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Genome-wide survey of the amino acid transporter gene family in wheat (*Triticum aestivum* L.): Identification, expression analysis and response to abiotic stress

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ABSTRACT

Amino acid transporters (AATs), which transport amino acids across cell membranes, play important roles in alleviating plant damage under stresses and in plant growth. To data, little is known about the *AAT* genes in wheat because of its complex genome. In this study, a total of 296 *AAT* genes were identified from the latest wheat genome sequence (IWGSC v1.1) and classified into 12 distinct subfamilies based upon their sequence composition and phylogenetic relationship. The expansion of the wheat AAT family was mainly the results of whole-genome duplication (WGD) and tandem events. The unequal expansion of different subfamilies brought new features to *TaAATs*. *TaAATs* were highly expressed and exhibited distinct expression patterns in different tissues. On the basis of homology and expression pattern analysis, we identified several wheat AAT family members that may affect grain quality. In addition, *TaAAP3*, *TaATLa2* and *TaATLb13* exhibited sustained expression in response to drought and high-temperature stress. These genes are involved in the response of wheat to abiotic stress by regulating the transport and distribution of amino acids. Overall, our results help to understand the complexity of *TaAATs* and provide a theoretical basis for further identification and utilization of *AATs* in wheat and other crop species.

1. Introduction

Amino acids not only are indispensable for the formation of proteins but also the main carrier for nitrogen exchange in plants [1,2]. Acquisition of organic nitrogen depends to a large extent on the transport of amino acids, especially in the processes of seed germination and seedling growth, mainly by amino acid transporters (AATs) [3,4]. Moreover, studies have confirmed that AATs play important roles in determining the nutritional quality and protein content of seeds [5–7]. AATs are essential membrane proteins responsible for the transport of amino acids across cellular membranes in higher plants and functions in multiple physiological processes of plant growth and development, including long-distance transport of amino acids, absorption of amino acids from the soil and the responses to pathogens and abiotic stresses [8–13]. The *AAT* gene family has been systematically identified and characterized in several plant species; for instance, there are 63 genes in *Arabidopsis*, 85 in rice, 189 in soybean, and 72 in potato, indicating that the *AAT* gene family is widespread in higher plants [14–17]. This gene family in plants includes the amino acid-polyamine-choline (APC) family and the amino acid/auxin permease (AAAP) family, both of which belong to the APC transporter superfamily. The APC family is composed mainly of three subfamilies - amino acid/choline transporters (ACTs), cationic amino acid transporters (CATs) and polyamine H^+-symporters (PHSs), with tyrosine-specific transporters (TTPs) also identified in some plant species [18–20]. The AAAP family contains at least six subfamilies, including lysine and histidine transporters (LHTs), γ-aminobutyric acid transporters (GATs), proline transporters (ProTs), amino acid permeases (AAPs), auxin transporters (AUXs), and aromatic and neutral amino acid transporters (ANTs) [18,20,21].

Although the AAT family has been identified by genome-wide scanning in multiple plant species, the most detailed characterizations of its functions have been mainly performed in *Arabidopsis*. Many *AAT* genes alleviate damage to plants under water stress by promoting the transport of stress-related compounds and compatible solutes [22]. For example, *AtProTs* are responsible for the transport of various substrates, such as proline, glycine betaine and γ-aminobutyric acid (GABA), among which AtProT1 can rapidly transport proline under water stress to reduce plant damage [23]. Although ProTs have similar subcellular localization and substrate specificity, in tomato, LeProT1 transports glycine and GABA with different affinities, while in *Arabidopsis*, *AtProTs* are specifically expressed in tissues with elevated proline content [23,24]. Moreover, *AtProT2* also plays a crucial role in the transport of

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compatible solutes to the root tip region, as evidenced by studies in barley [23,25]. In addition, *AAT* genes also affect plant growth and development through the regulation of auxin [26]. For example, *AtAUX1* promotes the root tropism and lateral root formation by mediating the influx of auxin into the roots [26,27].

In addition to alleviating plant damage under water stress by promoting the transport of stress-related compounds and compatible solutes, many *AAT* genes directly affect embryonic development and seed protein content. The Arabidopsis AAP subfamily contains eight members: *AtAAP1*-*AtAAP8*. Notably, detailed characterization of *AtAAP*s via heterologous expression systems has demonstrated that six *AtAAP*s preferentially transport neutral and charged amino acids with different specificities and affinities [28,29]. *AtAAP1* is expressed specifically in cotyledons and endosperm, regulating the transport of amino acids to root cells or developing embryos, and is therefore essential for seed yield and storage protein synthesis [10,30]. *AtAAP2* affects the transport of amino acids from xylem to phloem [31]. Analysis of the aap6 mutant confirmed that *AtAAP6* affects interaction with aphids by modulating the amino acid content in the sieve elements of *Arabidopsis* [20]. *AtAAP8* is considered to play an important role in the uptake of amino acids in embryos and endosperms during early embryonic development [32]. In addition, the functions of *AAP* genes have been studied in other species and have been confirmed to be related to grain development and protein content [5–7]. For example, the content of storage proteins in seeds of *Vicia narbonensis* and *Pisum sativum* overexpressing *VfAAP1* significantly increased [6]. Nitrogen supply during barley grain development is dependent mainly on the specific expression of *HvAAP3* [33]. Moreover, the quantitative trait locus (QTL) qPC1, which controls rice grain protein content (GPC), was confirmed to be associated with the expression of *OsAAP6* [7].

Bread wheat (*Triticum aestivum* L.) is one of the most important crop species worldwide, occupying 17% of cultivated land and accounting for approximately 35% of global staple foods, and it is also an important protein source for humans [34,35]. Genetically, wheat is an allohexaploid species with a complex origin and evolutionary history. The large and complex genome, which is over 17 Gbp and comprises three homologous sub-genomes (A, B and D), and poses an enormous challenge for wheat genomic research. Recently, the release of the high-quality genome sequence of hexaploid wheat Chinese Spring (CS) based on the chromosomal strategy laid the foundation for the identification of wheat gene families at the genomic level [36]. Although Wan et al. studied the temporal and spatial expression characteristics of the wheat AAT family and deduced their functions in nitrogen transport, the identification of the AAT family was incomplete and unsystematic, as it was based on the draft genome sequence and lacked an analysis of gene structure and evolutionary characteristics [37]. In the present study, a genome-wide scan was conducted to identify the wheat *AAT* gene family. Then, the chromosome localization, gene structure, conserved protein motifs, duplication pattern, and selective pressure of the putative wheat *AAT* genes were systematically analyzed, and a phylogenetic tree was constructed. Finally, on the basis of published transcriptomic data and real-time PCR data, the expression characteristics of these genes in different tissues/organs and under different abiotic stresses were analyzed. Our work once again emphasizes the positive role of gene duplication and selection pressure in the expansion and functional diversification of the genes in *AAT* family. Combining these genes' temporal and spatial expression characteristics and their responses to abiotic stresses, this study will provide a basis for further functional analysis of the wheat *AAT* genes, as well as an improved understanding of the molecular mechanisms underlying the wheat *AAT* genes' regulation of wheat growth and stress responses.

2. Materials and methods

2.1. Identification of the AAT gene family in wheat

Several methods were used to discover putative AAT family members in the wheat genome. First, wheat whole-genome protein sequences (IWGSC RefSeq v1.1) were downloaded from the Wheat URGI database (https://wheat-urgi.versailles.inra.fr) to construct a local protein database. The known protein sequences of 337 *AAT* genes from *Arabidopsis thaliana*, rice (*Oryza stativa*) and soybean (*Glycine max*) were then used as seed sequences to search the wheat protein database via the local BLASTP program with an e-value of 1e−5 and an identity of 50% as the threshold. Furthermore, the hidden Markov model (HMM) profiles of the AAT domain (PF00324 and PF01490) were downloaded from the PFAM database (http://pfam.xfam.org/), and all putative AAT proteins predicted by BLASTP were further screened by conserved domains using the HMMER search tool [38]. An NCBI conserved domain database (CDD) search was also used to check the conserved protein domains of these candidate genes (https://www.ncbi.nlm.nih.gov/cdd). After the redundant sequences were removed, the remaining sequences were submitted to InterProScan (http://www.ebi.ac.uk/interpro/scan.html) to reconfirm the presence and integrity of the AAT conserved domains. Last, all putative *AAT* gene family members in wheat were identified after the sequences that did not contain the entire conserved domains were removed. Information about these putative wheat *AAT* genes (*TaAAT*s), including full-length cDNA accessions, coding sequence length, and gene structure information, was obtained from the gff3 genome annotation file. The Gene Structure Display Server GSDS (http://gsds.cbi.pku.edu.cn/) online tool, was used to determine the structures of these genes, and the Computer pI/MW tool in the ExPASy database (https://web.expasy.org/compute_pi/) was used to calculate the biochemical parameters, including the theoretical isoelectric point (pI) and molecular weight (MW) [39]. Moreover, the TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used with the default settings to predict the putative transmembrane (TM) regions in each TaAAT protein, and the Cell-PLoc software was used to predict the subcellular location of the proteins [40].

2.2. Chromosomal localization, duplication and selective pressure of AAT genes in wheat

The *TaAAT* genes were mapped onto the chromosomes by identifying their chromosomal positions obtained from the gff3 wheat genome annotation file. The Multiple Collinearity Scan (MCScanX) toolkit program was used to investigate gene duplication, and manual screening was performed according to the mature method described by Wang et al. [41,42]. TBtools tool was subsequently used to visualize the chromosome localizations and duplicated regions of all *TaAAT* genes [43].

Natural selection shapes the various functions of duplicated genes. To assess the impact of sequence duplication on function, KaKs_Calculator 2.0 was used to calculate the nonsynonymous (Ka) and synonymous (Ks) ratio (Ka/Ks) of each aligned codon in the pairs of duplicated *AAT* genes [44].

2.3. Phylogenetic analysis and multiple sequence alignment

To clarify the evolutionary relationship between *AAT* gene family members in wheat and other angiosperms and to classify their subfamilies, *AAT* gene family members of three monocotyledonous species, maize (*Zea mays*), rice (*Oryza stativa*), *Brachypodium distachyon*, and two dicotyledonous species, potato (*Solanum tuberosum*) and *Arabidopsis thaliana*, were compared using a phylogenetic tree together with *TaAAT* genes. Multiple-sequence alignment of the protein sequences was performed by MUSCLE 3.8 [45]. The phylogenetic tree was constructed using the maximum likelihood (ML) method by PhyML 3.1 (http://www.atgc-montpellier.fr/phyml/versions.php) with the JTT model from IQ-TREE [46]. iTOL v3 (http://itol.embl.de/#) was used to display the phylogenetic tree.

The Multiple EM for Motif Elicitation (MEME) program was used to determine the conserved protein motifs of these genes, and the parameters of which were as follows: the optimal motif width was between 6 and 200 residues, allowing the presence of any number of repeating

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motif sites, and the maximum number of motifs was 20 [47]. The amino acid sequence conservation of TaANT subfamily members was analyzed via DNAMAN software, and the conserved motifs and TM regions were manually annotated.

