Identification, Systematic Evolution and Expression Analyses of The AAAP Gene Family in *Capsicum Annuum*

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Abstract

Background: The amino acid/auxin permease (AAAP) family represents a class of proteins that transport amino acids across cell membranes. Members of this family are widely distributed in different organisms and participate in processes such as growth and development and the stress response in plants. However, a systematic comprehensive analysis of *AAAP* genes of the pepper (*Capsicum annuum*) genome has not been reported.

Results: In this study, we performed systematic bioinformatics analyses to identify AAAP family genes in the *C. annuum* 'Zunla-1' genome to determine gene number, distribution, structure, duplications and expression patterns in different tissues and stress. A total of 53 *CaAAAP* genes were identified in the 'Zunla-1' pepper genome and could be divided into eight subgroups. Significant differences in gene structure and protein conserved domains were observed among the subgroups. In addition to *CaGAT1*, *CaATL4*, and *CaVAAT1*, the remaining *CaAAAP* genes were unevenly distributed on 11 of 12 chromosomes. In total, 33.96% (18/53) of the *CaAAAP* genes were a result of duplication events, including three pairs of genes due to segmental duplication and 12 tandem duplication events. Analyses of evolutionary patterns showed that segmental duplication of AAAPs in pepper occurred before tandem duplication. The expression profiling of the *CaAAAP* by transcriptomic data analysis showed distinct expression patterns in various tissues and response to different stress treatment, which further suggest that the function of *CaAAAP* genes has been differentiated.

Conclusions: This study of *CaAAAP* genes provides a theoretical basis for exploring the roles of *AAAP* family members in *C. annuum*.

Background

Plants obtain nitrogen by absorbing ammonia, nitrate, amino acids, and soluble peptides from the soil. Nitrogen absorption and transport is mediated by several types of transport proteins, including ammonium transport proteins (AMTs), nitrate transport proteins (NRTs), amino acid transport proteins (AATs) and peptide transport proteins (PTRs)[1]. In plants, AATs are transmembrane (TM) proteins that transport amino acids from the extracellular environment to the intracellular environment [2]. According to conserved sequence and structure motifs, the plant AAT superfamily consists of the amino acid/auxin permease (AAAP) and amino acid-polyamine-choline (APC) gene families [3]. The AAAP subfamily includes eight subclasses of transporters: amino acid permeases (AAPs), lysine-histidine transporters (LHTs), proline transporters (ProTs), γ-aminobutyric acid transporters (GATs), putative auxin transporters (AUXs), similar to ANT1-like aromatic and neutral amino acid transporters (ANTs), and amino acid transporter-like (ATLa and ATLb) subfamilies [4, 5]. *AAAP* genes are widely present in plants, including *Arabidopsis* (46 genes) [6], rice (58 genes) [7], maize (71 genes) [8], poplar (71 genes) [9], potato (52 genes) [10], moso bamboo(55 genes) [11] and *Medicago truncatula*(86 genes) [12].

The first amino acid transporter protein (AtAAP1/NAT2) isolated from plants belongs to the AAP family. There are eight members in *Arabidopsis*, and AtAAP transports neutral, acidic and cationic amino acids with different specificities and affinities [13, 14]. *AtAAP1* is highly expressed in *Arabidopsis* cotyledons and the endosperm, and mediates uptake of amino acids to developing embryo or root cells [15–17]. *AtAAP2* is localized to the plasma membrane and the phloem, and the *aap2* mutant exhibits altered xylem-phloem transfer of amino acids, which affects metabolism and results in increased seed yield and oil content in *Arabidopsis* [18]. *AtAAP3* is exclusively expressed in roots and *AtAAP4* is primarily expressed in source leaves, stems, and flowers, *AtAAP5* has been observed in all tissues [19]. In the *aap6* mutant, the amino acid content of the *Arabidopsis* sieve elements was reduced but not affect leaves aphid herbivores [20]. *AtAAP8* participates in the early seed development in *Arabidopsis* [21]. *OsAAP3* and *OsAAP5* regulate tiller number and grain yield in rice [22, 23], and overexpression of *OsAAP6* increases grain protein content and improves rice nutritional quality [24]. In addition, there are reports of AAP subfamily members in other species, including *StAAP1* [25], *PvAAP1* [26], *PtAAP11* [27], *VfAAP1* and *VfAAP3* [28].

AtLHT1 localizes on the surface of roots in young seedlings and in pollen and mediates uptake of amino acids from the root to the mesophyll cells through the xylem [29, 30]. Under conditions of nitrogen deficiency in particular, overexpression of *AtLHT1* can increase the efficiency of nitrogen utilization [30]. *AtLHT2* localizes to the tapetum of *Arabidopsis* anthers [31]. *AtLHT6* is expressed in buds, flowers, and roots; *AtLHT4* expression is increased in developed buds compared to mature flowers; and expression of *AtLHT5* peaks in flowers [32, 33]. *OsLHT6* is specifically expressed in new shoot meristems [7], and *PgLHT* plays an important role in the growth and development of the ginseng root system [34]. The GAT subfamily mainly transports γ-aminobutyric acid (GABA) and GABA-related compounds; the highest expression of *AtGAT1* is observed in flowers and under conditions of elevated GABA [35]. *AtANT1* is expressed in all organs, with the highest abundance in flowers and cauline leaves, and mediates transport of aromatic and neutral amino acids, arginine, indole-3-acetic acid, and 2, 4-dichlorophenoxyacetic acid [36]. *AtAUX1* is a high-affinity transporter of indoleacetic acid (IAA), and *AtAUX1* and *AtLAX3*(a homolog of *AtAUX1*) are mainly expressed in roots and promote lateral root formation [37, 38]. The expression of *OsAUX* subfamily members is also tissue-specific: *OsAUX4* is preferentially expressed in new shoot meristems, and *OsAUX2* and *OsAUX5*

are specifically expressed in young roots, which suggests a role in the formation and development of root systems [7]. *MtLAX2*, a functional homolog of *AtAUX1*, is required for nodule organogenesis [39]. The ProTs subfamily is responsible for transporting proline, glycinebetaine (GB) and GABA. *AtProT1* is expressed in the phloem or phloem parenchyma cells, which indicates a role in the long-distance transport of proline [40]. By contrast, *AtProT2* is only expressed in root epidermis and cortical cells; *AtProT3* is more highly in leaf epidermal cells [40]. *HvProT2* is constitutively expressed in both leaves and roots, and heterologous expression experiments have shown that the affinity of *HvProT2* is highest for glycinebetaine [41]. *AtAVT3* and *AtAVT4* encode amino acid efflux proteins located in the vacuolar membrane, where they mediate transport of alanine and proline [42].

Pepper is an annual or perennial plant that belongs to the Solanaceae family; it is an important vegetable crop in China, which is number one in the world in terms of planting area and output (http://www.fao.org/faostat/en/). The pepper Zunla-1 (*C. annuum* L.) genome contains 34,476 protein-coding loci on 12 different chromosomes. Although the roles of many AAAPs in plants have been well characterized, members of the AAAP gene family in pepper have not been studied. We used bioinformatics to identify the AAAP gene family members in pepper and systematically analyzed the chromosome distribution, gene structure, evolution characteristics, and expression patterns of *AAAP* genes to provide a theoretical basis for exploring the roles of AAAPs in pepper.

