among them, 22 homologous genes were found in *Zunla-1* (v2.0) and *CM334* (v1.55), and 24 homologous genes were found in *Capsicum chinense* (v1.2). The *CaAAT* proteins consist of 317 to 621 amino acids, with molecular weights ranging from 35,843.95 to 69,821.78 Da. The theoretical isoelectric points (pI) of the *CaAAT* proteins range from 5.33 to 9.55, with 36 *CaAAT* genes having pI less than 7 and 32 homologous genes having pI greater than 7, showing a relatively dispersed distribution. The prediction of *CaAATs*' subcellular localization showed that most of them were distributed in the cytoplasm, five proteins (*CamAAT13*, *CamAAT20*, *CazAAT11*, *CazAAT17*, and *CacAAT12*) existed in mitochondria and three proteins (*CamAAT15*, *CazAAT13* and *CacAAT02*) were distributed in chloroplasts. Interestingly, three proteins (*CamAAT14*, *CazAAT12*, and *CacAAT24*) may be existed in either mitochondria or chloroplasts. This distribution of *CaAATs* is consistent with the role in the tricarboxylic acid cycle. According to the predictive analysis of TM region, most *CaAATs* do not contain transmembrane structures, four (*CamAAT05*, *CazAAT03*, *CazAAT05*, and *CacAAT17*) containing one transmembrane region and only one (*CacAAT19*) containing two transmembrane regions (Table 1).

Table 1
The information of the AAT gene family.

Gene Rename	Sequence ID	Chromosome	Number of aa	Molecular Weight (Da)	pI	Subcellular Prediction	TM Domain
CamAAT01	CA00g09810	Chr00	353	40176.33	8.65	Cytoplasm	0
CamAAT02	CA00g16570	Chr00	383	42330.71	6.38	Cytoplasm	0
CamAAT03	CA01g02040	Chr01	485	54867.71	5.93	Cytoplasm	0
CamAAT04	CA01g02050	Chr01	484	54819.7	5.83	Cytoplasm	0
CamAAT05	CA01g03950	Chr01	548	59965.09	7.1	Cytoplasm	1
CamAAT06	CA02g24710	Chr02	472	53477.06	8.23	Cytoplasm	0
CamAAT07	CA03g17110	Chr03	325	36530.24	6.34	Cytoplasm	0
CamAAT08	CA03g34590	Chr03	399	44086.85	5.65	Cytoplasm	0
CamAAT09	CA04g06460	Chr04	413	46229.02	8.51	Cytoplasm	0
CamAAT10	CA04g16840	Chr04	474	53904.36	8.79	Cytoplasm	0
CamAAT11	CA06g27380	Chr06	433	48134.25	5.5	Cytoplasm	0
CamAAT12	CA07g14280	Chr07	420	46503.69	5.55	Cytoplasm	0
CamAAT13	CA07g14630	Chr07	425	47053.71	7.21	Mitochondrion	0
CamAAT14	CA08g03040	Chr08	423	46665.32	6.62	Chloroplast, Mitochondrion	0
CamAAT15	CA08g06160	Chr08	456	50238.57	9.55	Chloroplast	0
CamAAT16	CA08g07680	Chr08	486	54816.65	7.92	Cytoplasm	0
CamAAT17	CA09g16680	Chr09	438	49420.61	6.11	Cytoplasm	0
CamAAT18	CA10g01500	Chr10	433	47525.14	6.71	Cytoplasm	0
CamAAT19	CA10g05940	Chr10	455	51220.59	6.73	Cytoplasm	0
CamAAT20	CA10g10790	Chr10	423	47990.83	8.65	Mitochondrion	0
CamAAT21	CA12g15660	Chr12	433	48109.07	6.34	Cytoplasm	0
CamAAT22	CA12g18900	Chr12	422	47177.72	6.56	Cytoplasm	0
CazAAT01	Capana01g000175	Chr01	485	54851.71	5.93	Cytoplasm	0
CazAAT02	Capana01g000176	Chr01	484	54819.7	5.83	Cytoplasm	0
CazAAT03	Capana01g000451	Chr01	548	59965.09	7.1	Cytoplasm	1
CazAAT04	Capana02g002931	Chr02	472	53477.06	8.23	Cytoplasm	0
CazAAT05	Capana03g002485	Chr03	531	58701.34	6.76	Cytoplasm	1
CazAAT06	Capana04g000721	Chr04	473	53393.59	8.83	Cytoplasm	0
CazAAT07	Capana04g001943	Chr04	462	51382.14	8.52	Cytoplasm	0
CazAAT08	Capana06g000029	Chr06	407	45076.56	5.33	Cytoplasm	0
CazAAT09	Capana07g001709	Chr07	421	46739.91	5.91	Cytoplasm	0

Gene Rename	Sequence ID	Chromosome	Number of aa	Molecular Weight (Da)	pI	Subcellular Prediction	TM Domain
CazAAT10	Capana07g001710	Chr07	420	46503.69	5.55	Cytoplasm	0
CazAAT11	Capana07g001814	Chr07	425	47053.71	7.21	Mitochondrion	0
CazAAT12	Capana08g000430	Chr08	462	50748.01	8.8	Chloroplast, Mitochondrion	0
CazAAT13	Capana08g000820	Chr08	453	49732.85	9.26	Chloroplast	0
CazAAT14	Capana08g000980	Chr08	486	54816.65	7.92	Cytoplasm	0
CazAAT15	Capana09g000213	Chr09	317	35843.95	5.95	Cytoplasm	0
CazAAT16	Capana10g000085	Chr10	433	47525.14	6.71	Cytoplasm	0
CazAAT17	Capana10g001251	Chr10	423	48008.93	8.87	Mitochondrion	0
CazAAT18	Capana10g001736	Chr10	455	51220.59	6.73	Cytoplasm	0
CazAAT19	Capana10g001745	Chr10	500	56116.89	5.99	Cytoplasm	0
CazAAT20	Capana12g000452	Chr12	414	46169.58	6.94	Cytoplasm	0
CazAAT21	Capana12g000857	Chr12	436	48363.09	6.67	Cytoplasm	0
CazAAT22	Capana12g001792	Chr12	481	51415.9	8.73	Cytoplasm	0
CacAAT01	CC.CCv1.2.scaffold1196.21	Chr08	486	54875.67	7.18	Cytoplasm	0
CacAAT02	CC.CCv1.2.scaffold1372.3	Chr08	453	49794.86	9.26	Chloroplast	0
CacAAT03	CC.CCv1.2.scaffold138.39	Chr03	360	40274.67	7.03	Cytoplasm	0
CacAAT04	CC.CCv1.2.scaffold225.134	Chr12	422	47162.71	6.56	Cytoplasm	0
CacAAT05	CC.CCv1.2.scaffold3.39	Chr04	495	55340.73	8.74	Cytoplasm	0
CacAAT06	CC.CCv1.2.scaffold303.74	Chr04	470	52932.09	8.73	Cytoplasm	0
CacAAT07	CC.CCv1.2.scaffold328.9	Chr12	441	47142.93	8.45	Cytoplasm	0
CacAAT08	CC.CCv1.2.scaffold38.4	Chr10	368	41715.7	8.95	Cytoplasm	0
CacAAT09	CC.CCv1.2.scaffold449.81	Chr01	484	54732.63	5.93	Cytoplasm	0
CacAAT10	CC.CCv1.2.scaffold449.82	Chr01	485	54823.7	5.93	Cytoplasm	0
CacAAT11	CC.CCv1.2.scaffold454.4	Chr09	344	38629.58	8.48	Cytoplasm	0
CacAAT12	CC.CCv1.2.scaffold475.37	Chr07	455	50379.91	7.56	Mitochondrion	0
CacAAT13	CC.CCv1.2.scaffold617.11	Chr07	421	46777.01	5.76	Cytoplasm	0
CacAAT14	CC.CCv1.2.scaffold617.12	Chr07	420	46503.65	5.45	Cytoplasm	0
CacAAT15	CC.CCv1.2.scaffold617.13	Chr07	422	46957.12	5.4	Cytoplasm	0
CacAAT16	CC.CCv1.2.scaffold656.17	Chr12	399	44256.1	5.41	Cytoplasm	0
CacAAT17	CC.CCv1.2.scaffold683.12	Chr01	548	60021.2	8.24	Cytoplasm	1
CacAAT18	CC.CCv1.2.scaffold734.49	Chr02	472	53474.04	8.41	Cytoplasm	0
CacAAT19	CC.CCv1.2.scaffold774.34	Chr09	621	69821.78	5.85	Cytoplasm	2