2.4. Identification of cis-regulatory elements and prediction of three-dimensional modeling

The sequence 2000 bp upstream of the start codon was considered the promoter region for each gene, and the promoter sequences were extracted from each genome using the SAMtools program [48]. The putative transcriptional response elements within these gene promoters were predicted using the PlantCARE serve (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), a database of plant cis-acting regulatory elements [49].

To determine the differences in the structure of different AAT subfamily proteins and their effects on functions, the three-dimensional structure of a representative AAT protein from each subfamily was determined via the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2) [50].

2.5. Spatiotemporal expression patterns of AATs in wheat

To explore the spatiotemporal expression patterns of *TaAAT*s, published wheat transcriptomic data were downloaded from expVIP (http://www.wheat-expression.com) [51]. These data include read count values from 14 tissues at three important stages of wheat growth and development: the seedling stage, flag leaf stage and milk grain stage. The tissues used at the seeding stage included radicle (SRA), coleoptile (SC), stem axis (SSA), first leaf blade (SFL), roots (SR) and shoot apical meristem (SSAM) tissues; the tissues at the flag leaf stage included flag leaf blade (FFL), shoot axis (FSA), roots (FR) tissues; and the tissues at the milk grain stage included flag leaf blade (MFL), peduncle (MP), awns (MA), glumes (MGL), and grain (MG) tissues. In order to analyze the roles of *TaAAT*s in the development of wheat grain, a transcriptome dataset containing all stages of wheat embryo, endosperm and seed coat development was used to determine essential *AAT* genes that may affect wheat grain development [52]. The normalized expression levels of *TaAAT*s were expressed by calculating transcripts per million (TPM) values standardized by the R package edgeR [53]. The $\log_2$ (TPM + 1) values were displayed in a heat map to visualize the tissue expression characteristics of *TaAAT*s.

2.6. Plant materials, abiotic stress treatments and quantitative real-time PCR

For stress treatments, the wheat seeds (cultivar 'Chinese Spring') were collected from the experimental field of our own laboratory in College of Agronomy of Northwest A&F University. All the seeds planted in a greenhouse at Northwest A&F University under a temperature of 22 °C, and a 16 h light (12,000 lx)/an 8 h dark photoperiod was used for seed growing. Wheat seedlings at the three-leaf stage of wheat were used for the abiotic stress treatments; the seedlings were exposed to 20% polyethylene glycol (PEG-6000) and 35 °C temperature for 1 h and 6 h to simulate drought and heat stress, respectively [17]. In addition, long-term soaking of seedlings in NaCl solution (200 mM; 6 h, 12 h, 24 h, 48 h) was used to simulate salt stress [54]. The plant leaves and roots were then collected for RNA extraction by an RNAiso Plus Kit (Takara, Dalian, China). To verify the spatiotemporal expression characteristics of *TaAAT*s, a total of 13 tissues derived from four important growth stages of wheat (cultivar 'Chinese Spring') were used for RNA extraction. A PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) and a SuperReal PreMix Color Kit (SYBR Green) (Tiangen Biotech, Beijing, China)were used for the synthesis of the first strand complementary cDNAs and the quantitative real time-PCR (qRT-PCR), respectively. TaActin was used as an internal control, and all the special primers for qRT-PCR were designed using Primer Premier 6.0 software (http://www.premierbiosoft.com/primerdesign/); all primers are listed in Table S2. These primers cannot distinguish three homologous copies, so the expression level of the selected gene would be amplified. The experiment included three independent biological replicates for each sample, and the $2^{-\Delta\Delta Ct}$ method was used to evaluate the expression level.

3. Results

3.1. Identification of AAT gene family in wheat

Initially, a total of 307 putative wheat *AAT* transcripts were identified in the wheat genome by local BLASTP and HMMER searches. Twenty of the transcripts corresponded to 10 genes, and we selected the longest transcripts as candidates. One transcript containing incomplete conserved domains was omitted. In total, 296 putative *AAT* genes with high confidence were ultimately identified in the wheat genome. Compared with other plant species reported, wheat had the largest *AAT* gene family,

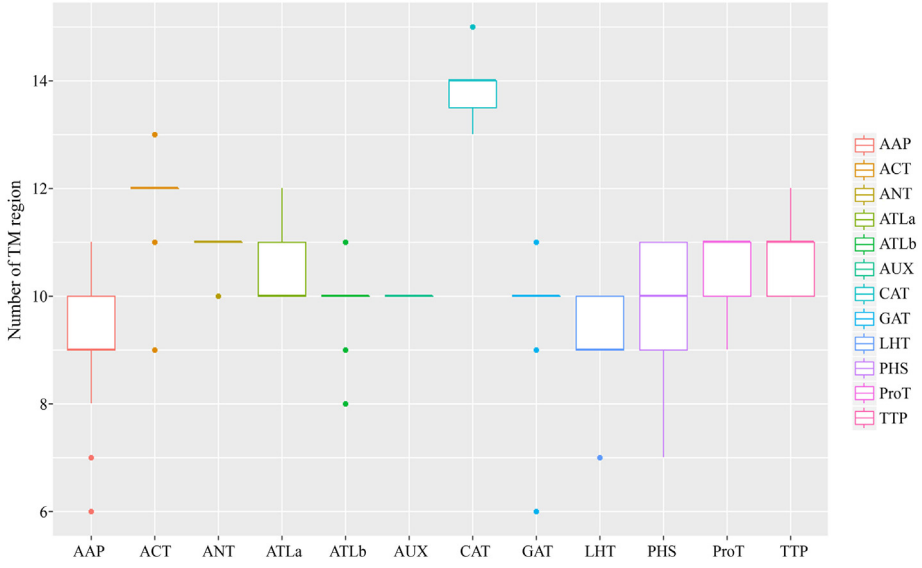

Fig. 1. Distribution of numbers of putative transmembrane (TM) regions in the wheat amino acid transporter (AAT) family. The X-axis lists the 12 AAT subfamilies in wheat, and the Y-axis represents the range of numbers of TM regions in each subfamily. In the boxplot, the middle line indicates the median, and the box indicates the range from the 25th to the 75th percentile of the total number of TM regions. The top and bottom points represent the maximum and minimum values, respectively.

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Table 1
Comparison of the gene abundance in 12 *AAT* gene subfamilies of different plant species.

		Monocots				Eudicots		
		Wheat	Rice	Maize	*Brachypodium*	Soybean	*Arabidopsis*	Potato
AAAP	AAP	66	19	24	19	35	8	8
	LHT	24	6	15	8	24	10	11
	GAT	14	4	2	3	19	2	3
	ProT	9	3	2	2	7	3	4
	AUX	15	5	6	3	16	4	5
	ATLa	18	7	7	6	16	5	8
	ANT	18	4	3	5	6	4	5
	ATLb	40	10	17	7	30	10	8
APC	ACT	21	7	7	6	7	1	1
	CAT	31	11	14	11	19	9	9
	PHS	31	9	7	7	9	5	8
	TTP	9	0	3	3	1	2	2
Total		296	85	107	80	189	63	72

which might be the result of its allohexaploid genome and complex evolutionary process. Similar methods were used to identify the genes of AAT family in maize and *Brachypodium*, with 107 AAT genes identified in maize and 80 in *Brachypodium* (Table 1). On the basis of subfamily classification and chromosomal localization, these 296 *AATs* in wheat were renamed, and detailed information, including gene structure and protein properties, is listed in Table S1. The length of the putative wheat AAT proteins ranged from 318 to 1002, with pIs ranging from 5.00 to 8.96 and MWs ranging from 33.5 to 52.5 kDa. The protein characteristics of the homologous *AAT* genes from different wheat subgenomes did not significantly differ.

To better understand the TM structure of the AAT family, TMHMM Server 2.0 was used to predict the putative TM regions. The number of TM regions in TaAAT proteins ranged from 6 to 15, and *AAT* genes belonging to the same subfamily exhibited a similar distribution (Fig. 1, Table S1). Among them, CAT subfamily members contained the most TM regions, ranging from 13 to 15, while the AAP and LHT subfamily members contained the fewest TM regions, ranging from 7 to 11 and 7 to 10, respectively (Fig. 1). In addition, it was found that 19 groups of homologous genes derived from the A, B and D subgenomes had mutations that affected the TM number, accounting for approximately 20% (19 of 93) of the total complete homologous gene groups identified in

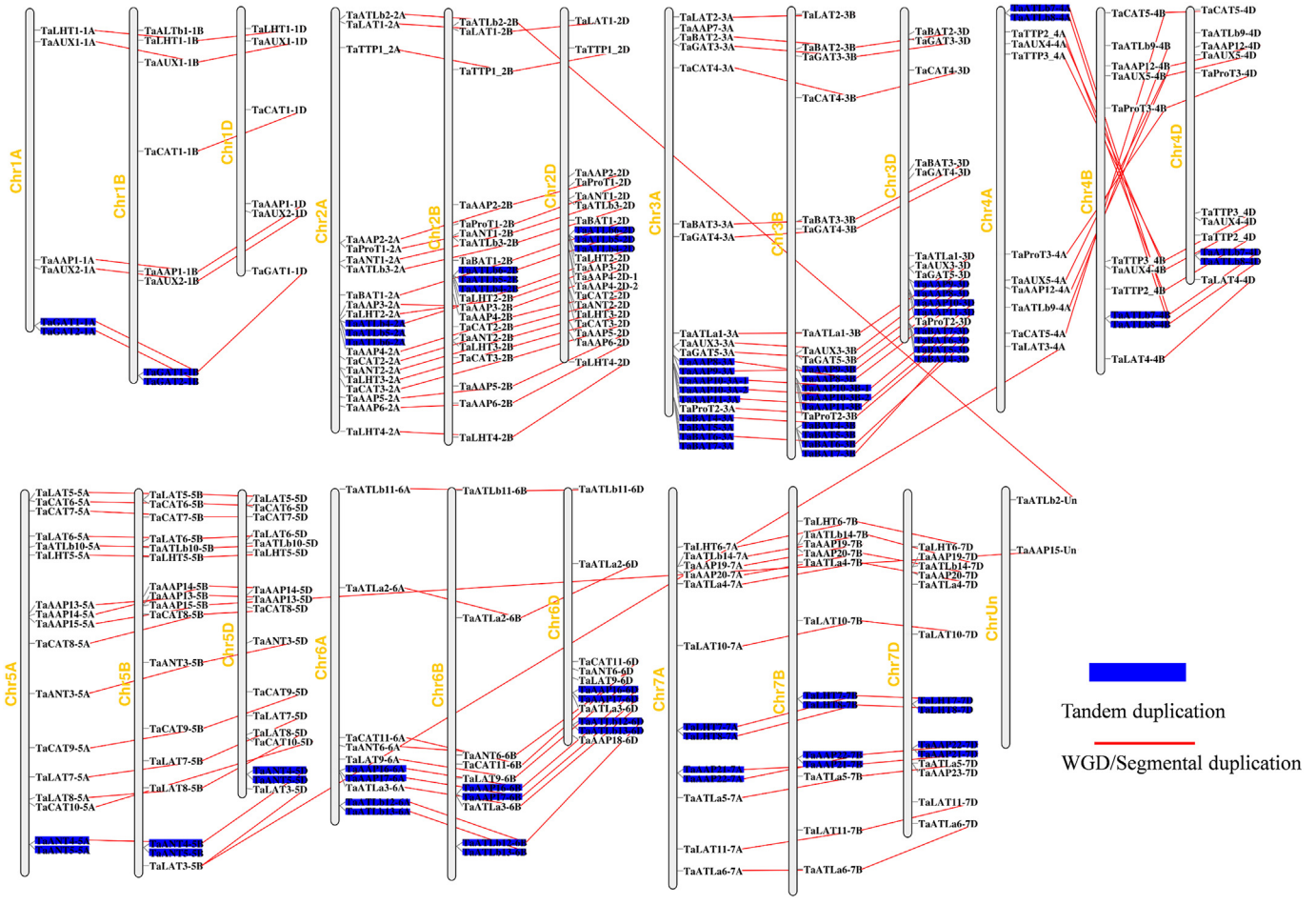

Fig. 2. Chromosomal localization and gene duplication events of *TaAAT* genes. The chromosome numbers are shown on the left side of each strip. Homologous genes are linked by red lines. The blue shadows indicate tandem duplications. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Comparison of the proportion of tandemly duplicated genes in 12 *AAT* gene subfamilies of different plant species.

