Genome-Wide Identification and Evolutionary Analysis of Argonaute Genes in Hexaploid Bread Wheat

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Argonaute (AGO) proteins play a pivotal role in plant growth and development as the core components of RNA-induced silencing complex (RISC). However, no systematic characterization of AGO genes in wheat has been reported to date. In this study, a total number of 69 TaAGO genes in the hexaploid bread wheat (Triticum aestivum cv. Chinese Spring) genome, divided into 10 subfamilies, were identified. Compared to all wheat genes, TaAGOs showed a significantly lower evolutionary rate, which is consistent with their high conservation in eukaryotes. However, the homoeolog retention was remarkably higher than the average, implying the nonredundant biological importance of TaAGO genes in bread wheat. Further homoeologous gene expression bias analyses revealed that TaAGOs may have undergone neofunctionalization after polyploidization and duplication through the divergent expression of homoeologous gene copies, to provide new opportunities for the generation of adaptive traits. Moreover, quantitative real-time polymerase chain reaction (qRT-PCR) analyses indicated that TaAGO gene expression was involved in response to heat, drought, and salt stresses. Our results would provide a theoretical basis for future studies on the biological functions of TaAGO genes in wheat and other gramineous species.

1. Introduction

Small RNA- (sRNA-) guided gene silencing is crucial for the maintenance of genome stability and plant response to environmental alterations. Argonaute (AGO) family proteins function as the major components in this RNA silencing machinery. They associate with sRNAs such as short interfering RNAs (siRNA) and microRNAs (miRNAs), to form RNA-induced silencing complexes (RISC), which induce sequence-specific degradation or repress the translation of mRNAs [1]. AGO family proteins are highly conserved in eukaryotes, containing several typical domains such as the N-terminal, Piwi Argonaute Zwille (PAZ), MID, and PIWI domains. The N-terminal domain participates in the loading and unwinding of small RNA duplexes, whereas the PAZ and MID domains anchor the 3' and 5'-ends of the sRNA, respectively. The PIWI domain is structurally similar to RNaseH and is responsible for the cleavage of its target miRNA.

In Arabidopsis, a total number of 10 AGO proteins, which are divided into three phylogenetic clades: AGO1/AGO5/AGO10, AGO2/AGO3/AGO7, and AGO4/AGO6/AGO8/AGO9, have been detected. AGO1 regulates plant development through the miRNA-mediated pathway. A loss of function in AGO1 led to abnormal inflorescence and filamentous structures in the Arabidopsis mutant plants [2]. AGO10 regulates the differentiation of shoot apical meristem (SAM) by sequestering AGO1 from miR165/166, leading to promoted expression of their target homeodomain-leucine zipper (HD-ZIP) proteins [3]. AGO2 and AGO3 are similar to each other and may have redundant functions. AGO2 plays a role in antibacterial immunity by binding to miR393b*, which inhibits the translation of MEMB12, a gene repressing the exocytosis of PR1 [4]. No AGO3 orthologs have been detected in rice and maize [5]. In Arabidopsis, rice, and maize, AGO7 predominantly binds to miR390, which cleaves TAS3 precursor RNA [6–8]. The cleavage products are converted into double-stranded RNAs by the Suppressor
of Gene Silencing 3 (SGS3) and RNA-directed RNA polymerase 6 (RDR6) and then diced by Dicer-like 4 (DCL4) into tasiRNAs, which control the transition from juvenile to adult phase at the vegetative stage [9].AGO4/6/9 is involved in the de novo methylation of CG, CHG (H=A, C, T), and CHH sequence contexts in plants [10]. Mutation in AGO4 clade genes (AGO4, AGO6, AGO8, or AGO9) induces supernumerary female gametophytic precursors in Arabidopsis [11].

In rice, 19 OsAGO5s have been identified, of which 14 were preferentially expressed at reproductive stage. Collinearity analysis revealed that segmental and tandem duplication contributed to the expansion of AGO family in rice, and duplicated genes have possessed diverged functions during evolution [12]. In pepper, 12 CaAGOs were detected. CaAGO4b, CaAGO7, and CaAGO10a were significantly repressed by salt stress, whereas CaAGO2 and CaAGO5s were induced by cold stress, suggesting a potential role of AGOs in abiotic stress resistance [13]. In Saccharum spontaneum, 21 SsAGOs were detected based on the genome-wide identification and phylogenetic analyses. Among them, SsAGO18a was preferentially expressed in the stem, and SsAGO2b, SsAGO5a, SsAGO5c, SsAGO6b, and SsAGO10c were significantly induced by osmotic stress [14]. In Populus trichocarpa, among the 14 detected AGOs, PtAGO10a, PtAGO1b, and PtAGO4c were upregulated in stems, while PtAGO4b and PtAGO5a were downregulated [15]. In potato, among the 14 identified AGOs, StAGO15 was activated upon pathogen infection [16]. In Citrus sinensis, 13 AGOs were identified, and the repression of RNA-directed DNA methylation (RdDM) pathway is necessary for fruit abscission [17]. Therefore, AGO proteins could contribute to a diverse set of functions in plants.