Gene Rename	Sequence ID	Chromosome	Number of aa	Molecular Weight (Da)	pI	Subcellular Prediction	TM Domain
<i>CacAAT20</i>	<i>CC.CCv1.2.scaffold780.7</i>	Chr10	433	47520.04	6.27	Cytoplasm	0
<i>CacAAT21</i>	<i>CC.CCv1.2.scaffold83.33</i>	Chr05	455	51125.51	7.57	Cytoplasm	0
<i>CacAAT22</i>	<i>CC.CCv1.2.scaffold857.44</i>	Chr03	394	43430.22	5.55	Cytoplasm	0
<i>CacAAT23</i>	<i>CC.CCv1.2.scaffold924.7</i>	Chr05	500	56136.92	5.81	Cytoplasm	0
<i>CacAAT24</i>	<i>CC.CCv1.2.scaffold98.8</i>	Chr08	462	50699.95	8.8	Chloroplast, Mitochondrion	0

Phylogenetic Analysis of CaAAT Genes

To further investigate the homology of *CaAAT* genes in other species, we additionally obtained the corresponding AAT genes from the whole genome data of tomato, tobacco, eggplant, *Arabidopsis thaliana*, maize, rice, and cotton. A phylogenetic tree (Fig. 2) was constructed based on the Neighbor-Joining (NJ) method in MEGA7.0. We identified a total of 133 homologous genes in the above-mentioned species, and we renamed these homologous genes for subsequent analysis (Table S2). The phylogenetic tree shows that the AAT gene family in these plants can be divided into four subfamilies: I, II, III, and IV. Interestingly, most of the *CaAAT* genes belong to subfamily I, while *CazAAT13*, *CamAAT15*, and *CacAAT02*, along with *OsP37833*, *ZmGOT1* and *AtASP3-1*, belong to subfamily IV. *OsP37833* and *ZmGOT1* have been reported to alleviate salt stress in poaceae (*Aeluropus lagopoides*) and *Dendrobium officinale*, respectively. This suggests that *CazAAT13*, *CamAAT15*, and *CacAAT02* may have similar functions in alleviating salt stress and can serve as candidate genes for further investigation.

Analysis of Conserved Domain, Gene Structure and Conserved Protein Motif of AAT Family Genes

Based on the phylogenetic tree, we analyzed the conserved motifs and domains of *CaAAT* homologous genes from three cultivars of peppers, which are closely related to the functions of the genes. MEME analysis revealed ten conserved motifs, *CaAAT* homologous genes generally contain Motif 6. The types and distribution of conserved motifs among *CaAAT* genes in the same subfamily are relatively consistent; however, the types and distribution of conserved motifs in different subfamilies vary greatly. These different conserved motifs may lead to functional differences in *CaAAT* genes in different subfamilies (Fig. 3B). The prediction results of the main conserved domain Aminotran_1_2 (AAminotran_1_2) using Batch CD Search and SMART are basically consistent, and the distribution positions of Aminotran_1_2 domains of all *CaAAT* homologous genes are similar (Fig. 3C-D). Among them, *CazAAT13*, *CamAAT15*, and *CacAAT02* contain only Motif 6 and all have an additional low-complexity region at the front of the sequence. This structural distinction may explain their separation from other *CaAAT* genes in the phylogenetic tree of multiple species (Fig. 2). *CaAATs* only have one typically conserved Aminotran_1_2 domain and are essentially devoid of transmembrane domains. The Aminotran_1_2 domain sites within the same subfamily are highly conserved, indicating that *CaAATs* play an important role in the evolutionary process.

Chromosomal Locations and Collinearity of CaAATs

The *CazAAT* gene family is evenly distributed on the *Zunla* genome, except for Chr05 and Chr11, and is distributed on all other chromosomes (Fig. 4). The *CazAAT* gene family exhibits three collinearity relationships: *CazAAT01*, *CazAAT14* and *CazAAT18* form one relationship; *CazAAT04* and *CazAAT06* constitute another; and *CazAAT09* and *CazAAT16* were linked in the third relationship (Fig. 4). *CazAAT13* did not show any collinearity relationship, indicating a more distant genetic relationship with other *CazAAT* genes.

Analysis of Cis-acting Elements of AAT Gene Promoter in Pepper

Due to the important role of cis-acting elements in gene transcription regulation, we predicted the cis-acting elements of the *CazAAT* genes. According to the results, *CazAAT* genes mainly include three types of cis-acting elements with different functions (Fig. 5). Firstly, the cis-acting elements related to stress resistance mainly include anaerobic and hypoxic elements such as ARE and GC motif and drought resistant elements such as MBS. Secondly, the homeopathic elements related to hormone response mainly include auxin responsive elements such as TGA element, gibberellin responsive elements such as P-box, abscisic acid responsive elements such as ABRE, and MeJA responsive elements such as TGACG and CGTCA motif. Finally, it also includes photoresponsivity elements such as G-box motif and I-box elements (Table S3).