		Monocots		Eudicots	
		Wheat	Rice	*Arabidopsis*	Potato
AAAP	AAP	39.39%	52.63%	31.42%	25%
	LHT	25%	0%	8.33%	18.18%
	GAT	28.59%	0%	31.57%	0.00%
	ProT	0%	0%	28.57%	100%
	AUX	0%	0%	0%	0%
	ATLa	0%	0%	37.50%	50%
	ANT	33.33%	0%	0%	40%
	ATLb	52.50%	50%	6.66%	0%
APC	ACT	57.14%	71.42%	71.42%	0%
	CAT	0%	0%	10.52%	44.44%
	PHS	0%	0%	0%	25%
	TTP	0%	NA	0%	0%

this study, which confirmed that during the process of wheat genome doubling, the TM number of the homologous genes from the different subgenomes varied widely to achieve functional synergy and suitability.

All 296 AATs identified were transmembrane proteins and mainly distributed on plasma membrane, vacuolar membrane, chloroplast membrane and golgi apparatus membrane (Table S1). Results from the subcellular location and prediction via Cell-PLoc software indicated that genes of different subfamily varied in subcellular localization (Table S1). For example, genes of AAP, ATLa, AUX, GAT, LHT and ProT subfamilies were located on the plasma membrane, while genes of ANT, ATLb and ACT subfamilies were located on the vacuolar membrane.

3.2. Chromosomal distribution and duplication analysis of AAT genes in wheat

To study the relationship between *AAT* gene family expansion and gene duplication in wheat, 294 of the 296 *TaAAT*s were mapped onto 21 chromosomes of wheat, with the remaining two genes mapped onto an unattributed scaffold (Fig. 2). With the exception of the chromosomal inversion of chromosomes 4A and 4B, the number and distribution of *TaAAT*s from the different subgenomes were highly similar, confirming that the AAT family has been relatively conserved since the formation of hexaploid wheat formed. Moreover, the chromosome distribution of wheat AAT family genes was significantly heterogeneous, with relatively high density in specific chromosomal regions, such as the end of chromosome groups two and three, whereas the top of chromosome group six contained very few *TaAAT*s. In terms of their overall distribution among chromosome groups, chromosome group two contained the most (61) *AAT* genes, while chromosome group one had only 20, and the other chromosome groups had 31 to 56 (Fig. 2).

Gene duplication is generally considered to be the major factor leading to gene family expansion and functional diversification. After two naturally interspecific hybridization events of three diploid species and doubling, current hexaploid wheat was produced. In principle, each wheat gene usually has three homologous loci caused by polyploidization [55]. Through sequence similarity analysis and chromosome location analysis, 93 homologous genes consisting of three copies from the A, B, and D subgenomes were found in the wheat genome, accounting for approximately 94% of all putative *TaAAT*s. Although *TaLAT3*, *TaATLb2* and *TaAAP15* did not match our expectations in terms of their chromosomal locations, the three sets were still considered homologs on the basis of their high sequence similarity. Moreover, six *AAT* genes contained only two copies among the A, B, or D homologous chromosomes, which accounted for approximately 4% of all putative *TaAAT*s, and five *AAT* genes contained only one copy. Only 8% of the *AAT* genes were lost, and the genes with the TM region mutation reached 20%, indicating that the functional adaptation of the wheat AAT family genes during wheat polyploidization was driven mainly by sequence mutation rather than copy number variation.

Among the 296 wheat *TaAAT* genes, 25.33% (75 of 296) originated from tandem duplication events (Fig. 2). The 75 tandemly duplicated genes could be divided into 32 groups, of which 24 groups contained 2 genes each, 5 groups contained 3 genes each and 3 groups contained 4 genes each. With the exception of the two sets of tandemly duplicated genes only in the A and B subgenomes, all of the tandemly duplicated genes had homologous copies in all three subgenomes, indicating that most tandem duplication events occurred before wheat polyploidization. To assess the effect of tandem duplication events on the expansion of AAT subfamilies, we compared the proportions of tandemly duplicated genes in the AAT subfamilies of wheat, rice, *Arabidopsis* and potato subfamilies (Table 2). In all these species, the proportion of tandemly duplicated genes in the AAP subfamily was very high, ranging from 25% to 52.63%. In addition, for other subfamilies, dicotyledons and monocotyledons significantly differed. For example, a high proportion of genes in the ATLb subfamily in monocots were tandemly duplicated, while members of the ProT subfamily, the ATLa subfamily, and the CAT subfamily in dicots had high proportion of tandemly duplicated genes.

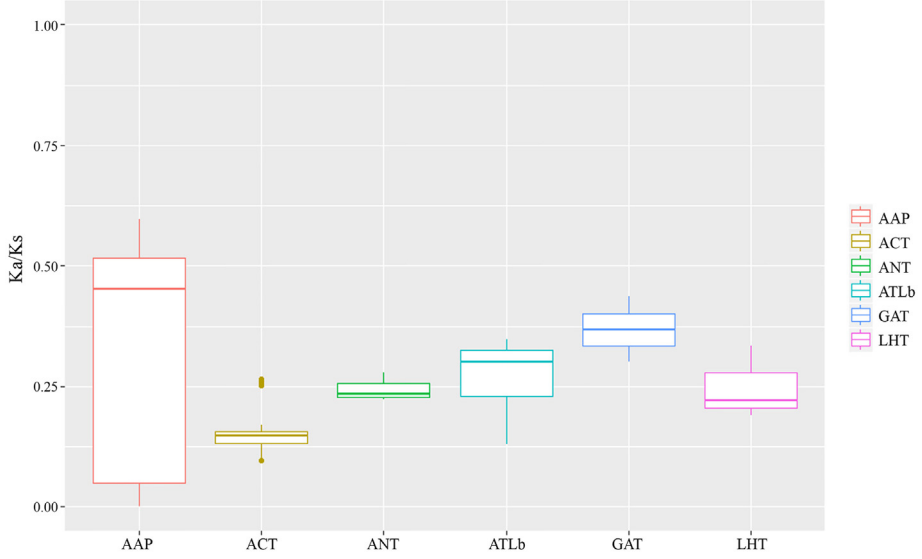

Fig. 3. Effects of tandem duplication events on the functional differentiation of wheat amino acid transporter (*AAT*) gene subfamilies. The Y-axis indicates the rates of nonsynonymous (Ka) and synonymous (Ks) substitutions (Ka/Ks). The X-axis shows the subfamilies containing tandemly duplicated *AAT* genes.

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3.3. Selective pressure analysis of AAT genes in wheat

Gene duplication and natural selection largely shape the diversity of gene functions. In view of the tremendous impact of tandem duplication events on the expansion of the AAT family in wheat, we used the Ka/Ks value to evaluate the selection pressure of tandemly duplicated gene pairs (Fig. 3). Fifty tandemly duplicated gene pairs distributed in the same homologous group were selected for Ka/Ks analysis. The average value of all gene pairs was 0.27, which indicated that all the genes were under purifying selection to maintain important biological roles. Notably, the median value and dispersion of Ka/Ks in the wheat AAP subfamilies were significantly greater than those in other subfamilies, which indicated that a considerable number of *TaAAP*s have evolved new features under weak selection pressure with gene replication and sequence diversity (Fig. 3). All the above results indicate that chromosome doubling and tandem duplication were keys to the expansion of the wheat AAT family. In addition, tandem duplication events played an important role in the functional differentiation of the wheat AAP subfamily genes.

3.4. Structural characteristics of 296 AAT genes in wheat

Numerous studies have confirmed that one of the representative traces of family evolution involves gene structural characteristics [56–58]. On the basis of the annotated genome structure information, we investigated all 296 wheat *AAT* genes (Fig. 4). Homologous genes derived from different subgenomes exhibited similar intron/exon distributions in their gene structure, suggesting that the *AAT* homologous genes in various subgenomes of wheat are extremely functionally conserved. Notably, the gene structure of the paralogous genes of the same subfamily somewhat differed, indicating that there is also a very significant functional differentiation among members of the same subfamily. For example, the structure of six of the 13 tandemly duplicated genes in the AAP subfamily significantly varied, which was consistent with previous Ka/Ks results, again confirming that the production of new functions between tandemly duplicated genes was key to the functional diversification of the wheat AAP subfamily genes. Twenty-nine *AAT* genes did not contain introns, while the rest contained 1–16 introns, which was consistent with the results of studies of other species.