Results

Identification of AAAP genes in pepper

To explore the AAAP protein family in pepper, we used one domain (PF01490) searche of Pepper Genome Database2 (http://peppersequence.genomics.cn/page/); the HMM profile was used as a query and each putative AAAP protein sequences was verified by SMART, CDD and Pfam analyses. A total of 53 AAAP genes were identified and renamed in pepper according to their affinities within gene subfamilies; *CaGAT1, CaANL4* and *CaVAAT1*, were not anchored to chromosomes (Table 1). Gene lengths ranged from 669 (*CaLHT4*) to 2,532 bp (*CaAAP4*), the molecular weight varies from 24.43 kDa (*CaLHT4*) to 93.22 kDa (*CaAAP4*). Thus, the gene length span of the *CaAAAP* gene family is large and correlated with molecular weight. The isoelectric points (pIs) of CaAAAP proteins ranged from 4.27(CaVAAT5) to 10.06(CaANT5); the majority of proteins (83%) had pIs more than 7.0, which indicates that AAAP proteins in pepper may represent a class of basic protein.

Table 1
The general information and sequence characterization of 53 *CaAAAP* genes.

S.N.	Gene[a]	Locus[b]	Location[c]	ORF(bp)[d]	Exon[e]	Protein[f]			TM region[g]
						Size (aa)	MW(d)	pI	
	AAP group								
1	*CaAAP1*	Capana07g002429	Chr07:220179435–220181692	1335	7	444	49460.8	8.72	11
2	*CaAAP2*	Capana07g002430	Chr07:220188828–220192330	1869	10	622	68584.1	8.46	14
3	*CaAAP3*	Capana07g002431	Chr07:220195003–220198004	1410	7	469	51763.2	8.45	10
4	*CaAAP4*	Capana07g002432	Chr07:220225817:220233681	2532	13	843	93224.4	8.88	17
5	*CaAAP5*	Capana04g000780	Chr04:14469803:14475148	1446	7	481	52757.7	8.81	10
6	*CaAAP6*	Capana12g000826	Chr12:27187513:27194231	1467	7	488	53825.3	8.94	9
7	*CaAAP7*	Capana08g002210	Chr08:143014796:143019992	1419	7	472	51747.8	9.12	11
8	*CaAAP8*	Capana04g001588	Chr04:67204663:67207202	1434	6	477	52413.8	8.27	10
9	*CaAAP9*	Capana06g001752	Chr06:50038242:50040303	1419	6	472	51681.9	7.84	10
10	*CaAAP10*	Capana05g001770	Chr05:174328892:174330262	1020	3	339	37207.9	6.86	7
	LHT group								
11	*CaLHT1*	Capana02g003614	Chr02:162887482:162890584	1350	8	449	50428	8.6	9
12	*CaLHT2*	Capana02g003615	Chr02:162905774:162912940	1266	7	421	47443	8.24	9
13	*CaLHT3*	Capana02g003616	Chr02:162914284:162921151	1332	8	443	49858.6	9.08	11
14	*CaLHT4*	Capana04g002888	Chr04:215599914:215604069	1227	9	408	46113.8	8.27	7
15	*CaLHT5*	Capana04g001881	Chr04:130533897:130536648	1329	6	442	49917.6	8.06	10
16	*CaLHT6*	Capana11g000230	Chr11:5761051:5762379	1329	1	442	49944.5	9.1	10
17	*CaLHT7*	Capana03g001379	Chr03:25005836:25008812	1329	7	442	49012.6	9.4	11
18	*CaLHT8*	Capana05g000336	Chr05:7406911:7414486	1065	7	354	39858.5	9.42	7
19	*CaLHT9*	Capana11g002248	Chr11:216341951:216346445	1311	8	436	48573.9	9.03	8

[a] Systematic designation given to pepper *AAAPs* in this study.

[b] Locus identity number of *AtAATs* assigned by Pepper Genome Database2 (http://peppersequence.genomics.cn/page/).

[c] Chromosomal localization of pepper AAAP genes..

[d] Length of the open reading frame.

[e] Number of extrons obtained from GSDS by comparing sequences between transcript and genome (Gene Structure Display Server; http://gsds.cbi.pku.edu.cn/)..

[f] Protein characterization of CaAAAPs obtained from EXPASY server (http://web.expasy.org/protparam/).

[g] Number of transmembrane segments possessed by CaAAAPs, predicted by the TMHMM Server v2.0.

S.N., serial number; ORF, open reading frame; bp, base pair; aa, amino acids; MW, molecular weight; pI, isoelectric point; TM, transmembrane; NA, not available.

S.N.	Gene[a]	Locus[b]	Location[c]	ORF(bp)[d]	Exon[e]	Protein[f]			TM region[g]
						Size (aa)	MW(d)	pI	
20	*CaLHT10*	Capana04g000478	Chr04:7738487:7744000	1581	5	526	57977.4	9.61	9
21	*CaLHT11*	Capana04g000098	Chr04:1109665:1112218	1338	5	445	49110.6	8.68	10
22	*CaLHT12*	Capana08g002793	Chr08:152269921:152272976	1713	5	570	61963	9.55	9
23	*CaLHT13*	Capana11g000398	Chr11:11019799:11021033	708		235	25865.9	9.01	2
24	*CaLHT14*	Capana04g000106	Chr04:1178475:1183677	669		222	24427.3	8.47	3
	GAT group								
25	*CaGAT1*	Capana00g003418	Chr00:545297054:545303475	1365	7	454	49950.5	8.68	10
26	*CaGAT2*	Capana11g000210	Chr11:5435275:5440152	1092	6	363	39923.8	9.98	9
	ProT group								
27	*CaProT1*	Capana05g001989	Chr05:191409867:191415970	1320	7	439	47836.8	9.73	12
28	*CaProT2*	Capana05g001990	Chr05:191424542:191430181	1347	7	448	49162.1	9.4	11
29	*CaProT3*	Capana03g002827	Chr03:118029421:118036334	1344	7	447	49190.2	9.61	12
	AUX group								
30	*CaAUX1*	Capana09g001555	Chr09:181029189:181033262	1467	7	488	54841.3	8.15	10
31	*CaAUX2*	Capana10g001370	Chr10:147549183:147556929	1467	7	488	54912.8	8.56	10
32	*CaAUX3*	Capana04g001744	Chr04:99262090:99266939	1317	8	438	49663.3	8.25	9
33	*CaAUX4*	Capana08g002704	Chr08:150979738:150984984	1482	8	493	55541.5	8.75	10
	ANT group								
34	*CaANT1*	Capana02g002432	Chr02:144978448:144979728	1281	1	426	46665.9	7.92	11
35	*CaANT2*	Capana02g002433	Chr02:144981268:144982602	1335	1	444	48548.7	4.74	11
36	*CaANT3*	Capana02g002434	Chr02:144983909:144985192	1284	1	427	46457.5	4.82	11
37	*CaANT4*	Capana04g002414	Chr04:201839016:201840293	1278	1	425	46811.9	7.45	11
38	*CaANT5*	Capana03g004210	Chr03:248829547:248830964	930	2	309	33786.3	10.06	10

[a] Systematic designation given to pepper *AAAPs* in this study.