Expression of *CazAAT11* and *CazAAT13* in Pepper under Salt Stress

qRT-PCR analysis showed that both *CazAAT11* and *CazAAT13* exhibited an upregulation trend in response to salt stress in roots. Among them, *CazAAT13* responded more rapidly, with its expression level gradually increasing between 3 to 12 hours and stabilizing by 24 hours, while *CazAAT11* only showed an increase in expression level at 24 hours. Interestingly, the application of exogenous Asp moderated the upregulation trend of *CazAAT13* during the 3 to 12 hours of salt stress, but accelerated the response of *CazAAT11* until the effect of exogenous Asp gradually diminished after 24 hours (Fig. 6A, D). In stems, *CazAAT11* showed a strong response to both salt stress and exogenous Asp at 12 hours, while the expression level of *CazAAT13* was less affected by these treatments, although it still showed an upregulation trend after 24 hours (Fig. 6B, E). In leaves, both *CazAAT11* and *CazAAT13* continuously respond to salt stress from 6 hours onwards, with their expression levels steadily increasing. Exogenous Asp still demonstrated a slowing effect on the upregulation trend of *CazAAT13*, while exerting a stronger role in upregulating the expression level of *CazAAT11*. This differential response may be related to the method of Asp application (Fig. 6C, F). These findings effectively highlight the roles of *CazAAT11* and *CazAAT13* genes, as well as the impact of exogenous Asp, in enhancing salt stress resistance in pepper, consistent with the transcriptome expression trends, further supporting their involvement in stress adaptation.

The response of *CazAAT* genes in pepper under abiotic stress.

To further explore the response of *CazAAT* to abiotic stress, we investigated the expression of *CazAAT* in pepper leaves under four types of stress. As depicted in Fig. 7, the expression levels of the majority of *CazAAT* genes were upregulated under the four stresses, with the most notable response being to salt stress. When confronted with different stresses, the response times of each *CazAAT* gene varied, mostly reaching the peak at 6 hours or 72 hours. Additionally, *CazAAT09*, *CazAAT11* and *CazAAT13* exhibited significant responses to all four stresses (Fig. 7E). It is speculated that *CazAATs*, particularly *CazAAT11* and *CazAAT13*, might play a positive regulatory function in the resistance of peppers to cold stress, heat stress, salt stress, and osmotic stress.

Co-expression Patterns of *CazAAT* Genes in Pepper Under Abiotic Stress.

To explore the co-expression of *CazAAT* genes in pepper under abiotic stress, we calculated the correlation between the expression levels of *CazAAT* genes and all other genes in the genome. We identified the genes co-expressed with each *CazAAT* gene. Using the String database, the interaction relationships between the *CazAAT* genes and their co-expressed genes were clarified, and the results were visualized with. Notably, *CazAAT09*, *CazAAT11*, and *CazAAT13* were found to be central nodes in the co-expression network in all four stress conditions (Fig. 8). Additionally, the co-expression networks of the four stress conditions were closely associated with the 2-oxoglutarate metabolic process, aspartic acid metabolic process, α -amino acid metabolic process, and glutamate metabolic process (Table S4). These findings further demonstrate that *CazAAT11* and *CazAAT13* can alleviate the damage caused by abiotic stress by regulating the carbon and nitrogen metabolism of pepper.

Discussion

Pepper is a widely cultivated crop, but it is susceptible to damage from salt stress during its growth [39]. When adapting to salt stress, pepper primarily regulates gene expression. However, there is relatively little research on the role of the *AAT*

gene in pepper adaptation. In the current study, based on bioinformatics analysis, a total of 68 AAT genes were identified across three cultivars of peppers: 22 in "Zunla-1," 22 in CM334, and 24 in *Capsicum chinense*. According to the existing transcriptome results from our research group, six AAT genes in Zunla-1 were upregulated in response to salt stress. Systematic evolutionary analysis and subcellular localization results indicate that among the six differentially expressed AAT genes, four (*CazAAT04*, *CazAAT14*, *CazAAT16*, and *CazAAT20*) are located in the cytoplasm and belong to subfamily I, which includes most AAT homologous genes. The other two genes, *CazAAT11* and *CazAAT13*, are located in the mitochondria and chloroplasts, respectively; *CazAAT11* belongs to subgroup II, along with *AtASP1-1* and *AtASP1-2*, while *CazAAT13* belongs to subgroup IV, alongside *AtASP3-1*. These localization results are consistent with previous studies [18]. Notably, *CazAAT13* contains a significant number of light-responsive elements, suggesting that it may alleviate the effects of abiotic stress by regulating plant photorespiration [40, 41].

According to the analysis of the conserved domains and cis-acting elements of *CazAAT11* and *CazAAT13*, on the one hand, *CazAAT11*, *CazAAT13*, and their homologous proteins all contain the conserved domain of Aminotransferase_1_2, which is closely associated with glycolysis-related supplementary reactions and anaerobic response elements (ARE). This indicates that *CazAAT11* and *CazAAT13* may increase plant energy supply levels under abiotic stress by regulating glycolysis and the TCA cycle, thereby mitigating damage [20, 42, 43]. On the other hand, it was found that *CazAAT11* and *CazAAT13* also contain cis-acting elements associated with stress and plant hormone signaling, and their expression levels were upregulated in leaves, suggesting that *CazAAT11* and *CazAAT13* play important roles in responding to abiotic stress.

Previous studies in Arabidopsis, soybean, rice, and tobacco have demonstrated that the AAT gene located in mitochondria and chloroplasts plays a central role in N metabolism. Overexpression of AAT in plastids can reduce Asp levels and increase the content of asparagine (Asn), glutamate (Glu), and alanine (Ala) [19, 44–46]. Additionally, studies on cucumbers have also shown that the AAT gene plays an important role in Glu supplementation and the increase of free proline (Pro) levels, and free Pro levels positively correlating with the plant's salt tolerance [21]. The accumulation of salt resistant free amino acids is also beneficial for plant osmotic regulation [22, 47]. Supplementing exogenous Asp alleviated the wrinkling of pepper leaves, which may be due to supplementing substrates for AAT, promoting the production of amino acids such as Glu and Pro, and alleviating the damage of salt stress to pepper leaves.

According to the qRT-PCR results, the expression of *CazAAT13* in roots was rapidly upregulated under salt stress, likely due to the direct effect of the salt solution. In contrast, the upregulation shows a delay in leaves, although *CazAAT13* showed a slight upregulation peak at 3 hours, which possibly due to the sensitivity and vigorous metabolism of young leaves. However, the upregulation of *CazAAT11* expression in roots, stems, and leaves occurred later. The study by Kang et al. also showed that *CazAAT11* and *CazAAT13* exhibited an upregulated expression trend in response to salt stress in peppers [38]. Interestingly, the application of exogenous Asp slowed the upregulation trend of *CazAAT13* expression, but accelerated the upregulation of *CazAAT11*. This suggests that *CazAAT11* and *CazAAT13* may have different preferences in the metabolic feedback regulation of Asp. Specifically, *CazAAT11* may prefer the metabolism of Asp, while *CazAAT13* may be more involved in the generation of Asp. The delayed upregulation of *CazAAT11* may be associated with a deficiency of Asp, while *CazAAT13* may play a role in Asp supplementation. Following exogenous Asp application, *CazAAT11* immediately exhibited immediate and significant upregulation, while the upregulation of *CazAAT13* was alleviated. These findings further support the hypothesis that *CazAAT11* and *CazAAT13* alleviate salt stress damage in peppers by regulating Asp metabolic pathways. In addition, *CazAAT11* and *CazAAT13* also respond to cold stress, heat stress, and osmotic stress. *CazAAT11* and *CazAAT13* may also participate in functional pathways such as the 2-oxoglutarate metabolic process and aspartate metabolism, playing a crucial role in regulating carbon and nitrogen metabolism in peppers, thereby alleviating the damage caused by abiotic stress. In summary, *CazAAT11* and *CazAAT13* can be considered as candidate genes for improving abiotic stress resistance in peppers, providing valuable targets for future research.