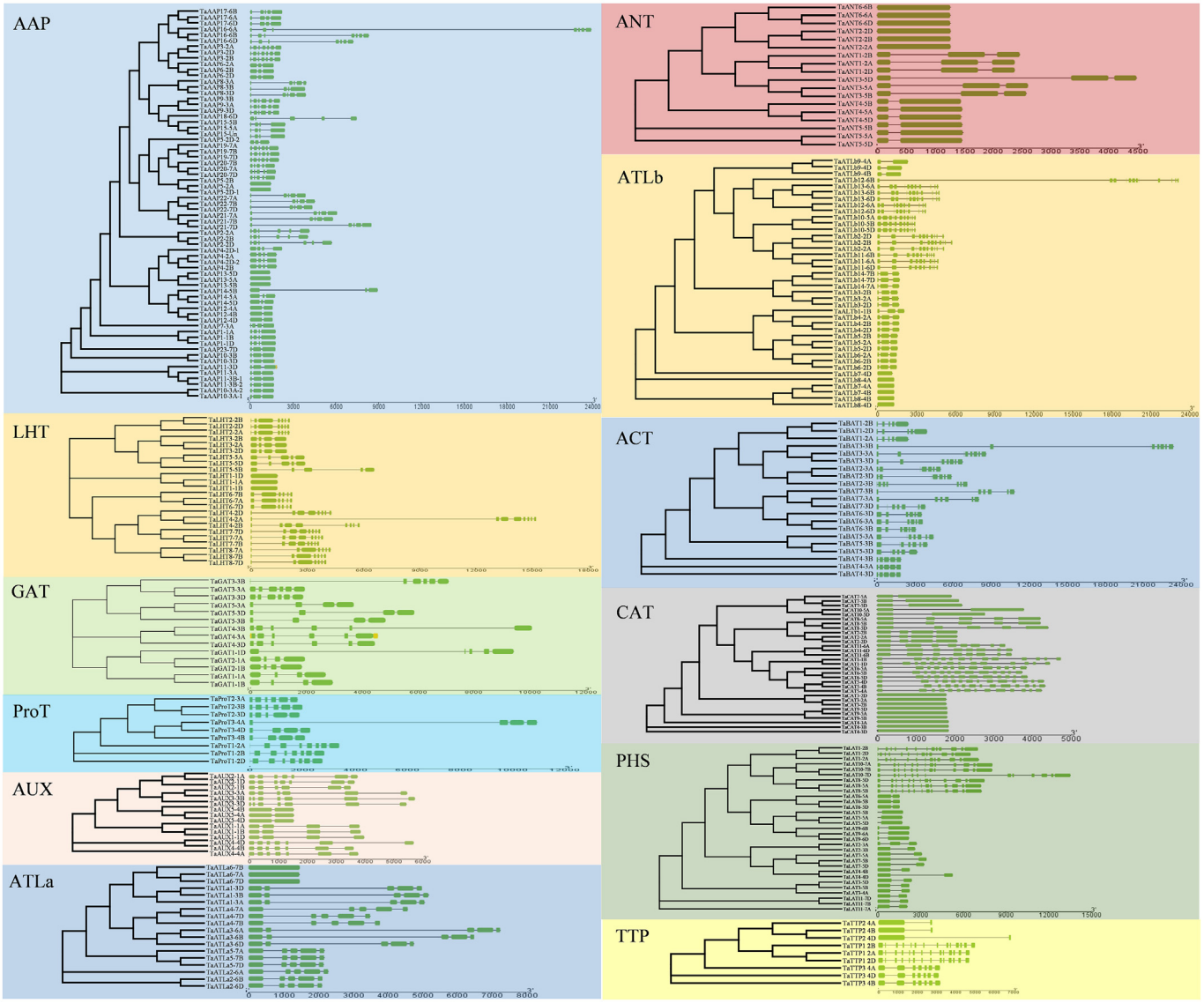

Fig. 4. Gene structure of *TaAAT* genes in each subfamily. Different color backgrounds represent different subfamilies of the wheat AAT family. The phylogenetic trees are constructed using the maximum likelihood method by PhyML 3.1 with the JTT model from IQ-TREE. The gene structure information is obtained from gff3 wheat genome annotation file. Green boxes represent exons, and the block line represents introns. The untranslated regions (UTRs) are indicated by yellow boxes. The sizes of introns and exons can be estimated by the scale at the bottom. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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To explore the evolutionary relationships among AAT members of different subfamilies better, conserved motifs were also predicted by MEME (Fig. S1). Similar to that which occurred for the gene structure, the distribution of conserved motifs in different gene families or subfamilies was also conserved or varied. For example, motif 1 was found in both the AAAP and APC family members (although it was incomplete in APC family members), which confirmed that Motif 1 was relatively conserved during the expansion of the AAT family and was retained in both families. In contrast, motifs 2 and 9 were found only in members of the AAAP family, and motif 10 was found mainly in members of the APC family, suggesting that functional differentiation of the *AAT* gene family was accompanied by loss of and variation in conserved motifs. In addition, some motifs were found to be distributed only in a few subfamilies. For example, motif 3 was specific to the AAT subfamily, motif 12 was specific to the ACT and CAT subfamilies, and motif 8 was specific to the AAT and ANT subfamilies. There were large differences between members of different families or subfamilies, while members of the same subfamily shared similar conserved motifs.

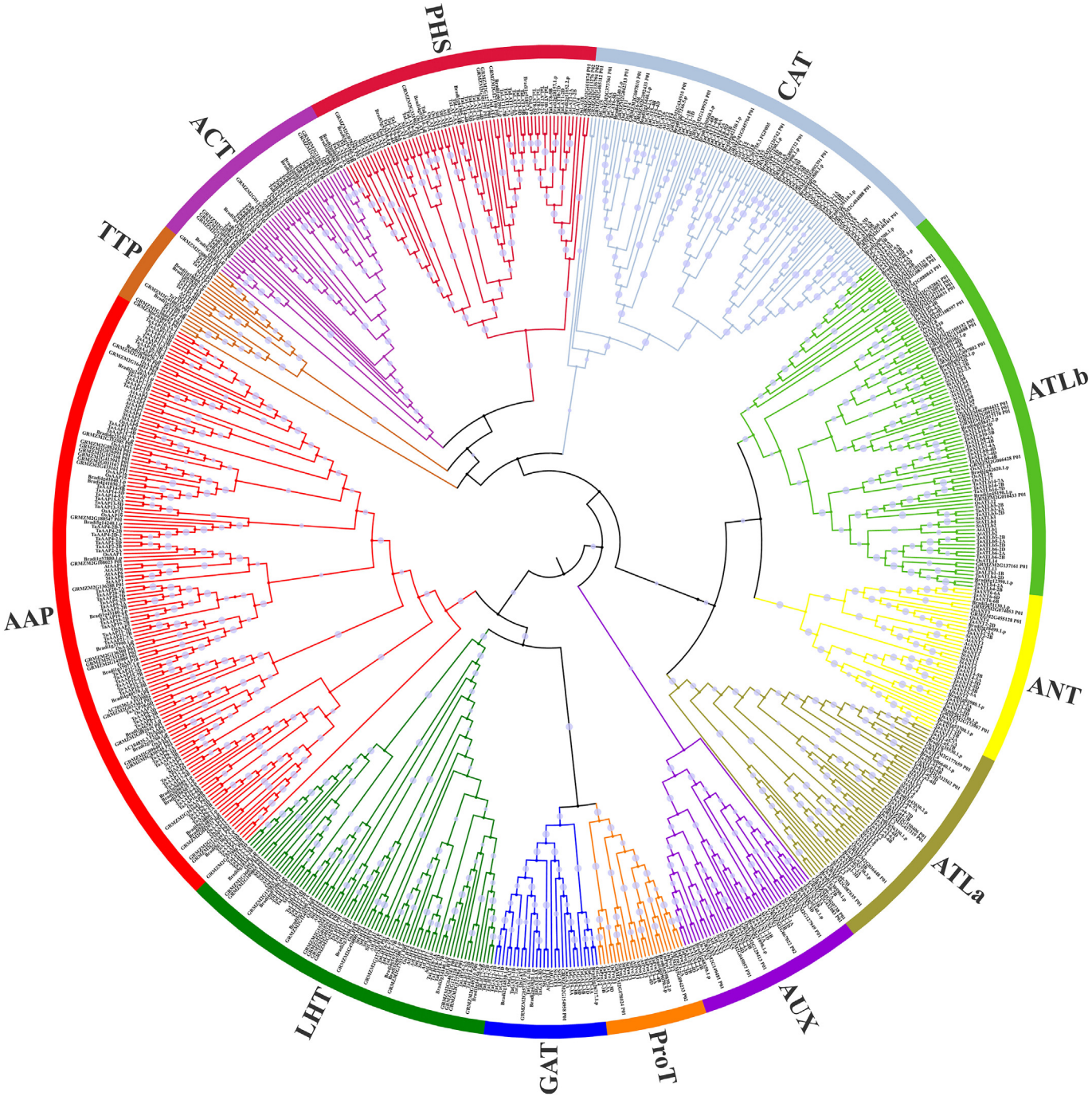

Fig. 5. Phylogenetic tree of amino acid transporter (AAT) proteins in wheat, maize, *Brachypodium*, *Arabidopsis* and soybean. Multiple sequence alignment was performed by MUSCLE, and the phylogenetic tree was constructed by PhyML in accordance with the maximum likelihood (ML) method. Different colored branches represent different subfamilies of the wheat AAT family. The gene ID starting with Os-, At-, St-, Ta-, Bradi- and GRMZM- represent the AAT genes in rice (*Oryza stativa*), *Arabidopsis thaliana*, potato (*Solanum tuberosum*), wheat (*Triticum aestivum*), *Brachypodium distachyon* and maize (*Zea mays*).The AAT gene IDs for wheat, rice, *A. thaliana* and potato are named according to the IDs in previous publications, and the gene IDs for *B. distachyon* and maize which were identified in this study are displayed according to the original gene number in the reference genome.

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3.5. Phylogenetic analysis and multiple-sequence alignment

To reveal the subfamily classification of the AAT family in wheat better, a phylogenetic tree comprising 707 protein sequences of AAT family members from rice, maize, *Brachypodium*, *Arabidopsis*, potato and wheat AATs was constructed in accordance with the ML method (Fig. 5). The ML tree showed that all AAT proteins could be clearly divided into 12 independent branches with high confidence. The AAAP family contained 204 AAT proteins from wheat, while the APC family contained 92 AAT proteins. The AAAP family consisted of eight distinct subfamilies: AAPs (66), lysine/histidine transporters (LHTs, 24), GABA transporters (GATs, 14), ProTs (9), AUXs (15), amino acid transporter-like a (ATLa, 18) proteins, aromatic and ANTs (18) and amino acid transporter-like b (ATLb, 40). The APC family consists of four distinct subfamilies: CATs (31), ACTs (21), PHSs (31) and TTPs (9). A given subfamily's genes from monocotyledonous and dicotyledonous plants were distributed on the same branch, confirming that the main features of the AAT family had formed before the differentiation of monocotyledonous and dicotyledonous plants.