[b] Locus identity number of *AtAATs* assigned by Pepper Genome Database2 (http://peppersequence.genomics.cn/page/).

[c] Chromosomal localization of pepper AAAP genes..

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[g] Number of transmembrane segments possessed by CaAAAPs, predicted by the TMHMM Server v2.0.

S.N., serial number; ORF, open reading frame; bp, base pair; aa, amino acids; MW, molecular weight; pI, isoelectric point; TM, transmembrane; NA, not available.

S.N.	Gene[a]	Locus[b]	Location[c]	ORF(bp)[d]	Exon[e]	Protein[f]			TM region[g]
						Size (aa)	MW(d)	pI	
	ATLa group								
39	*CaATL1*	Capana06g001998	Chr06:75940086:75942122	846	3	281	30550.9	4.94	3
40	*CaATL2*	Capana03g000522	Chr03:7178172:7179590	1419	1	472	51153.5	5.43	10
41	*CaATL3*	Capana05g002081	Chr05:197860240:197862960	1302	6	433	47459.1	8.35	11
42	*CaATL4*	Capana00g004937	Chr00:676629079:676631743	1320	5	439	47898.6	8.55	11
43	*CaATL5*	Capana04g000715	Chr04:12477359:12484737	1344	5	447	48657.5	8.36	11
44	*CaATL6*	Capana02g000804	Chr02:93929776:93933801	1407	5	468	50795.2	8.78	11
45	*CaATL7*	Capana02g003206	Chr02:157224580:157228911	1383	5	460	49954.5	8.55	11
	ATLb group								
46	*CaVAAT1*	Capana00g004212	Chr00:618994856:618996151	1296	1	431	46618.7	7.96	8
47	*CaVAAT2*	Capana04g001726	Chr04:93008498:93010474	1281	3	426	46958.9	7.71	10
48	*CaVAAT3*	Capana12g002556	Chr12:222093246:222094767	1017	2	338	36992	7.91	9
49	*CaVAAT4*	Capana05g002349	Chr05:207916251:207920239	1140	3	379	41865.5	9.04	9
50	*CaVAAT5*	Capana03g003057	Chr03:162840327:162847744	1395	9	464	51484.7	4.27	7
51	*CaVAAT6*	Capana10g001696	Chr10:173666186:173669984	1608	11	535	57908.7	5.19	10
52	*CaVAAT7*	Capana03g002859	Chr03:127734852:127743213	1338	7	445	48690.8	4.98	5
53	*CaVAAT8*	Capana12g002523	Chr12:220748120:220761121	1989	15	662	73214.4	5.85	8

[a] Systematic designation given to pepper *AAAPs* in this study.

[b] Locus identity number of *AtAATs* assigned by Pepper Genome Database2 (http://peppersequence.genomics.cn/page/).

[c] Chromosomal localization of pepper AAAP genes..

[d] Length of the open reading frame.

[e] Number of extrons obtained from GSDS by comparing sequences between transcript and genome (Gene Structure Display Server; http://gsds.cbi.pku.edu.cn/)..

[f] Protein characterization of CaAAAPs obtained from EXPASY server (http://web.expasy.org/protparam/).

[g] Number of transmembrane segments possessed by CaAAAPs, predicted by the TMHMM Server v2.0.

S.N., serial number; ORF, open reading frame; bp, base pair; aa, amino acids; MW, molecular weight; pI, isoelectric point; TM, transmembrane; NA, not available.

We studied the exon/intron arrangement of the coding sequences of *CaAAAP* genes in their genome sequences and found that 13.21% (7/53) of pepper *AAAP* genes contained a single exon, 3.77% (2/53) had a single intron, and 83.02% had 1 to 14 introns (Fig. 1).Prediction of TM regions showed that most CaAAAPs (77.36%) had 8–11. Similar numbers of TMs regions were found in several subfamilies (e.g., 10 TMs in the AUX subfamily and 11 TMs in the ANT and ATLa subfamilies; Table 1 and Additional file 1:Figure S1). Thus, members of the same subfamily have a conserved structure.

Phylogenetic and structural analyses of AAAP proteins in pepper

To further understand the homology between the AAAP gene families of pepper and other plant species (Table 2), we constructed an unrooted phylogenetic tree of full-length AAAPs from pepper, potato, rice and *Arabidopsis* was constructed (Fig. 2). We found that the genes *CaAAAP*, *StAAAP*, *OsAAAP* and *AtAAAP* were divided into eight distinct subfamilies, which indicates that the AAAP gene family has

eight subfamilies in angiosperms. In pepper, the LHT subfamily was the largest (26.42%; 14 genes), whereas the GAT subfamily comprised only two genes. The numbers of genes in the subgroups ProT and ANT were the same as or similar to those in potato, rice, and *Arabidopsis*, which indicates that CaAAAPs of these two subgroups have not changed greatly in the pepper genome.

Table 2
Comparative analysis of Amino acid/auxin permease (AAAP) proteins between Capsicum and other plant species.

Specie	AAAP subfamily								# of AAAP proteins	# of Proteins	# % of AAAP proteins	Reference
	AAP	LHT	GAT	ProT	AUX	ANT	ATLa	ATLb				
A. thaliana	8	10	2	3	4	4	5	10	46	25,498	0.18	6
P.edulis	16	8	6	3	7	2	6	7	55	31,987	0.17	11
O. sativa	19	6	4	3	5	4	7	10	58	35,825	0.16	7
Z. mays	15	24	2	2	5	3	6	14	71	39,591	0.18	8
M.truncatula	26	18	4	3	5	3	13	14	86	44,623	0.19	12
P. trichocarpa	17	13	7	3	8	4	8	11	71	45,000	0.16	9
S.tuberosum	8	11	3	4	5	5	8	8	52	39,031	0.13	10
C.annuum	10	14	2	3	4	5	7	8	53	34,476	0.15	

AAP: amino acid permease; LHT: lysine and histidine transporter; GAT: g -aminobutyric acid transporter; ProT: proline transporter;AUX: auxin transporter; ANT: aromatic and neutral amino acid transporter; ATL: amino acid transporter-like

Conserved domains of pepper AAAP proteins were analyzed with the MEME server and a total of 20 conserved motifs were identified (Fig. 1, Additional file 3: Table S1). Motifs 1 (44/53), 2 (42/53), and 7 (49/53) were widespread among members of the CaAAAP family. Some subfamilies included several specific motifs. For example, the LHT and GAT subfamilies contained motifs 3, 12, 13, and 14, whereas motif 5 was only found in the LHT, AAP, GAT, and ProT subfamilies. Motifs 9, 10, and 17 were only present in the AUX subfamily; motifs 15 and 18 were only present in the ANT subfamily; motifs 16 and 19 were only present in the ATLa subfamily. Similar numbers of motifs were found in the ProT and AUX subfamilies (Fig. 1), which suggests that the structures of these subfamilies are highly conserved.

Chromosomal location and duplication analyses

We used Mapchart 2.30 mapping to identify the chromosomal location of AAAP genes in the pepper genome (Fig. 3). In addition to *CaGAT1, CaANL4* and *CaVATT1*, the remaining 50 genes were unevenly distributed on 11 of 12 chromosomes; no genes were mapped to chromosomes 1 (Fig. 3, Table 1). Most of the genes were mapped to the bottom of chromosomes 2, 5, 7 and 8, whereas the genes on chromosome 11 were mostly mapped to the top. A total of 58.5% (31/53) of genes were mapped to chromosome 2, 3, 4 and 5, which contained 8, 6, 11 and 6 genes, respectively. Only one gene was located on chromosome 9, and two to four genes were mapped to the remaining chromosomes (Fig. 3).