Conclusions

This study systematically identified and analyzed the AAT gene family in peppers at the whole-genome level, integrating genome and transcriptome data. A total of 68 *CaAAT* genes were identified across three pepper cultivars. Under salt stress, the expression levels of *CazAAT11* and *CazAAT13* in Zunla-1 were significantly upregulated. The application of exogenous aspartic acid further augmented the expression of *CazAAT11* but attenuated that of *CazAAT13*. This indicates that although both *CazAAT11* and *CazAAT13* are involved in alleviating salt stress damage in peppers, they demonstrate distinct tendencies in the metabolic feedback regulation of Asp. In addition, *CazAAT11* and *CazAAT13* also respond to cold stress, heat stress, and osmotic stress. This research provides valuable insights into the mechanisms of pepper's response to salt stress and offers a basis for further functional exploration of the *CazAAT11* and *CazAAT13* genes.

Declarations

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Author contributions

X.H. and D.Z.: Bioinformatic analysis, conducted experiments, data curation, methodology, validation, visualization and writing-original draft; X.L., Y.H., N.X., Z.Y. (Yike Zhong) and Z.P.: performed the experiments, formal analysis, validation, methodology; Z.C. and S.Y.: Bioinformatic analysis, validation; F.H., Q.S., L.Q. and H.Y.: Formal analysis, validation, visualization, writing-review & editing; B.L. and W.L.: provided pepper seeds, resources, funding acquisition, project administration; H.S. and Y.Z. (Yamin Zhang): Conceptualization, formal analysis, funding acquisition, investigation, project administration, resources, supervision, writing-original draft, conceived and designed the experiments. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data from this work can be found in Pepper Genome Platform and Solanaceae Genomics Network. The transcriptomic datasets analyzed during this study are available in the NCBI GEO repository under accession number GSE132824 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132824>) and the SRA project SRP187794.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

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Figures

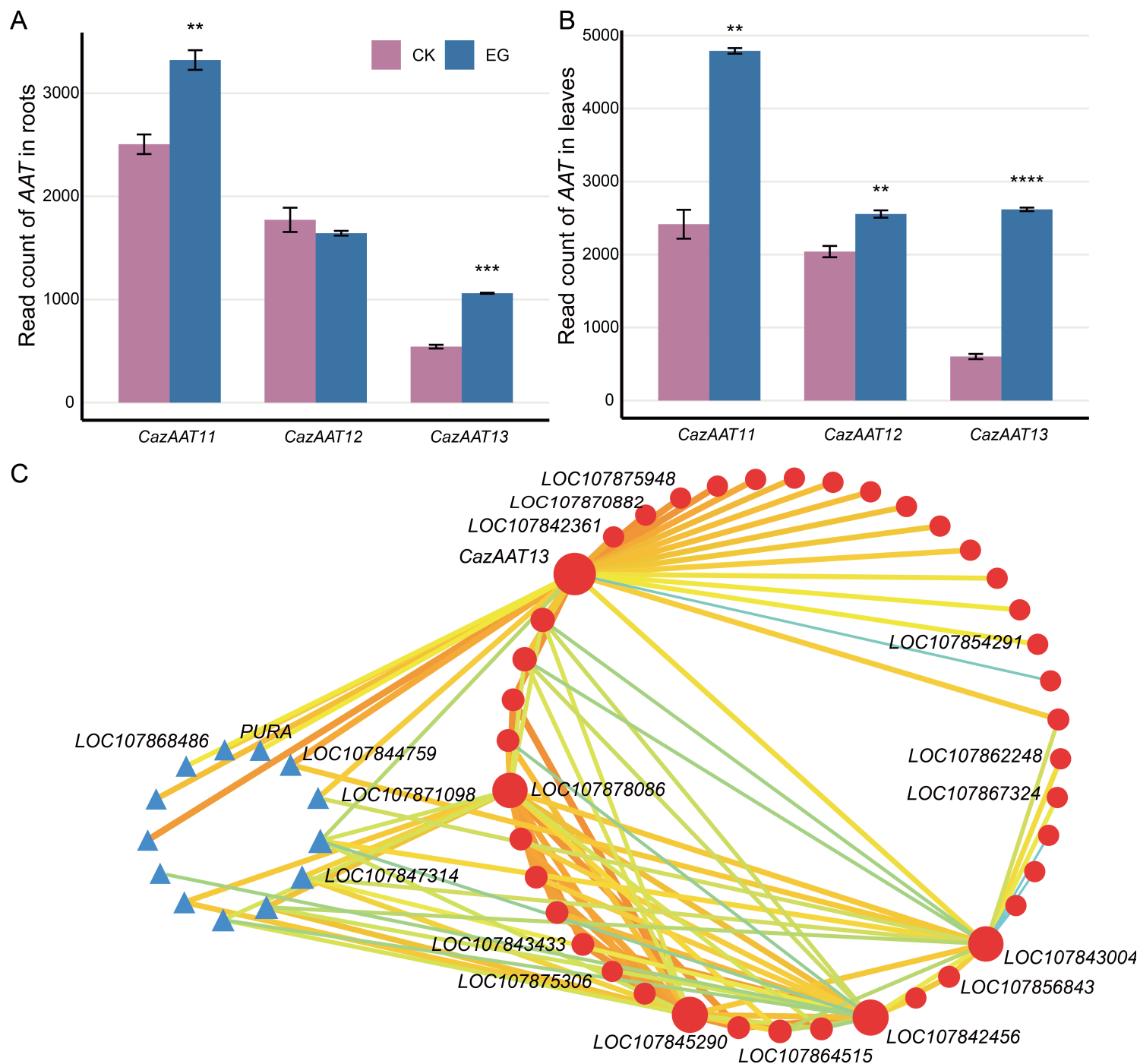


Figure 1

The response of AAT genes to salt stress in pepper P20914. Alterations in the expression of AAT genes in roots (A) and leaves (B) on the sixth day after treating. (C) Interactions network of differentially expressed genes under salt stress.

Note: CK, control check; EG, treat with 200mM NaCl solution.