The alignment of the TaANT members is shown in Fig. 6 as an example. All ANT family member protein sequences derived from the wheat A subgenome were used for multiple sequence alignments. The overall identity of the protein sequences of these genes was 61.97%. There were six conserved motifs within the TaANTs: motifs 1, 7, 9, 2, 13 and 5 (Fig. 6). There was a very strong correlation between the conserved motifs and the TM regions, in which motifs 1, 7, 2, 5, and 13 corresponded

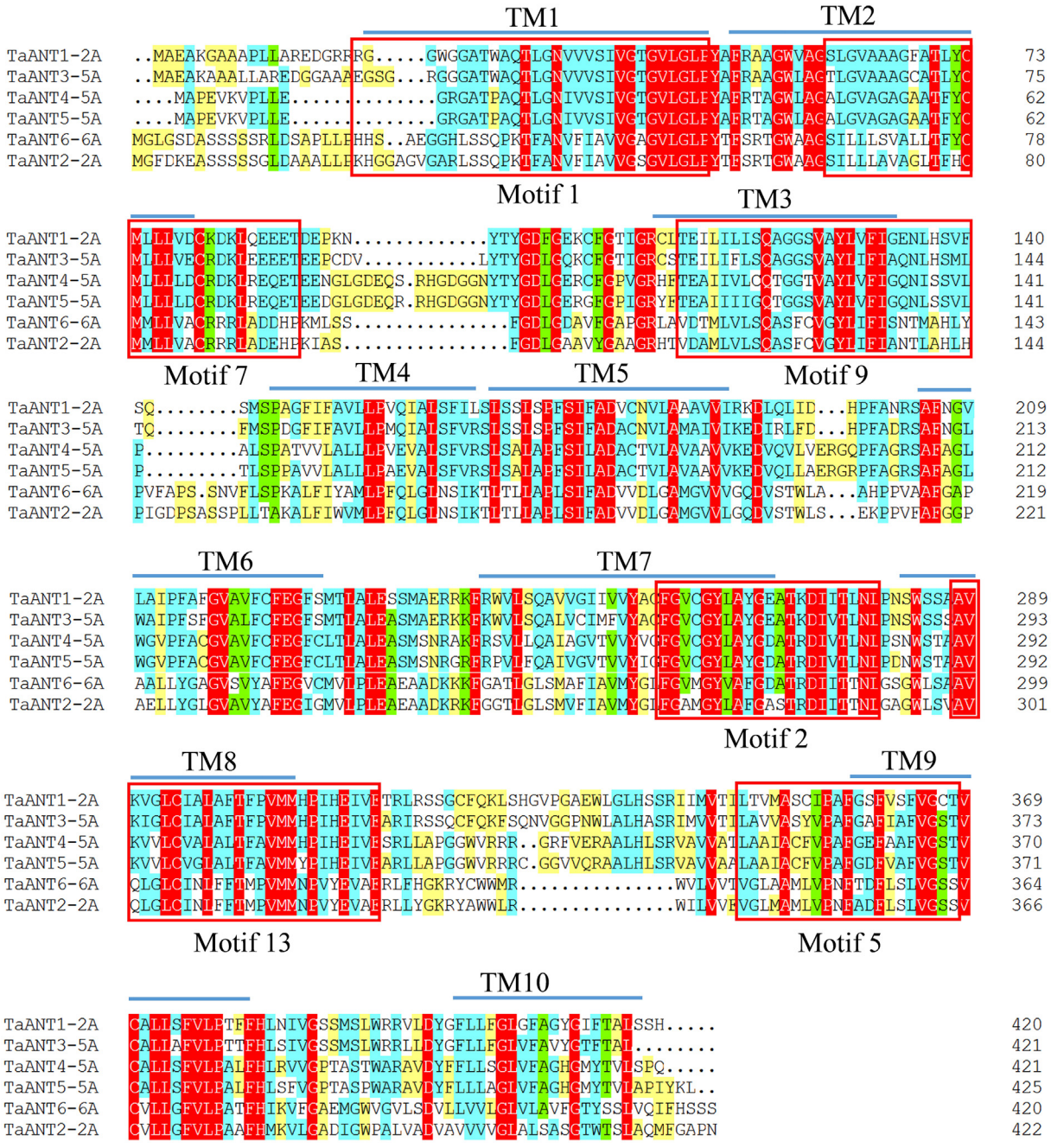

Fig. 6. Multiple sequence alignment of the transmembrane (TM) region of the aromatic and neutral amino acid transporter (ANT) subfamily members in wheat. The blue line represents the TM regions of TaANT proteins, and the red box represents the conserved motifs predicted by Multiple EM for Motif Elicitation (MEME). Identical (100%), conserved (75–99%), and blocks of similar (50–74%) amino acid residues are shaded in red, blue, and yellow, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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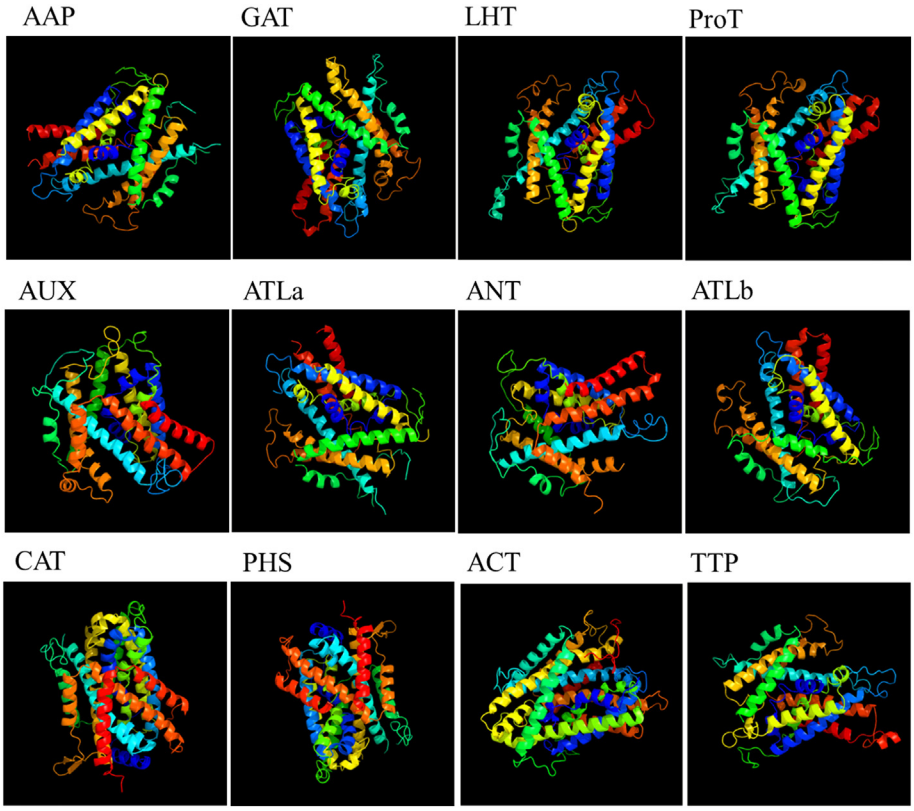

Fig. 7. Three-dimensional modeling of amino acid transporter (AAT) proteins in wheat. The AAT proteins were selected from 12 subfamilies for three-dimensional structure prediction and display, with a confidence level > 90%. The displayed AAT proteins are TaAAP2-2A, TaGAT1-1A, TaLHT1-1A, TaProT1-2A, TaAUX1-1A, TaATLa1-3A, TaANT1-2A, TaATLb9-4A, TaCAT2-2A, TaLAT1-2A, TaBAT1-2A and TaTTP1-2A.

to TM1, TM2, TM7, TM9 and TM8, respectively, and motif 9 corresponded to TM3. All of the conserved sequences were located near the TM domains, suggesting that the stabilization of the TM regions plays an integral role in the normal function of ANT family proteins.

3.6. Analysis of cis-regulatory elements and three-dimensional modeling

The cis-regulatory elements predicted within the promoter regions of all *AAT* genes were classified mainly into three clusters: tissue specificity, stress response, and hormone response. A large number of stress-responsive cis-regulatory regulatory elements were found within gene promoter regions, indicating that the *AAT* genes responded strongly to stress. In addition, gibberellin (GA), abscisic acid (ABA) and other hormone-responsive elements were detected, indicating that these genes might be involved in multiple hormone signal pathways. In addition, multiple transcription factor binding sites, such as those of MYBs, were detected within the promoter regions, confirming that these genes might be regulated by a variety of transcription factors.

All predicted AAT proteins contained multiple α-helices and coil structures (Fig. 7). The multiple α-helix structures ensured the efficient and stable TM transport of AAT proteins. Most AAT proteins had similar three-dimensional structures, and the closer the phylogenetic relationship was between genes, the closer the three-dimensional structures of the proteins were, as was the case for LHT and ProT (Fig. 7).

3.7. Spatiotemporal expression patterns of AATs in wheat

To determine the expression pattern of the *TaAAT* genes, we downloaded the transcriptome data from different tissues at different growth stages from the public expression database. The gene pairs containing three copies accounted for 94% of the total *AAT* genes, so for convenience of display, the average TPM value of the homologous gene pair expression was used for expression level analysis. The $\log_2$ (TPM + 1) value was used for the heat map display (Fig. 8). The results showed that the wheat AAT genes exhibited different expression patterns. Some genes of the AAP, ATLa, CAT, and AUX subfamilies, such as *TaAAP1*, *TaAAP14*, *TaATLa4*, *TaATLa5*, *TaAUX3*, *TaCAT6* and *TaCAT11*, were highly expressed in multiple tissues, while members of other families were highly expressed in specific tissues or specific organs. For example, *TaANT3* was expressed specifically in the peduncles, while *TaTTP1* was expressed specifically in the leaves. In addition, the same gene was differentially expressed at different developmental stages. For example, *TaBAT4* was expressed specifically in leaves only during the grain-filling stage and not during the seedling stage or flag leaf stage.

Fifty-one homologous genes were expressed in the grain tissues during grain development and were included in three clusters (Fig. 9). *TaAAT*s (*TaAAP8*, *TaATLb13*, *TaCAT11* and *TaLAT5*) in the first cluster were highly expressed in embryo, endosperm and seed coat during whole grain development. *TaAAT*s in the second cluster were expressed differently in different grain tissues. For example, *TaAAP20* was highly expressed in late leaf stage endosperm (LLSEM), while *TaATLa2* was highly expressed in mature embryo (MEO). *TaAAT*s in the third cluster mainly exhibited high expression levels in endosperm or seed coat. For example, *TaANT5*, *TaLHT3*, *TaBAT2*, *TaAAP14* and *TaAAP21* were highly expressed in seed coat; *TaLHT2*, *TaAAP19*, *TaATLb5* and *TaATLb11* exhibited high expression in endosperm.