To identify the duplication events of *AAAP* genes in pepper, we analyzed the 53 full-length AAAP protein sequences using MCScanX. According to the defined criterion of separation five or fewer genes with more than 50% similarity at protein level, 33.96% (18 of 53) originated from the duplication events (Fig. 3).Twelve genes (22.64%) were arranged in tandem duplication and organized into four groups. Two pairs of tandem duplicate genes were identified on chromosome 2; chromosomes 5 and 7 each contained one pair (Fig. 3). Three segmental duplication blocks were located on chromosomes 2, 4 and 12, representing 11.32% of all *CaAAAP* genes (6/53) (Fig. 3, Additional file 2: Figure S2). Therefore, compared to segmental duplications, tandem duplication events predominated in the expansion of AAAP genes in pepper. Furthermore, high-sequence similarity occurred in duplicated genes: *CaAAP1* and *CaAAP3*, which originated via tandem duplication, were 94.28% similar, whereas *CaANT1* and *CaANT4*, which were a result of segmental duplication, exhibited 81.79% similarity.

We further estimated nonsynonymous (Ka) and synonymous (Ks) nucleotide substitution rates in the coding sequences of paralog pairs to explore the selective pressures and duplication time of AAAP gene family members in pepper (Table 3). In general, Ka/Ks ratios less than 1 indicate purifying selection, and Ka/Ks ratios greater than 1 indicate positive selection [43]. The Ka/Ks ratios of all seven paralog pairs were < 1.0, which indicates that *CaAAAP* genes evolved under purifying selection (Table 3). We also estimated the dates of duplication events of paralog pairs using the formula T = Ks/2λ (assuming a clock-like rate (λ) of 6.96×10^{-9} synonymous substitutions

per years [44]); duplication events were estimated to have occurred 8.53 to 68.69 million years ago (Mya), with an average duplication time of 43.61 Mya. In addition, the two segmental duplications of *CaAAAP*s occurred from 54 to 58.87 Mya, and five tandem duplications occurred from 41.43 to 8.53 Mya.

Table 3
Ka-Ks calculation for each pair of AAAP paralogs in pepper. .

Paralog pairs	S-sites	N-sites	Ka	Ks	Ka/Ks	Selection pressure	Duplication type	Duplication time (Mya)
CaANT1-CaANT2	304.25	970.75	0.16	0.57	0.29	Purifying selection	Tandem	40.96
CaANT2-CaANT3	305.00	976.00	0.05	0.12	0.40	Purifying selection	Tandem	8.53
CaANT1-CaANT3	304.83	970.17	0.15	0.57	0.26	Purifying selection	Tandem	40.59
CaLHT1-CaLHT3	313.75	1015.25	0.13	0.58	0.22	Purifying selection	Tandem	41.43
CaAAP1-CaAAP3	316.08	1015.92	0.07	0.14	0.50	Purifying selection	Tandem	10.37
CaANT1-CaANT4	303.58	971.42	0.11	0.82	0.14	Purifying selection	Segmental	58.87
CaAAP5-CaAAP6	351.08	1091.92	0.11	0.75	0.15	Purifying selection	Segmental	54.00

S-Sites, number of synonymous sites; N-Sites, number of non-synonymous sites; Ka, non-synonymous substitution rate; Ks, synonymous substitution; Mya, million years ago.

Expression patterns of *CaAAAP* genes in various tissues

We investigated the expression profiles of all *CaAAAP* genes in roots, stems, leaves, floral buds, flowers and different developmental stages of fruits (Fig. 4, Additional file 4: Table S2). 48 (90.5%) of the *CaAAAP* genes were detected in at least one tissue (RPKM ≥ 1), and 19(35.8%) genes were detected in all tissues tested (RPKM ≥ 1). In particular, approximately half of the *CaAAAP* genes showed low expression in fruits. By contrast, approximately 80% *CaAAAP* genes showed high expression in flowers (RPKM ≥ 1). These results indicate that *CaAAAP*s play an important role in the growth and development of pepper, in particular in the flowers. The *CaAAAP* genes clustered into three distinct clades based on expression patterns (Fig. 4). Seven genes (*CaAAP2, CaAAP3, CaAAP5*, *CaAAP9, CaATL6, CaATL7*, and *CaVAAT8*) in group I were expressed at relatively high levels in all tissues. In addition to several genes exhibited relatively high expression in specific organs (such as *CaLHT3*, *CaLHT5*, *CaLHT8, VAAT1* and *VAAT6* in buds; *CaATL4* in fruits; *CaLHT9* and *CaGAT2* in roots; *CaLHT12* in roots, stems and leaves), the other genes in group II were expressed at relatively low levels in all tested tissues. Group III comprised 20 genes that were expressed at relatively high levels in most organs.

Differential expression profiling of *CaAAAP* genes in response to abiotic stress

To study whether *CaAAPs* are involved in responses to hormones and abiotic stresses in pepper, we investigated the expression levels of the *CaAAPs* in the roots and leaves of 40-day old seedlings in response to cold, heat, salt, osmotic, oxidative, ABA, IAA, GA3, JA and SA treatment (Fig. 5, Additional file 5: Table S3). In addition to *CaLHT2*, *CaLHT5*, *CaLHT7*, *CaLHT8*, *CaLHT13*, and *CaAAP10*, most *AAAP* genes were induced in at least one of the treatment as compared with the control (Fig. 5). Interestingly, some *AAAP* genes varied greatly between the leaves and roots in the response to abiotic or hormones stress. For instance, *CaAAP4*, *CaLHT9*, *CaLHT10*, *CaATL3*, *CaATL6*, *CaATL7*, *CaAUX3*, and *CaVAAT7* were found to be upregulated under cold, heat, osomotic, oxidative and salt in the roots, but downregulated in the leaves. There were 28, 10, 20, and 18 *CaAAAP* genes were also upregulated by ABA, GA3, IAA, and JA treatment in the roots respectively, but downregulated in the leaves. Whereas there were 4, 5, and 7 *CaAAAP* genes were observed to be upregulated in the leaves but downregulated in the roots under the cold, IAA and salt stress treatment, respectively. In contrast, the highest number of *CaAAAP* genes were upregulated in the SA response in the leaves and roots (33 genes).There were several stress-responsive cis-elements showing in the promoter regions of these members, such as ABRE, ARE, LTR, MBS, TGACG-motif, CGTCA-motif, TCA-element, GARE-motif, AuxRR-core, and TC-rich repeats (Additional file 6: Table S4). Among the 53 AAAP genes, the *CaAAP7* promoter had no these stress-responsive elements, while *CaVAAT2* had maximum 14 elements. These results reaveled that a number of *CaAAAP* genes might involved in regulating abiotic and hormone stress responses.