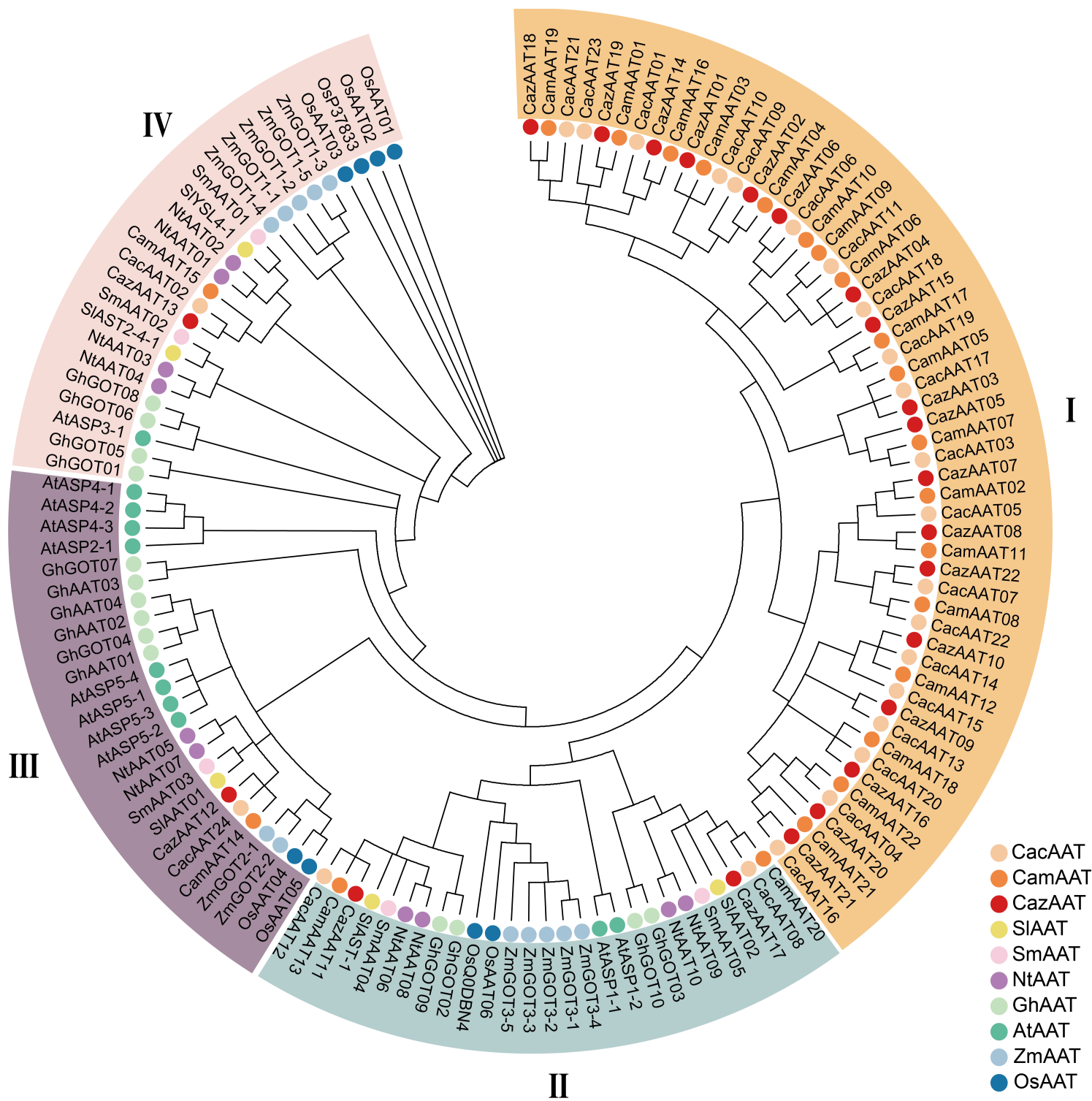


Figure 2

Phylogenetic relationships of AAT genes from pepper (Caz; Cac; Cam), *A. thaliana* (At), *Z. mays* (Zm), *S. lycopersicum* (Sl), *N. tabacum* (Nt), *G. hirsutum* (Gh), *S. melongena* (Sm) and *O. sativa* (Os).

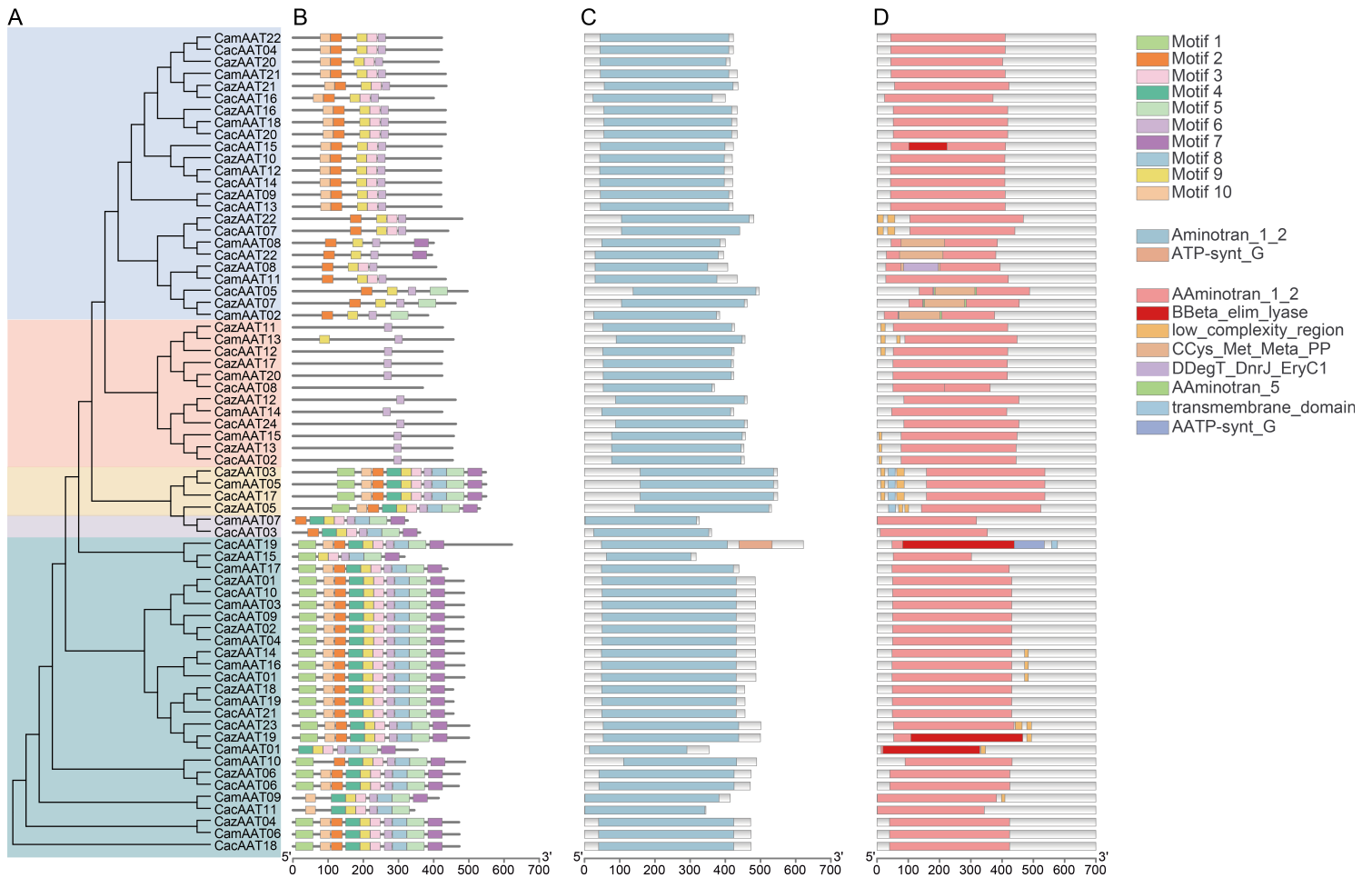


Figure 3

Phylogenetic tree, conserved motif and conserved domain of CaAAT genes. (A) Phylogenetic tree constructed according to the NJ method using MEGA 7 (B) motifs of three pepper species, and different motifs were represented by different colored boxes (C) Schematic diagram of conserved domains through Batch CD Search analysis (D) Schematic diagram of gene structures through SMART analysis.