To verify the reliability of the transcriptome data, expression level of 12 *AAT* genes were analyzed during wheat whole growth period (Fig. 10). The qRT-PCR results for the expression of the 12 genes showed high consistency with the respective gene expression levels from transcriptome data (Figs. 8 and 10). Some genes (*TaAAP3*, *TaAAP15*, *TaATLa2*, *TaAAP7*, *TaAAP17*, *TaANT5* and *TaLHT3*) were highly expressed in root. *TaANT3* and *TaLHT3* were highly expressed at seeding stage. *TaAAP15*, *TaAAP7*, and *TaAAP17* were highly expressed at flag leaf stage. *TaAAP3*

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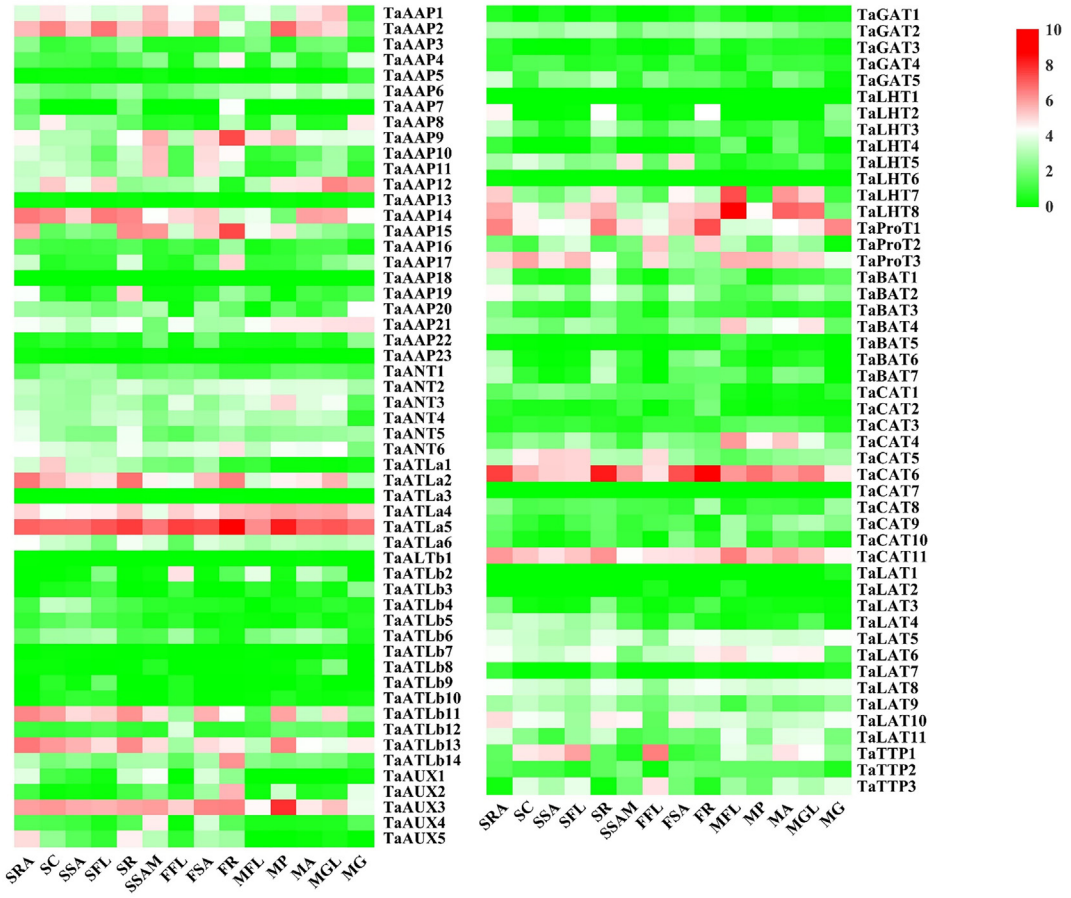

Fig. 8. Expression levels of wheat amino acid transporter (*AAT*) genes in 14 tissues. The transcriptomic data of the 14 tissues at three stages were used to reconstruct the expression patterns of wheat *AAT* genes. The tissues at the seeding stage included radicle (SRA), coleoptile (SC), stem axis (SSA), first leaf blade (SFL), roots (SR) and shoot apical meristem (SSAM) tissues; the tissues at the flag leaf stage included flag leaf blade (FFL), shoot axis (FSA), root (FR) tissues; and the tissues at the milk grain stage included flag leaf blade (MFL), peduncle (MP), awn (MA), glume (MGL), and grain (MG) tissues. The samples are listed at the bottom of each lane, and the color scale is shown at the right.

and *TaATLa2* had similar expression levels at seeding stage and flag leaf stage. *TaAAP2* was highly expressed in grain tissues. *TaAAP18* was constitutively expressed in various tissues.

3.8. Gene duplication and expression patterns of duplicated AAT genes

Gene duplication events can serve as a key mechanism for increasing gene family diversity, particularly through nonfunctionalization, subfunctionalization, and new functionalization of duplicated genes. Subfunctions and new functional duplication lead to functional diversity within the family, and these new genes can be expressed in tissues different from those of their progenitor cells or at different developmental stages [59,60]. Gene replication and diversification events are well documented in *Arabidopsis* [61]. We observed that most of the repeated *AAT* genes in wheat were differentially expressed in different tissues/organs at developmental stages (Fig. 9). On the basis of the gene expression pattern, we observed three functional variations in homologous gene pairs in wheat. For example, we observed nonfunctionalization in the *TaAAP8*/*TaAAP9* gene pair. Specifically, *TaAAP9*s were expressed in root, peduncle and shoot apical meristem, while *TaAAP8*s were hardly expressed in any tissue. *TaAAP21*s were expressed in all tissues, while *TaAAP22*s were not. Subfunctional phenomena were also observed, such as the expression levels of *TaLHT7*s being significantly weaker than those of *TaLHT8*s, and similar phenomena were also observed for the *TaAAP16*/*TaAAP17* genes. In addition, gene duplication has produced new functions. Collectively, our results show that gene duplication events play integral roles in the generation of new functions and the retention of key functions during species evolution. In addition, the expression profiles of repeated wheat AAT proteins showed that most of the proteins had undergone subfunctionalization. These observations are consistent with those in other plant species, in which closely related genes have different expression patterns.

3.9. Response of TaAATs to abiotic stresses

To explore the response of the AAT family genes to abiotic stresses, three major abiotic stresses (drought, heat, salt) were simulated, and 12 *TaAAT*s that were highly expressed in the leaves or roots were selected for qRT-PCR analysis (Fig. 12). Both *TaAAT*s that were highly expressed in the leaves and *TaAAT*s highly expressed in the roots responded differently to different abiotic stresses. The expression of *TaAAP2* in the leaves rapidly decreased under drought stress but decreased first and then increased with prolonged heat stress. The expression levels of *TaAAP3* and *TaATL3* increased slightly at 1 h of drought and heat stress and increased significantly at 6 h. Unlike the expression of these genes, the expression of *TaLHT8* was significantly upregulated at the early stage of drought stress (1 h) but was inhibited at 6 h. In addition, *TaATLa2* showed a sustained response to drought stress.

All six selected genes showed different degrees of response to salt stress, and their expression patterns could be roughly divided into two categories: upregulation and downregulation. The expression of *TaAAP7*, *TaAAP17*, *TaAAP18* and *TaLHT3* was upregulated under salt stress. *TaAAP7* and *TaLHT3* maintained high expression at 48 h after salt stress, while the expression of *TaAAP17* and *TaAAP18* was downregulated, which confirmed that the response of *TaAAT*s to salt stress was similar to that of drought and heat stress, with different response

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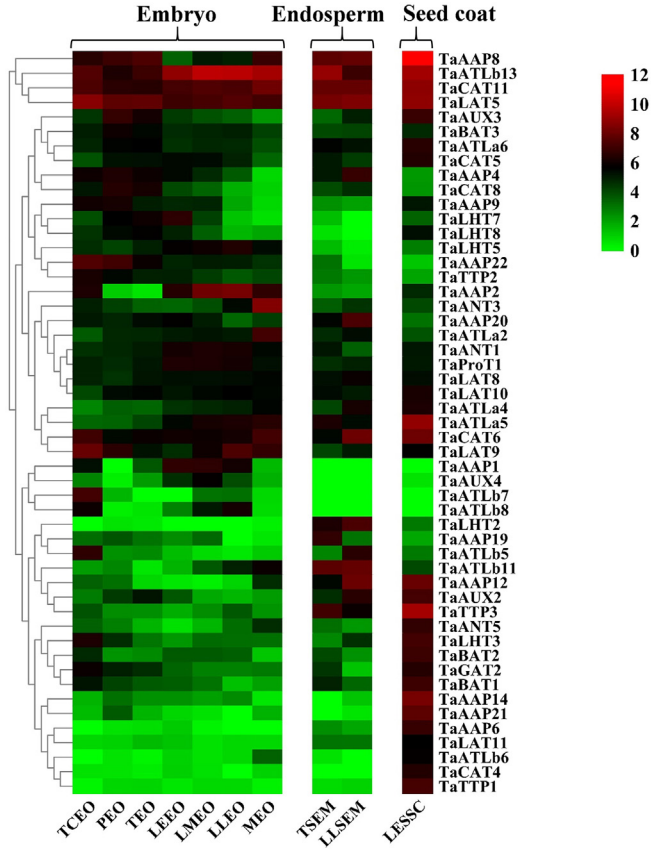

Fig. 9. Expression levels of wheat AAT genes in three grain tissues during grain development. Each column of the heat map represents a sample. The samples include two cell embryo (TCEO), pre-embryo (PEO), transition embryo (TEO), leaf early embryo (LEEO), leaf middle embryo (LEMO), leaf late embryo (LLEO), mature embryo (MEO), transition stage endosperm (TSEO), late leaf stage endosperm (LLSEO) and leaf early stage seed coat (LESSC). The samples are listed at the bottom of each lane, and the color scale is shown at the right.

intensities and durations to improve the adaptability of wheat to salt stress. Moreover, with prolonged salt stress, the expression levels of *TaANT5* and *TaBAT2* decreased continuously, suggesting that salt stress may seriously affect the functions of these two genes.

4. Discussion

Plant AATs play important roles in processes involving seed germination, seedling growth, grain quality formation and response to pathogens and abiotic stresses by shifting the transport and distribution of different amino acids [3–5,12]. Members of the *AAT* gene family were recently systematically identified and characterized in multiple species [15–17,29]. However, owing to the complexity of the wheat genome, members of the *AAT* gene family have not been thoroughly or systematically characterized in wheat. In the present study, we identified and characterized members of the *AAT* gene family in wheat through genome-wide analyses and studied their evolutionary model, tissue expression patterns and response to abiotic stress.

The number of reported *AAT* genes varies across various higher plants, ranging from 63 to 189, with 63 in *Arabidopsis* [29], 72 in potato [17], 189 in soybean [16] and 85 in rice [15]. In the present study, 297, 107 and 80 *AAT* genes were identified in wheat, maize and *Brachypodium*, respectively (Table 1). The major difference that exists between AAT family members of monocots and eudicots is due to the unequal expansion of different subfamilies. For example, there are significantly more AAP subfamily members in monocots than in eudicots, indicating that the expansion rate of the AAP subfamily differed after monocot and eudicot differentiation. The different numbers of *AAT* genes in the different species may be due to gene duplication events, including tandem, segmental or whole-genome duplication (WGD) events. In fact, 279 of the 296 identified *AAT* genes were copies of 93 homologous genes from the A, B and D subgenomes, accounting for 94% of all putative *AAT* genes, confirming that a WGD event was the main driver of AAT family expansion in wheat. Second, 25.33% (75 of 296) were related to tandem duplication events, and 30 of 32 tandemly duplicated gene groups had homologous copies in all three subgenomes, which suggested that most occurred before the formation of hexaploid wheat (Fig. 2). In general, the expansion of the *AAT* gene family in wheat mainly occurred via WGD and tandem duplication events.

To improve adaptability to the environment, the evolution of plants is usually accompanied by the generation of a large number of new genes and their subsequent functional differentiation [29]. Gene functional differentiation includes three main levels of variation: sequence variation, structural variation, and expression level variation. Natural selection is a key determinant of gene functional diversity [15]. Ka/Ks reflects the selection pressure of duplicated genes. Overall, the Ka/Ks values for all tandemly duplicated gene pairs evaluated were less than 1, confirming that these genes underwent purifying selection to maintain important biological functions (Fig. 3). On the other hand, the median value and dispersion of the Ka/Ks values of wheat AAP subfamily members were much greater than those of other subfamily members, which indicated that the AAP subfamily was more likely to generate new features during the expansion process. This finding is consistent with the large difference in the number of AAP subfamily members between monocots and eudicots (Table 1). In addition, approximately 20% (19 of 93) of the homologous *AAT* genes from the A, B and D subgenomes displayed variation in TM number, which was not due to the variation in conserved motifs to a large extent (Table 1, Table S1). These results collectively confirmed that the AAT family in wheat improved the diversity, coordination and adaptability of AAT family members through sequence variation of the different subfamily members and different subgenomic homologous genes during the expansion process.