Discussion

The AAAP gene family, which contains eight subfamilies, encodes integral TM proteins that play a pivotal role in various aspects of normal plant growth and development. This gene family has been identified in many plants, including *Arabidopsis* [6], rice [7], maize [8], poplar [9], potato[10], moso bamboo [11] and *Medicago truncatula* [12]. Although the role of AAAP genes in plants has been previously suggested, systematic study of the AAAP gene family in pepper has not been performed. We identified 53 *AAAP* genes in *C.annuum.Zunla-1* in this work, the *CaLHT* subfamily was the largest (14 genes), and the *CaGAT* subfamily was the smallest that comprised only two genes (Table 1). AAAP proteins account for 0.13–0.18% of the total proteins in many plant species studied (Table 2), and the percentage of CaAAAPs identified in the present study was 0.15%. Thus, the number of AAAP genes in most plants appears to be similar, regardless of genome size. Phylogenetic analyses showed that the pepper AAAP gene family can be divided into eight subfamilies, consistent with that in Arabidopsis, rice and potato (Fig. 2), which indicates that AAAP genes diversified before the split from dicot to monocot. In addition, gene structure analysis indicated the same subgroup had the same or similar numbers and types of exon/intron, TM regions, and motif compositions (Fig. 1, Table 1), which suggests that those groups have been relatively conserved during evolution.

In addition to *CaGAT1, CaVATT1* and *CaATL4*, the remaining 50 genes were unevenly distributed on 11 of 12 chromosomes, and most of the genes were mapped on chromosomes 2, 3, 4 and 5 (Fig. 3). Meanwhile, four groups of tandem duplicate genes were identified on three chromosomes(two, one, and one groups on chromosome 2, 5 and 7, respectively), and segmental duplication blocks were located on chromosomes 2, 4 and 12 (Fig. 3). Based on chromosomal distribution and phylogenetic and sequence similarity analyses, we identified seven pairs of paralogs in the pepper AAAP family (Table 3). Two pairs of paralogs (*CaANT1* and *CaANT4*, and *CaAAP5* and *CaAAP6*) participated in segmental duplications on different chromosomes. Five pairs (*CaANT1 and CaANT2*, *CaANT2 and CaANT3*, *CaANT1 and CaANT3*, *CaLHT1* and *CaLHT3*, and *CaAAP1 and CaAAP3*) were the result of a putative tandem duplication event. These results suggest that tandem gene duplication is the main cause of expansion of the CaAAAP gene family; similar results have been reported in potato and *Arabidopsis* [10, 45]. We estimate that the duplication time of two AAAP paralog pairs in pepper (*CaANT1* and *CaANT4*, and *CaAAP5* and *CaAAP6*) occurred 58.87 to 54 Mya and that of five of the paralogous gene pairs (*CaANT1* and *CaANT2*, *CaANT2* and *CaANT3*, *CaANT1* and *CaANT3*, *CaLHT2* and *CaLHT3*, and *CaAAP1* and *CaAAP3*) occurred 40.96 to 8.53 Mya (Table 3). This indicated that the segmental duplication of AAAPs in pepper occurred before tandem duplication. The pepper/potato separation occurred approximately 36 Mya [46], the duplication of most AAAP paralog pairs occurred before their separation from pepper and potato, and only two paralogous pairs were duplicated after the pepper/potato split. The Ka/Ks ratios of seven paralog pairs were < 1 (Table 3), which indicates that these paralog pairs evolved under purifying selection. Similar results have been reported in moso bamboo [11] and poplar [9], which have no paralog pairs in the AAAP family that underwent positive selection.

Gene duplication is generally considered a major source of gene family expansion and functional diversity during evolution [47]. In rice, 50% (29/58) of *AAAP* genes are duplicated gene [7], duplicated genes represented 32.69% (17/52) in potato [10] and 30.43% (14/46) in *Arabidopsis* [45], and the subgroups AAP and LHT had the most members of the eight selected species (Table 2). In the present study, 33.96% of *AAAP* genes (18/53) in pepper were duplicated genes, the subgroup LHT and AAP contained 35.71% and 60% of the duplicated genes, respectively (Fig. 3). It can be speculated that these two subfamilies have greater selection pressure during the evolution process, and it is easier to get new functions. However, the ANT subfamily comprised five members and duplicated genes accounted for 80%, whereas the ProT subfamily comprised three members and duplicated genes accounted for 66.67% (Fig. 3). Furthermore, the subgroups ANT and ProT had the same or similar genes in currently studied species (Table 2), which suggests that the subgroup ANT and ProT genes was conservative and the selection pressure relatively lower during the evolution process. Comparative analysis of the expression pattern of duplicated *CaAAAP* genes revealed that *CaANT2* and *CaANT3* (tandem duplicated genes) exhibited similar expression patterns in various development stages and stresses, which indicated that they may have overlapping functions (Fig. 4 and Fig. 5). However, most duplicated *CaAAAP* genes exhibited distinct expression patterns, such as *CaAAP5* and *CaAAP6* (segmental duplicated genes) (Fig. 4 and Fig. 5); as well as *CaAAP1* and *CaAAP3, CaLHT1* and *CaLHT3* (tandem duplicated genes) (Fig. 4 and Fig. 5). These results indicate that the expression and functional divergence of duplicated genes under selection pressure, contributing to adapt to the diversity of the environment.

RNA sequencing data showed that *CaAAAP* genes exhibited tissue-specific expression, *CaLHT3*, *CaLHT5*, *CaLHT8, VAAT1* and *VAAT6* were preferentially expressed in the buds, whereas *CaLHT9* and *CaGAT2* in roots; thus, these genes may be related to the reproductive and vegetative organ development in the pepper plant, respectively (Fig. 4). In addition, Seven genes (*CaAAP2, CaAAP3, CaAAP5*, *CaAAP9, CaATL6, CaATL7*, and *CaVAAT8*) were highly expressed in all tissues, suggesting that they may be involved in the pepper growth and development. Our data showed that *CaAAP5*, an orthologous of *StAAP1* and *AtAAP6*, was highly expressed in flowers, roots, leaves, and stems (Fig. 4). *AtAAP6* is responsible for the long-distance transport of amino acids [20]. *StAAP1*, which is highly expressed in leaves, is also responsible for the long-distance transport of amino acids [25]. Therefore, *CaAAP5* might be involved in the long-distance transport of amino acid. In *Arabidopsis*, *AtAUX1* and *AtLAX3* are highly expressed in roots [37, 48]. AUX subfamily genes are also mainly expressed in roots of rice and potato [7, 10]. In the study, AUX subfamily genes exhibited relatively high expression in roots, which indicates that *CaAUXs* might be involved in root growth and development.

It has been reported that AAAPs is regulated by low temperature, high salt, and/or drought stress treatments in many plants [49, 50]. *MtAAAP42*[12], *OsAAP15*, *OsATL6* and *OsANT3*[7] were upregulated under cold, drought and salt stresses, and *MtAAAP19* was induced by drought, salt and ABA stress treatments [12].The AAP subfamily gene *PeAAAP9* has low expression level in leaf, but it is strongly induced by drought, cold and salt stress treatment [11]. Our results showed that 47 genes were regulated in at least one of the treatment as compared with the control and the expression of 48 genes were observed in all tissue analysis (Fig. 4). For instance, *CaAAP6* was highly expressed under ABA, salt and osmotic(D-mannitose) stress treatment in the roots. However, low expression of this gene was observed in root, suggesting that *CaAAP6* may take part in abiotic stress signaling pathways. Interestingly, some *AAAP* genes varied greatly between the leaves and roots in the response to abiotic stress. *CaAAP4*, *CaLHT9*, *CaLHT10*, *CaATL3*, *CaATL6*, *CaATL7*, *CaAUX3*, and *CaVAAT7* were

found to be upregulated under cold, drought, and salt in the roots, but downregulated in the leaves, suggesting that these genes may play different roles in stress responses in pepper (Fig. 5).