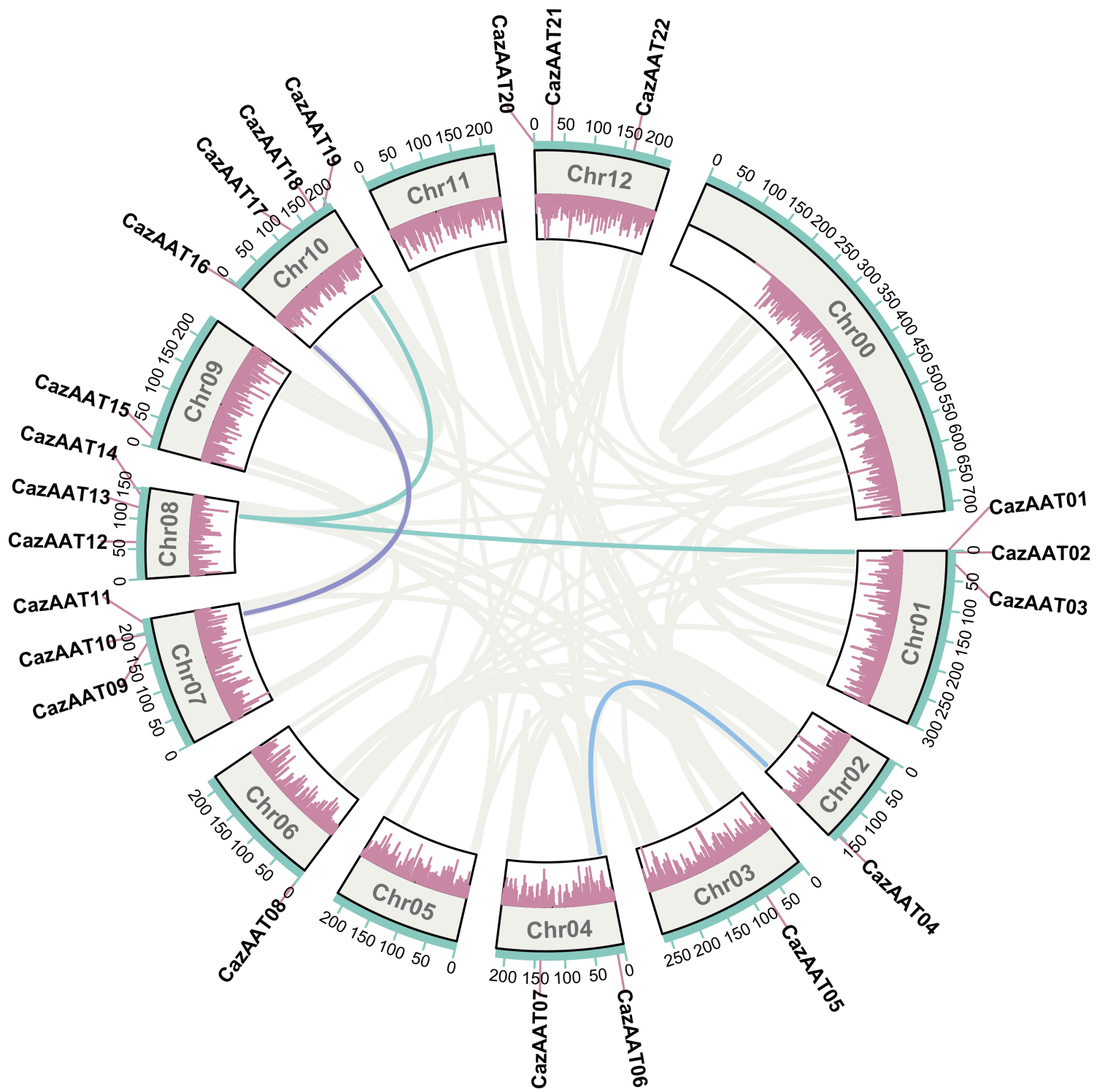


Figure 4

Chromosomal locations and collinearity of CazAAT genes.