Previous studies have confirmed that one of the representative traces of gene family evolution is the variation in gene structure [56–58]. The gene structure of members of the same AAT subfamily somewhat differed while maintaining conservation (Fig. 4). These differences were especially obvious in the members of the AAP subfamily. The number of introns within the *AAP* genes ranged from 0 to 6, and there were 6 pairs of significant structural variation in 13 tandemly duplicated gene pairs, which is consistent with our results concerning the differences in distribution of AAP subfamily members between monocots and eudicots, the large median value and the dispersion degree of Ka/Ks. Together, this confirmed that the expansion and functional differentiation of the AAP subfamily members have a positive effect on the evolution of the AAT family and the improvement of wheat adaptability. In addition, the analysis of the conserved motifs of the AAT family members revealed that some motifs, such as motif 1, were conserved across all subfamilies, while some motifs, such as motif 2, motif 9 and motif 10, were unique to different subfamilies (Fig. S1). The former may determine the important basic functions of AATs in wheat, while the latter may affect specific new functions. In essence, changes in gene structure, deletions and mutations of conserved motifs were also important reasons for the functional diversification of wheat AAT family members.

Variation in the expression level of newly duplicated genes, including nonfunctionalization, subfunctionalization, and new functionalization, is an important way for functional differentiation after gene family expansion [57]. The generation of subfunctionalization and new functionalization makes the newly duplicated genes different from the ancestral genes in terms of their expression level and spatial-temporal specificity of expression to perform different functions [59,60]. We observed all three types among the wheat *AAT* duplicated gene pairs. For

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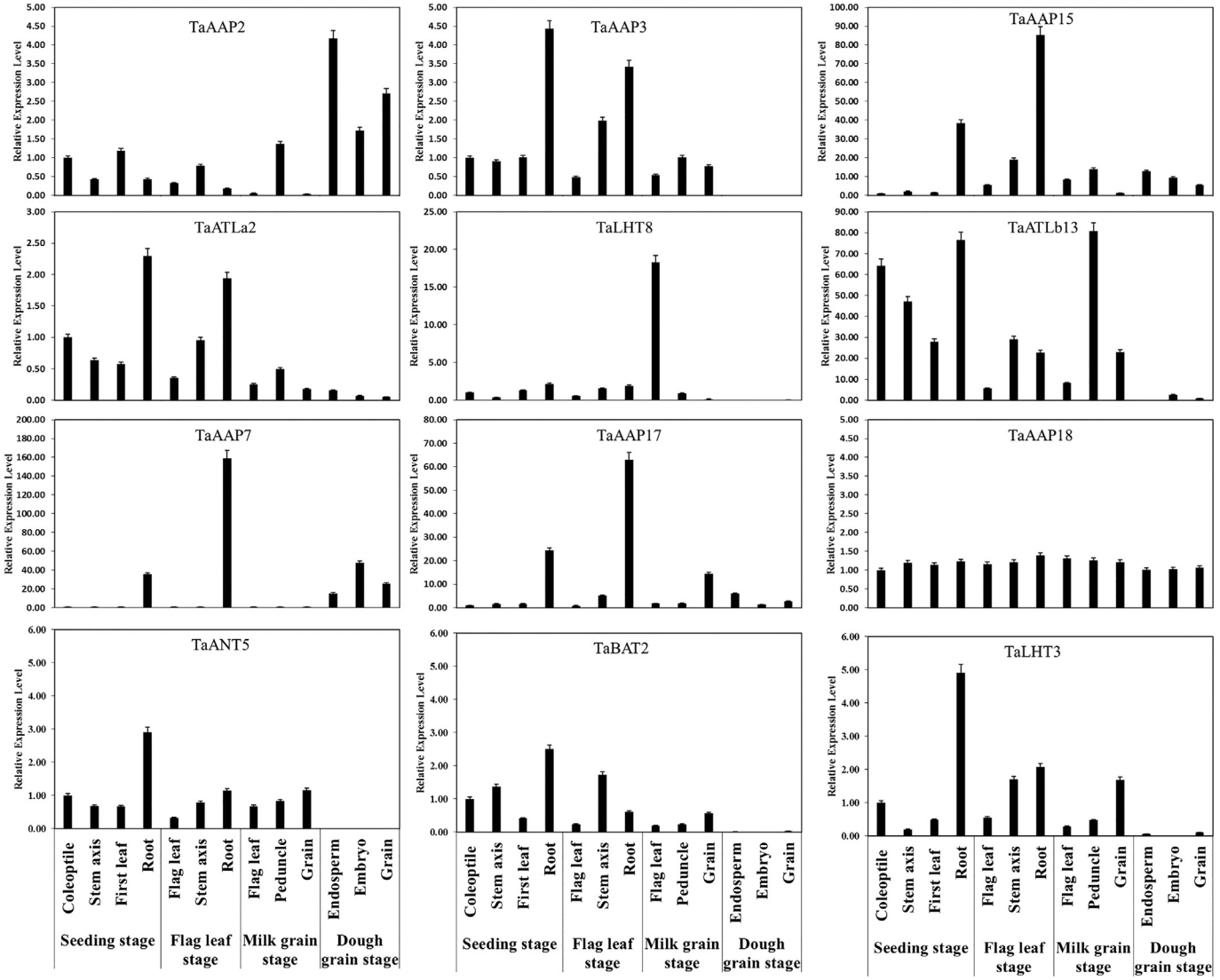

Fig. 10. Expression levels of 12 *TaAAT* genes in 13 tissues at four essential developmental stages of wheat. The tissue names and growth periods are listed in the bottom row. Bars represent the mean values of three replicates ± standard deviation (SD). All of the expression levels of the *TaAAT* genes were normalized to the expression level of *TaActin*.

example, the *TaAAP8*/*TaAAP9* gene pair showed significant nonfunctionalization of *TaAAP8*s, the *TaLHT7*/*TaLHT8* gene pair showed subfunctionalization of *TaLHT7*s, and *TaATLb12*s and *TaATLb13*s exhibited different spatiotemporal expression characteristics (Fig. 11). In addition, the expression levels of homologous genes from different subgenomes differed. For example, compared with *TaBAT4-3D*, *TaBAT4-3A* and *TaBAT4-3B* were expressed at higher levels in all tissues, while compared with *TaAAP16-6B*, *TaAAP16-6A* and *TaAAP16-6D* were expressed at higher levels. This indicated that during the formation of hexaploid wheat, the expression level of homologous *AAT* genes from different subgenomes changed, thereby enhancing the overall coordination of gene pairs. In actuality, the functional differentiation of the wheat AAT family is a result of the combined effects of the sequence, structure and expression levels of AAT family members, and the multilevel mutations of *AAT* homologous genes from different subgenomes have a very positive effect on the functional adaptation of *AAT* genes in wheat.

Analysis of the spatiotemporal expression patterns of *TaAAT* genes may provide useful information for determining their putative functions. *TaAAT* genes showed different expression patterns at different developmental stages in different tissues, and some genes, such as *TaAAP1*, *TaAAP14*, *TaATLa4*, *TaAUX3*, and *TaCAT6*, were highly expressed throughout the whole growth process, indicating that these genes were critical for the overall growth and development of plants. Some genes, such as *TaANT3* and *TaTTP1*, were highly expressed in specific tissues or organs. Other genes, such as *TaBAT4*, were expressed at specific developmental stages. The expression of these genes in specific tissues or organs at different stages indicated that these genes may perform specific functions in specific tissues at specific developmental stages. The qRT-PCR results confirmed the diverse expression levels of AAT family genes in different tissues at various developmental stages.

AAP members in *Arabidopsis* play critical roles in nutrient transport during seed development or in long-distance transport of amino acids [28,29]. *AtAAP1* is involved in the uptake of amino acids into root cells [10]. Our results also showed that *TaAAP19*, which is located on the same branch of *AtAAP1* in the phylogenetic tree, was highly expressed in the roots, suggesting that *TaAAP19* and *AtAAP1* may have similar roles and functions. Moreover, the expression characteristics of *TaAAP19*, *TaAAP7* and *TaAAP17* were similar, suggesting that wheat adaptability improved by an increase in the fault tolerance of *TaAAP*s. *AtAAP3* plays an important role in the uptake of amino acids in the xylem of *Arabidopsis* [61]. *TaAAP1* was highly expressed in SC and SSAM tissues, and considering its close relationship with *AtAAP3*, we speculated that the functions of *TaAAP1* were similar with those of *AtAAP3* in wheat. The GPC is an important determinant of nutritional quality in cereals, and studies based on *Arabidopsis* and rice have confirmed that both *AtAAP8* and *OsAAP6* are related to GPC [4,28]. Our

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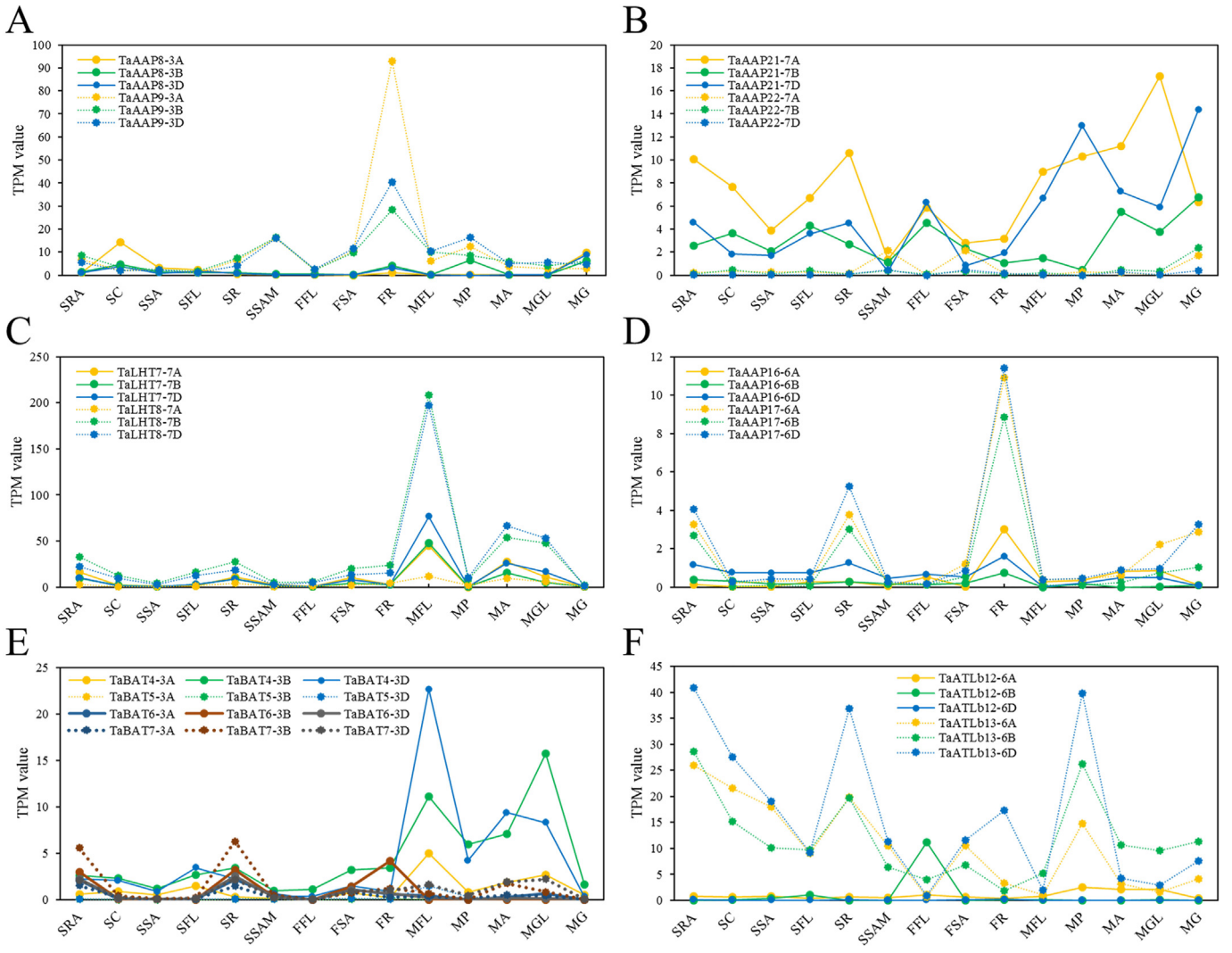