Conclusions

Overall, 53 AAAP gene family members were identified in the 'Zunla-1' pepper genome and could be divided into eight subgroups. Throughout its evolutionary history, *CaAAAP*s were highly conserved and expanded slowly. *CaAAAP* genes exhibit tissue-specific expression and coordinate to regulate growth and development in pepper.

Methods

Data retrieval and identification of gene families

All pepper protein sequences were obtained from the Pepper Genome Database2 (http://peppersequence.genomics.cn/page/). The HMM profile for the AAAP domain (PF01490) downloaded from the Pfam database (http://pfam.xfam.org) [51], was used to identify potential AAAP genes from the pepper genome with HMMER 3.2.1 (http://hmmer.janelia.org/). All candidate protein sequences were further verified for the presence of conserved domains with the online tools Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd/), SMART (http://smart.embl-heidelberg.de/), and pfam (http://pfam.xfam.org/). The results were integrated and redundant genes were discarded. Molecular weights and pIs of the proteins encoded by the identified genes were predicted with the online EXPASY serve (http://web.expasy.org/protparam/).

Phylogenetic tree, gene structure and conserved motif analyses of *CaAAAP* genes

Multiple sequence alignments analyses of AAAP amino acid sequences of *Arabidopsis*, rice, potato and pepper were performed with ClustalW. We built the phylogenetic tree using the neighbor-joining method with MEGA7 [52] and 1000 bootstrap replications, a Poisson model, and partial deletion gap parameters. We determined the exon/intron organization of *CaAAAP* genes by aligning the coding sequences with genomic sequences using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) [53]. Conserved motifs were generated with MEME (http://meme-suite.org/tools/meme) with the following parameters: zero or one motif in each sequence, 10 and 100 width of motifs, and a maximum of 20 motifs. Motifs were visualized with TBtools [54].

Chromosomal location and syntenic analyses

The physical positions of the *CaAAAP* genes were obtained from the pepper annotation file deposited in the Sol Genomics database, mapped to 12 chromosomes, and visualized with Mapchart v.2.32 [55]. For syntenic analyses of *CaAAAP* genes, we used MCScanX [56]with the default settings to identify gene pairs of segmental and tandem duplications within the pepper genome.

Expression patterns of *CaAAAP* genes in various tissues and different stresses

To study the expression patterns of pepper *AAAP* genes in the pepper plant, we downloaded transcriptome sequencing data from the NCBI (https://www.ncbi.nlm.nih.gov/geo/; accession no.GSE45037). These data covered a wide range of developmental stages of pepper: roots, stems and leaves from plants at the full-bloom stage; unopened flower buds (buds) and fully open flowers (flowers) from mature plants; and fruits lengths of 0-1, 1-3, 3-4, and 4-5 cm (F-Dev-1, F-Dev-1, F-Dev-3 and F-Dev-4, respectively); mature green fruit (F-Dev-5); fruit turning red (F-Dev-6); and fruit 3, 5, and 7 days after turning red (F-Dev-7, F-Dev-8, and F-Dev-9, respectively). Available RNA-sequencing data were normalized with RPKM method. A heat map representing digital expression profile of *CaAAAP* genes was created with R 3.6.3 with log-transformed values.

The gene expression data of pepper in roots and leaves under different stresses were downloaded from (http://pepperhub.hzau.edu.cn/) [57]. The 40-day-old seedlings were separately treated with 10 stress conditions in 0, 0.5, 1, 3, 6, 12 and 24 hours: cold stress (10℃), heat stress (42℃)；salt stress (200 mM NaCl), osmotic stress (400 mM D-mannitose), oxidative stress (30 mM H_2O_2), ABA stress (30 μM), IAA stress (2 μM), GA3 (2 μM), JA (10 μM) and SA (2 mM). Available RNA-sequencing data were normalized with FPKM method. Available RNA-sequencing data were normalized with FPKM method. A heat map representing digital expression profile of *CaAAAP* genes was created with R 3.6.3 with log-transformed values.

Abbreviations

KM: Reads Per Kilo bases per Million reads ; FPKM: Fragments Per Kilo bases per Million reads

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data obtained in the current study have been presented in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The study was conceived and designed by KL and XP, MH, ZW, and HW contributed to data collection and bioinformatics analysis. KL and XP participated in preparing and writing the manuscript. All authors contributed to revising the manuscript.