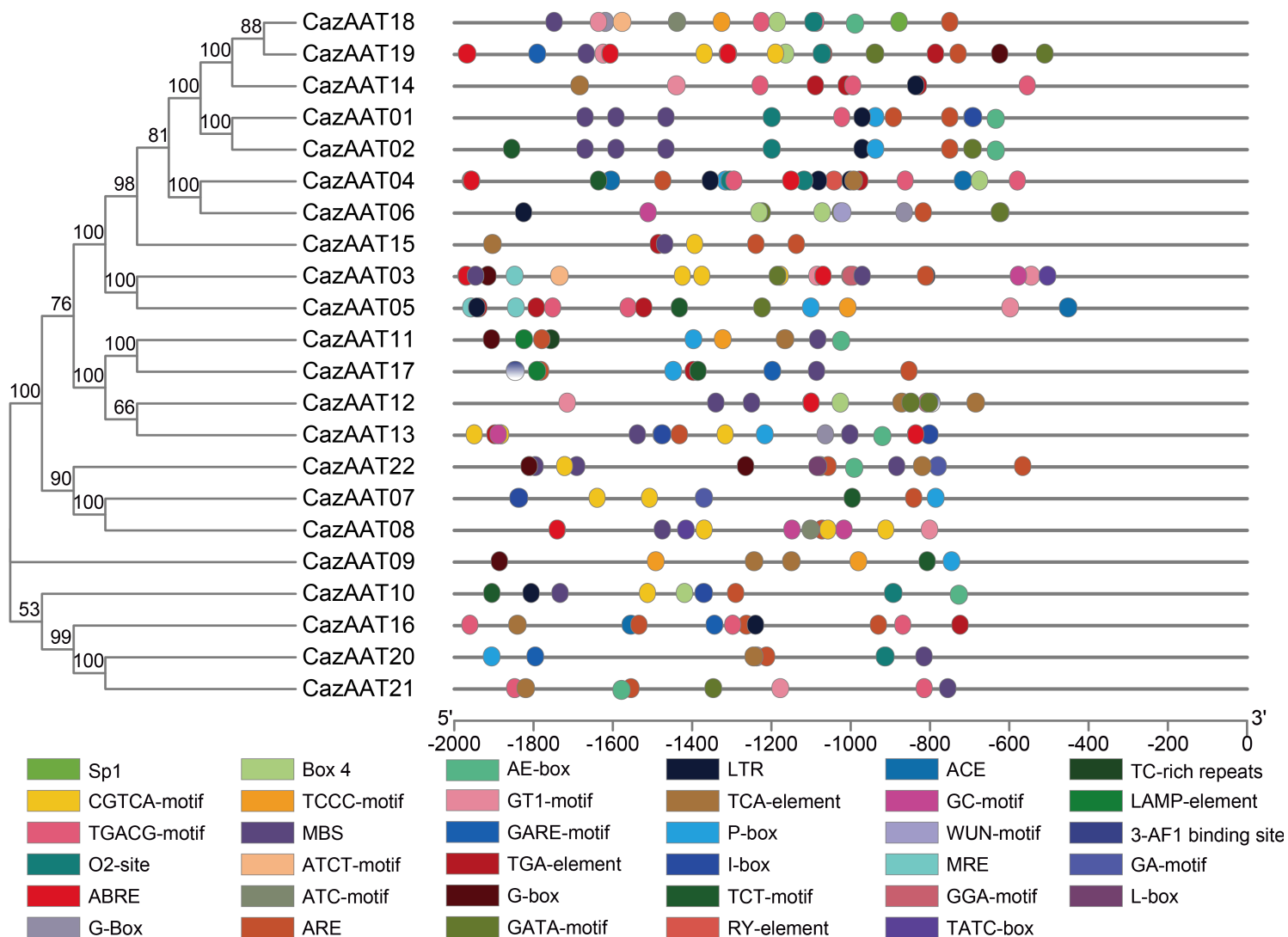


Figure 5

Analysis of Cis-acting element in the AAT gene family of *Zunla-1*.

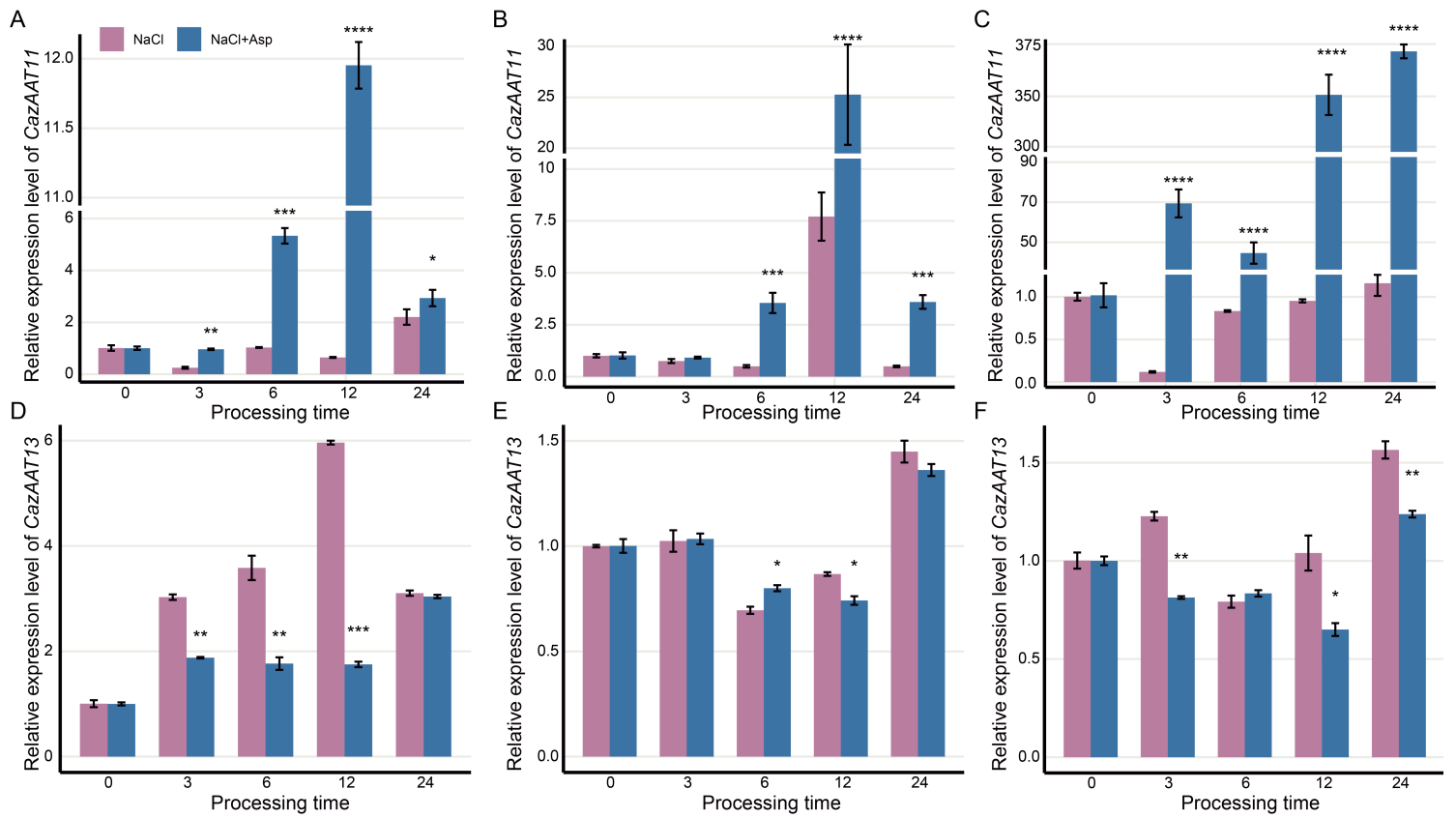


Figure 6

Expression of *CazAAT11* and *CazAAT13* in pepper under Salt Stress. The relative expression levels of *CazAAT11* (A-C) and *CazAAT13* (D-F) gene in roots (A, D), stems (B, E), and leaves (C, F), as well as the effect of exogenous Asp on *CazAAT11* and *CazAAT13* expression.