Fig. 11. Three trends of expression patterns of duplicated amino acid transporter (*AAT*) gene pairs in wheat. The X-axis indicates 14 tissues at three stages, and the Y-axis represents the transcripts per million (TPM) value. The full names for X-axis tissue abbreviations are shown in Fig. 8.

results also showed that *TaAAP8* was highly expressed in wheat grain, confirming that the functions of these genes were relatively conserved between monocots and eudicots. In our study, in addition to the homologous genes identified in other species, we also detected that several genes, such as *TaANT5*, *TaAUX5*, *TaLHT2*, and *TaBAT2*, were expressed specifically in root tissues. These genes obviously contribute to the uptake and transport of amino acids in the roots. Moreover, the high expression of *TaAAP4*, *TaAAP20*, *TaAUX2*, *TaProT1*, *TaLAT5* and *TaTTP3* in the grain is indispensable for the accumulation of protein.

Analysis of gene expression levels in embryo, endosperm and seed coat revealed the possible roles of AAT family genes in grain development. *TaAAP8*, *TaATLb13*, *TaCAT11* and *TaLAT5* were highly expressed in all grain tissues, indicating that these genes play an essential role during the entire grain development process. In addition, some genes including TaLHT2, TaATLb5 and TaATLb11 were highly expressed in the endosperm, suggesting these genes might be involved in formation of wheat quality. Previous studies showed that the contribution of wheat grain photosynthesis to wheat yield can reach 11%–40% [62]. Interestingly, *TaCAT11* and *TaTTP1* located on the chloroplast membrane were highly expressed in the seed coat, which was possibly be involved to photosynthesis of green seed coat.

Drought, heat and salinity are the three main abiotic stresses facing crops and may greatly reduce crop growth and productivity. The accumulation of osmotic active compounds in plants is an important way to balance osmotic potential under drought stress or salt stress, and the changes in the amino acid composition of plants are mainly regulated by AATs [63]. It has been proven that in many species, excessive accumulation of proline can enhance the tolerance of plants to osmotic stress [28,64,65]. In the present study, on the basis of the qRT-PCR analysis of the *AAT* genes that were highly expressed in wheat in response to different abiotic stresses, we identified multiple *AAT* genes that responded specifically/nonspecifically to different abiotic stresses, and these genes detected in response to the different abiotic stresses exhibited different response patterns. Some genes, such as *TaAAP3*, *TaATLa2*, and *TaATLb13*, showed sustained responses under drought and high-temperature stress. These genes may play important roles in maintaining normal amino acid transport in wheat under long-term drought and high-temperature stress. The expression of *TaAAP2* and *TaLHT8* was upregulated during the late stage of high-temperature stress and during the early stage of drought stress, respectively. The specific response of these two genes to various stresses at different stages increased drought and heat stress resistance. Similar to the response under high temperature and drought stress, under salt stress, the expression of different *TaAAT* genes also responded differently in terms of intensity and duration. The expression levels of *TaAAP7* and *TaLHT3* remained very high after 48 h of salt stress, while the expression of *TaAAP17* and *TaAAP18* was downregulated, confirming that the response of the former pair to salt stress can be maintained for a longer period of time, while the

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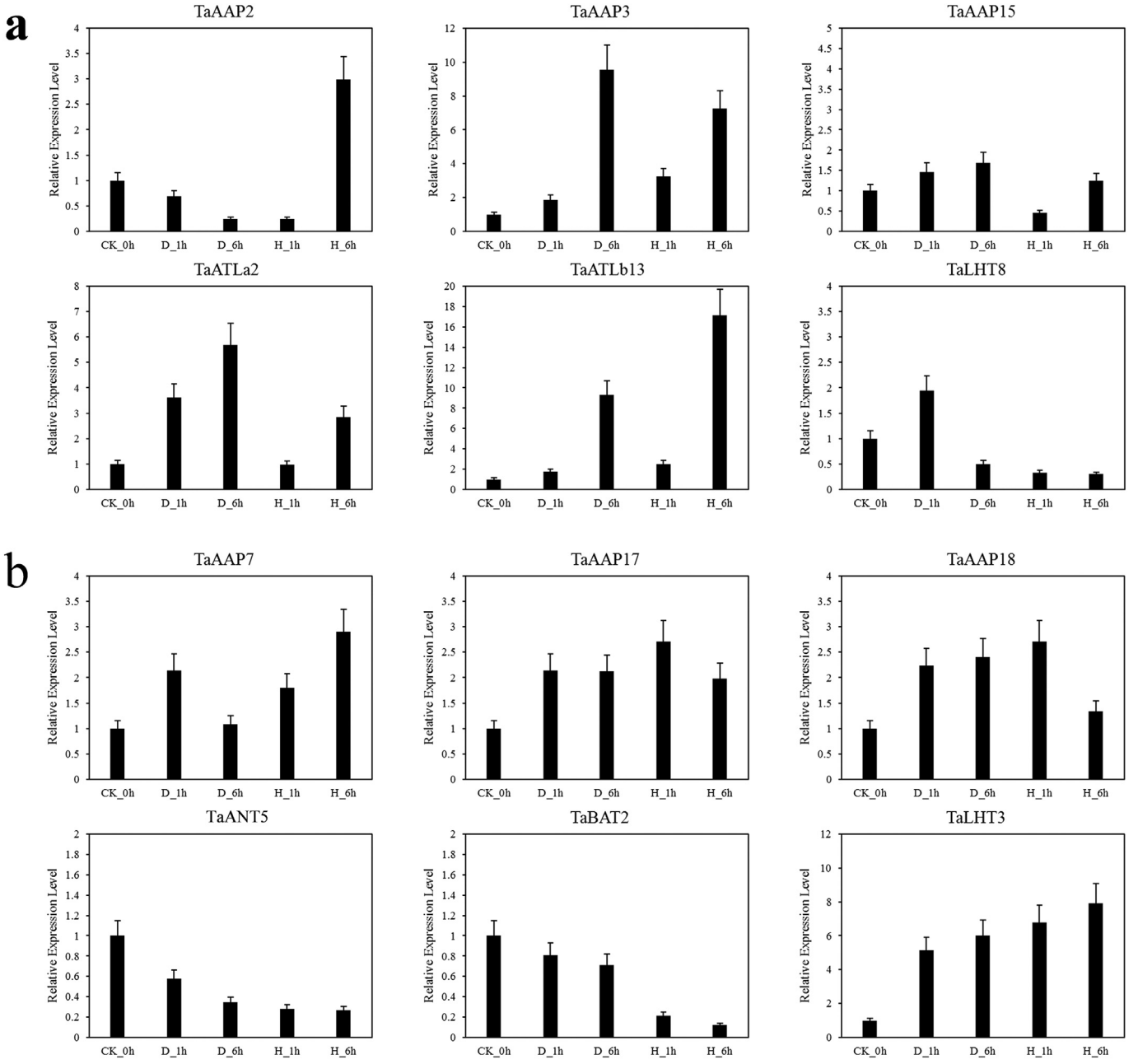

Fig. 12. Expression levels of 12 selected *TaAAT* genes in the leaves and roots in response to three different abiotic stresses. (A) Relative expression levels of *TaAATs* in response to drought (D) and heat stresses (H) for 1 h and 6 h in the leaves at the three-leaf stage. (B) Relative expression levels of *TaAATs* in response to salt stress (S) (NaCl, 200 mM) for 6 h, 12 h, 24 h and 48 h in the roots at the three-leaf stage. The bars represent the mean values of three replicates ± standard deviations (SDs). All of the expression levels of the *TaAAT* genes were normalized to the expression level of *TaActin*.

latter pair may play a role mainly in the early stage. Notably, *TaATLb13* located on vacuole membrane showed a stronger response to long-term drought and heat, which may increase the resistance of wheat to abiotic stress by transporting amino acids and regulating the vacuole osmotic potential. In general, *TaAATs* responded differently to abiotic stresses, including their readiness to respond, the generality of their response, and differences in their response duration, which may greatly improve the adaptability of wheat to abiotic stresses.

5. Conclusions

We identified 296 *AAT* gene family members in the wheat genome. Similar to those identified in other species, all wheat *AAT* genes could be classified into two families: the AAAP and APC families. The AAAP family could be subdivided into 8 subfamilies, while the APC family could be divided into 4 subfamilies. We demonstrate that the expansion of the wheat AAT gene family is primarily due to WGD and tandem duplication events, while tandem repeat events have greatly determined the functional differentiation of AAT family members. We systematically outlined the chromosomal distribution, gene structure, and conserved motifs of AAT family members in wheat and annotated all *AAT* genes, and we subsequently constructed a phylogenetic tree. We further evaluated the expression patterns of wheat *AAT* gene family members in different tissues and their responses to three conventional abiotic stresses: heat, drought and salt stress. We also identified several important candidate genes that may affect grain quality and root amino acid

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transport. Our work will provide a comprehensive framework for the study of the wheat AAT family and will also contribute to the functional analysis and utilization of wheat *AAT* genes.

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CRediT authorship contribution statement

Ruizheng Tian: Investigation, Writing - original draft, Writing - review & editing, Validation. **Yang Yang:** Investigation, Writing - original draft, Writing - review & editing, Validation. **Maohua Chen:** Methodology, Validation.

Declaration of competing interest

The authors declare that they have no competing interests. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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