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Figures

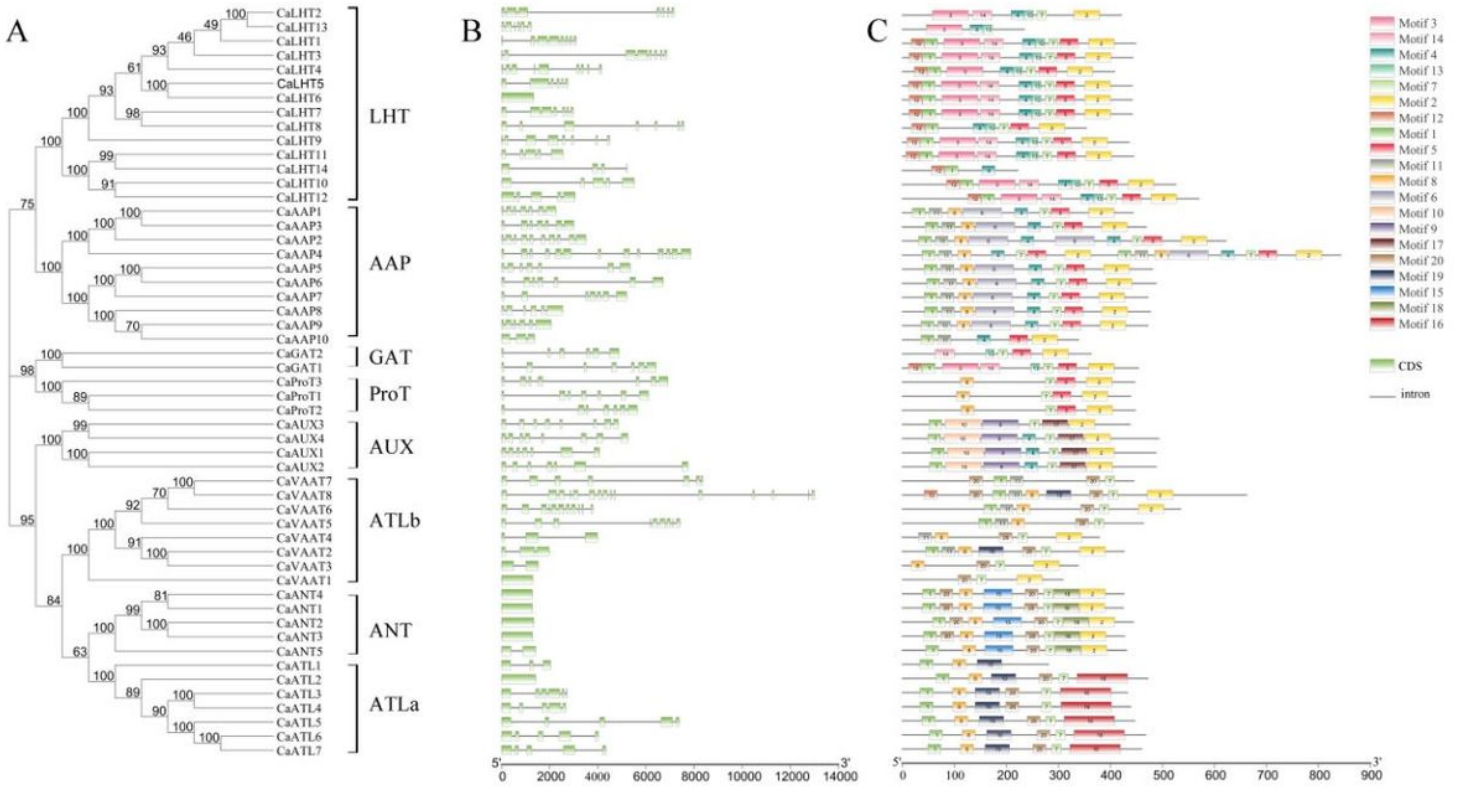

Figure 1

Phylogenetic relationship, gene structures and conserved motifs of CaAAAPs. (A) Phylogenetic tree of 53 CaAAAPs proteins. Neighbor-joining tree was constructed using MEGA7. Bootstrap support values from 1000 reiterations are indicated at each node. The 53 CaAAAPs in the tree were divided into eight subfamilies. (B) Exons and introns were indicated by green rectangles and gray lines respectively. (C) Conserved motifs of CaAAAPs proteins. Each colored box represents a specific motif in the protein identified using the MEME motif search tool. The order of the motifs corresponds to their position within individual protein sequences.

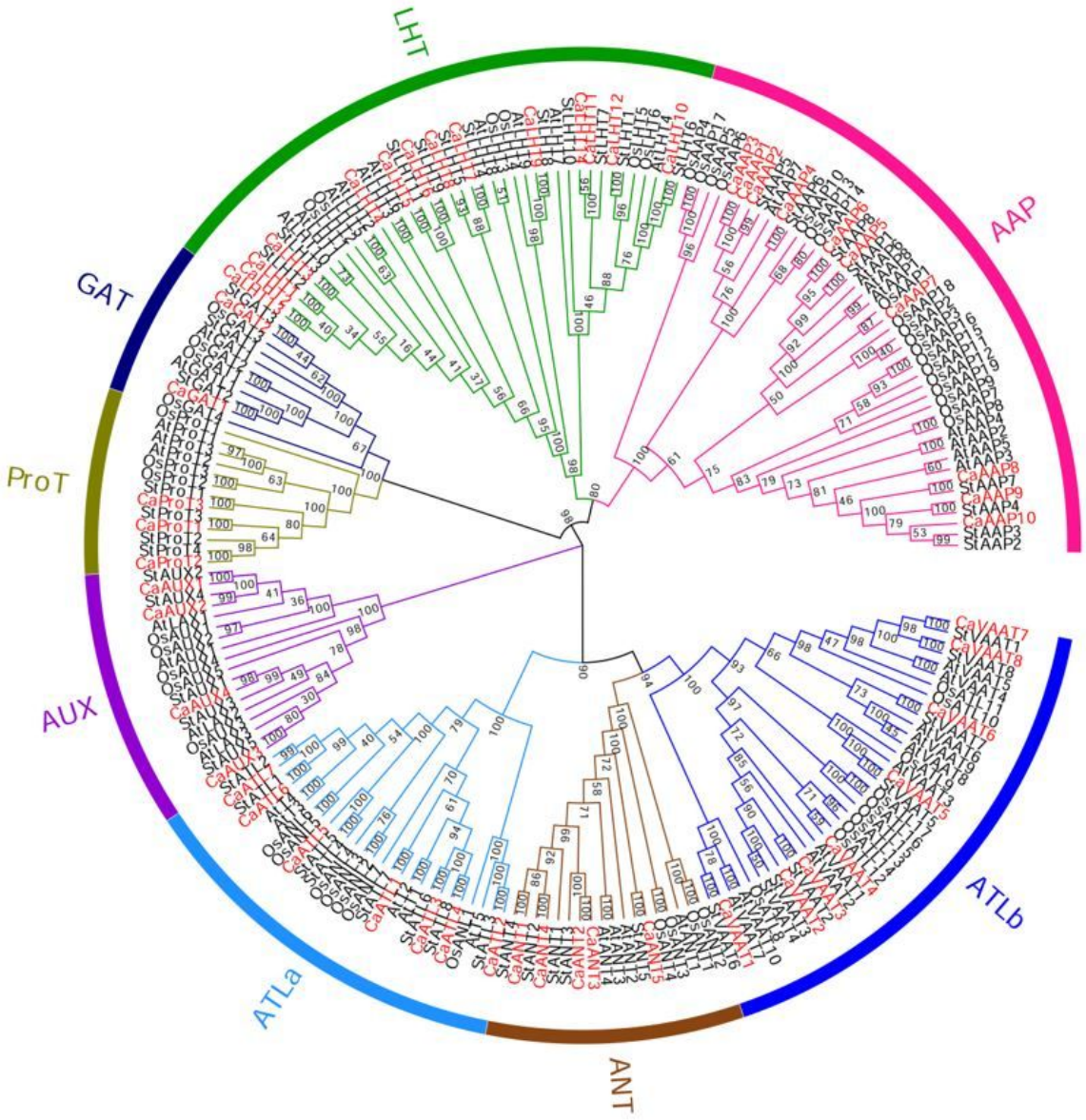

Figure 2

Phylogenetic relationships of pepper, potato, rice, and Arabidopsis AAAP proteins. Multiple sequence alignment of full-length proteins was performed by Clustal X1.83 and the phylogenetic tree was constructed using MEGA7 with the neighbor-joining method. The tree was divided into eight subgroups, marked by different color backgrounds.

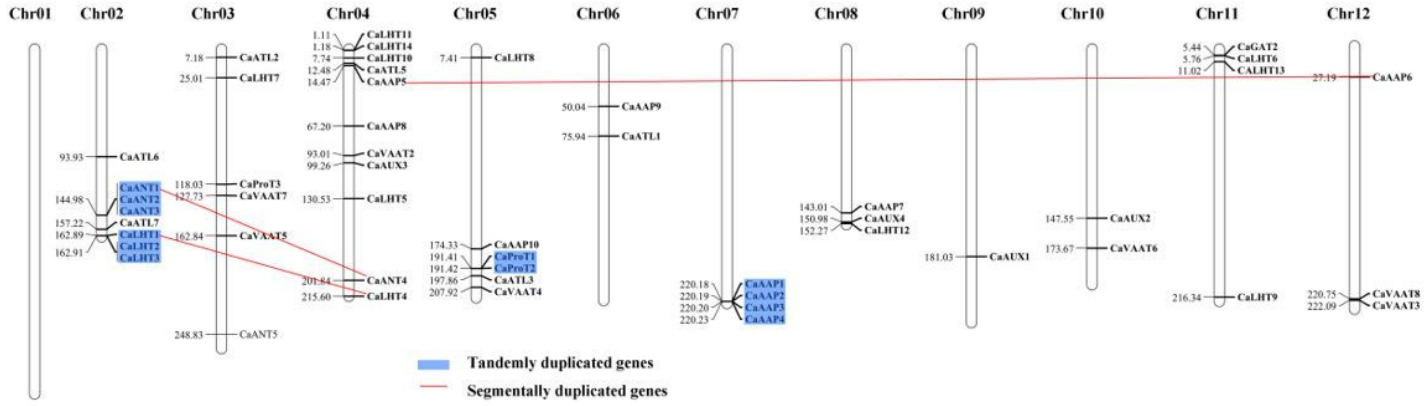

Figure 3

Chromosomal localization and gene duplication events of CaAAAP genes. Respective chromosome numbers are indicated at the top of each bar. Tandem duplicated genes are marked on a blue background. Segmental duplicated genes are shown by red line.