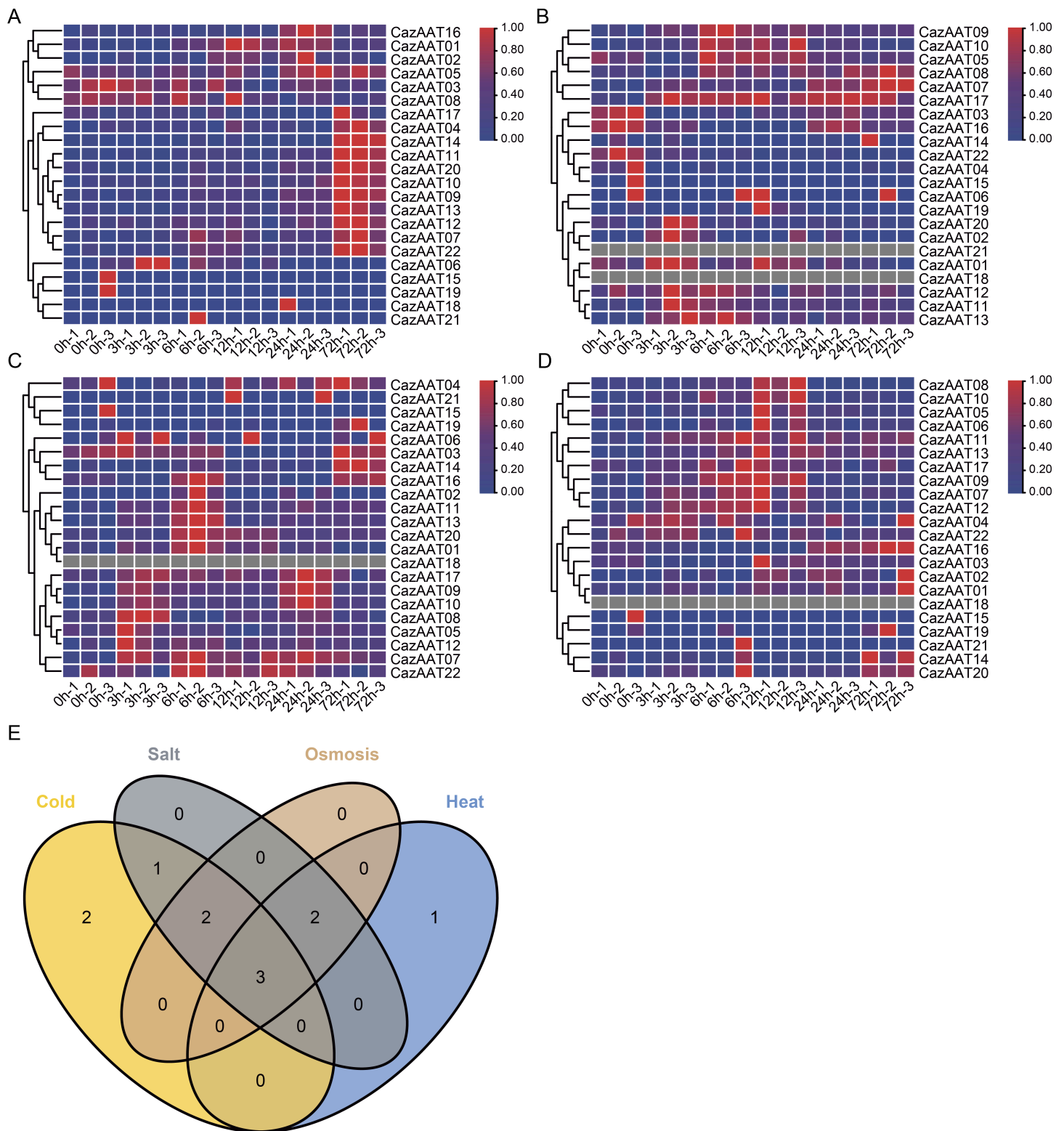


Figure 7

The response of CazAAT genes to abiotic stress in pepper. Alterations in the expression of CazAAT genes in peppers treated with cold (A), heat (B), salinity (C), and osmotic (D) stress at six different time points. (E) Overlapping CazAAT genes upregulated in pepper under different abiotic stresses.

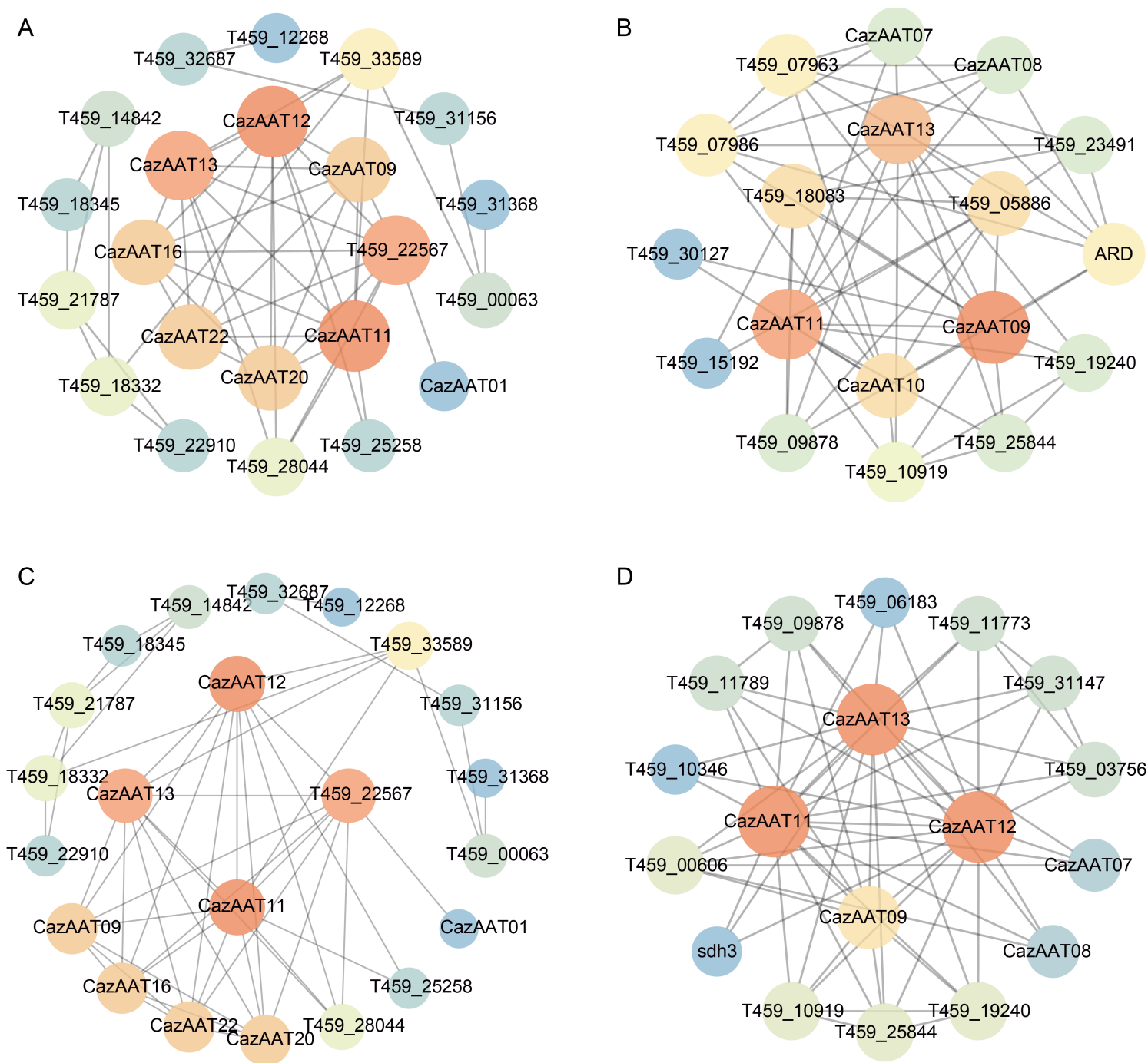


Figure 8

Co-expression patterns of *CazAAT* genes to abiotic stress in pepper. Co expression network based on *CazAAT* gene in peppers treated with cold (A), heat (B), salinity (C), and osmotic (D) stress.

Supplementary Files

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

- [SupplementaryInformationlegends.docx](#)
- [SupplementaryMaterial1.xlsx](#)
- [SupplementaryMaterial2.xlsx](#)

- [SupplementaryMaterial3.xlsx](#)
- [SupplementaryMaterial4.xlsx](#)