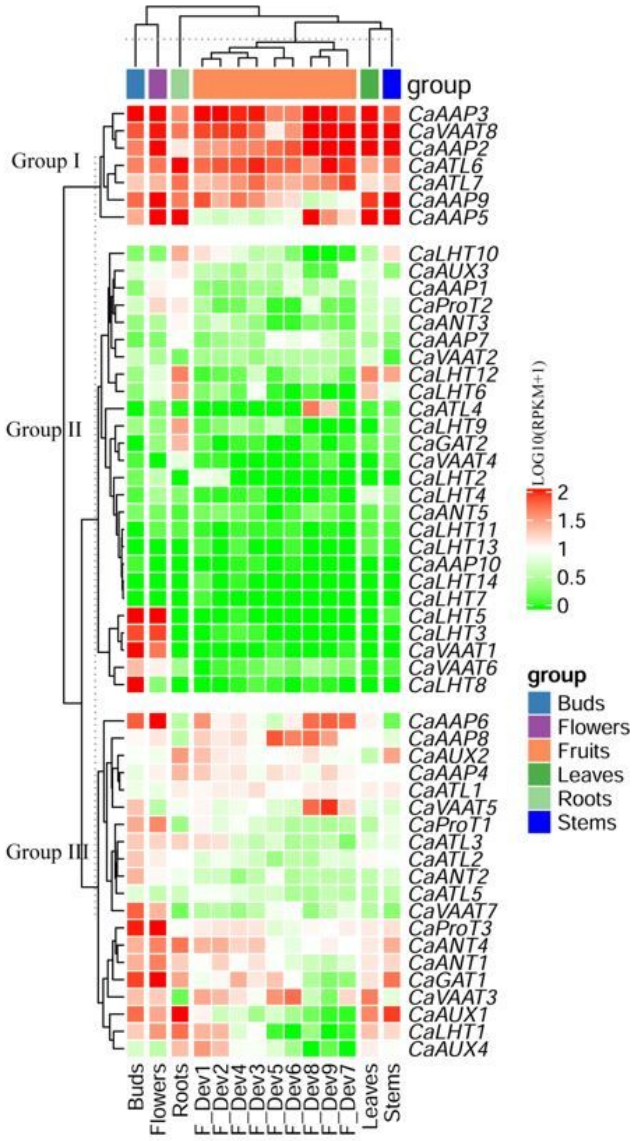

Figure 4

Expression profiles of CaAAAP genes in different tissues. The relative expression levels corresponding to log 10-transformed RPKM values after the addition of a pseudocount of 1 are shown. The scale represents the relative signal intensity of the RPKM values.

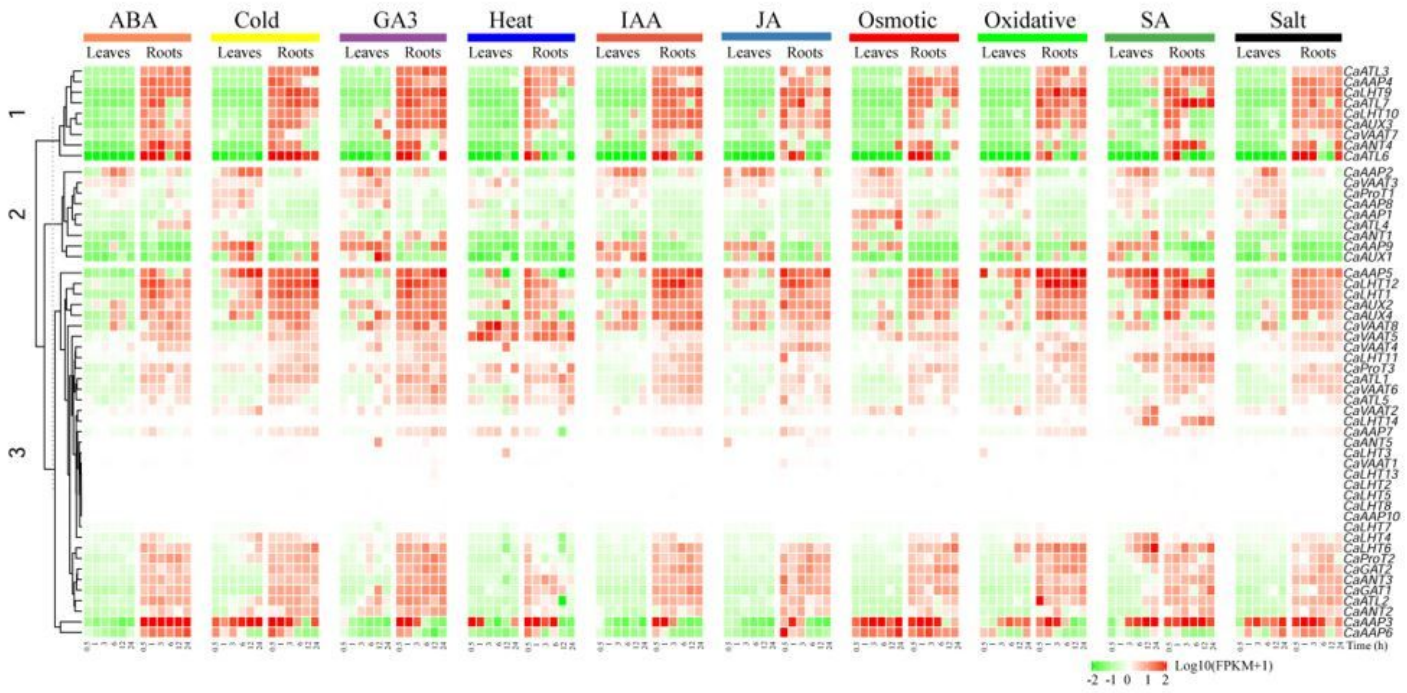

Figure 5

Expression patterns of CaAAAP genes in response to different hormone treatments and abiotic stress in the leaves and roots. Relative expression changes corresponding to log 10-transformed FPKM values between experimental and control tissues are shown. To avoid a situation where a FPKM equals 0, 1 was added to all the FPKM values. The scale represents the relative signal intensity of the FPKM values.

Supplementary Files

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

- Additionalfiles.docx
- renamed6c869.xlsx
- renamed4b105.xlsx
- renamedbc87d.xlsx
- renamed3fe5e.doc
- renamed9d279.tif
- renameda463f.tif