Hexaploid bread wheat has been grown as one of the most important cereal crops and provides about 30% of the staple food source for humankind [18]. Abiotic stresses have severely limited global wheat production [19, 20]. sRNA-guided gene silencing at the transcriptional and post-transcriptional levels has been found to have important functions in controlling growth and resistance to biotic and abiotic stresses [21]. To date, several AGO genes have been isolated from wheat. TaAGO1b and TaAGO4 were ubiquitously expressed in both vegetative and reproductive organs [22]. TaAGO5 is involved in the resistance to Diuraphis noxia infestation. Decreased expression of TaAGO5 led to increased susceptibility of wheat D. noxia feeding [23]. To further understand the possible functions and the evolutionary dynamic of AGO genes in wheat, we performed a systematic analysis on the structural conservatism, evolutionary relationship, and expression pattern of these genes. This work will provide an overview of the AGO family and a new perspective on their possible function and evolution in cereal crops.

2. Materials and Methods

2.1. Identification and Characterization of Candidate AGO Genes in Bread Wheat. To identify all the candidate AGO genes, the bread wheat (Triticum aestivum cv. Chinese Spring) high-confidence protein sequences were downloaded from https://urgi.versailles.inra.fr/download/iwgscl/IWGSC_RefSeq_Annotations/v1.0/. The Hidden Markov Model (HMM) profiles of conserved domains of AGO (PF16486, PF02170, and PF02171) were retrieved from the protein family database (Pfam, https://pfam.xfam.org/) and were used as queries to search for AGO candidate genes in the bread wheat genome as described previously [16]. All hits with a cut-off value lower than 1e-5 were retained and treated as candidate AGO genes. The candidate sequences were examined to confirm the existence of the conserved AGO domains using SMART (http://smart.embl-heidelberg.de/), Pfam (http://pfam.xfam.org/), and the Conserved Domain Database (CDD) in NCBI (https://www.ncbi.nlm.nih.gov/). Proteins with anonymous or truncated domains were eliminated to ensure the accuracy of the results. The resultant AGO genes were named according to their phylogenetic relationships and subgenome location (A, B, or D). In details, each gene name consists of the abbreviation for Triticum aestivum (Ta), followed by the subfamily name of rice or maize (e.g., AGO4a) and chromosomal location (e.g., 3A). Duplicated genes from the same chromosome were labeled with lower case letters (e.g., a). TTools, WoLF PSORT (https://wolfpsort.hgc.jp/), ExPASy (http://www.expasy.ch/tools/pi_tool.html), and MCScanX were used to generate exon-intron structures, subcellular localization, isoelectric point, molecular weight, and gene duplication type, respectively [24, 25]. The AGO genes in the progenitors, Triticum urartu (genome AA), Aegilops tauschii (genome DD), and Triticum turgidum L. ssp. durum (genome AABB), were detected using the same methods.

2.2. Evolutionary Rate Calculation of Bread Wheat Genes. The evolutionary rate was estimated using protein identity and nonsynonymous substitution ratios (Ka)/synonymous substitution ratios (Ks) as described previously [26]. The rice genome (Ostativa_323_v7.0) was downloaded from the Phytozome database v12.1 (http://phytozome.jgi.doe.gov/pz/portal.html), and orthologous gene pairs between bread wheat and rice were identified using the Blastp program (-evalue0.01; -max_target_seqs1). The protein identity between bread wheat and rice was normalized according to the length of alignment using Perl scripts. To calculate Ka, Ks, and Ka/Ks of orthologous gene pairs between bread wheat and rice, the sequences were aligned with ParaAT (-m clustalw2 -f pam1); then, the program “yn00” and the “Yang and Nielsen (2000) method” of Phylogenetic Analysis by Maximum Likelihood (PAML) software were used to get the value. The identity and Ka/Ks of the TaAGOs were extracted from all wheat genes using Perl scripts.

2.3. Phylogenetic and Chromosomal Distribution Analysis of TaAGO Genes. Phylogenetic tree was constructed with MEGA 5.0 based on the maximum likelihood method using 1000 bootstraps; the most suitable amino acid substitution model “WAG” was selected based on the results of “Find Best DNA/Protein Model.” The tree was visualized using iTOL (https://itol.embl.de/). Homoeologous TaAGOs among the A, B, and D subgenomes were identified using phylogenetic relationships and previous classification methods [27, 28].
The chromosomal distribution of the 69 TaAGOs was visualized using Circos [29].

2.4. Expression Analysis of TaAGOs Using RNA-seq Data and Quantitative Real-Time PCR (qRT-PCR). The expression levels of TaAGOs in seedlings, leaves, roots, stems, spikes, grains, stamens, and pistils were downloaded as Transcripts Per Million (TPM) from http://www.wheat-expression.com. A homoeolog expression pattern analysis for triads (a single gene copy per subgenome, i.e., A:B:D configuration of 1:1:1) with a total expression above 0.5 TPM was conducted. Homoeolog expression bias was classified as balanced (similar relative transcript abundance from A, B, and D homoeologs), A/B/D-suppressed (lower abundance of transcripts from A/B/D homoeologs), or A/B/D-dominant (higher abundance of transcripts from A/B/D homoeologs) patterns as described previously [28]. The ternary plot was generated using the R package “vcd.” Chinese Spring (Triticum aestivum L.) plants were grown in a growth chamber under 16 h/8 h light/dark period until anthesis stage. Flag leaves were dissected after 6 and 24 h of heat (42°C), PEG-6000 (25%), and NaCl (200 mM) treatment. Total RNA was extracted with Omega plant RNA kit (Omega Bio-tek, China). cDNA was synthesized using EasyScript (Trans). Glyceraldehyde3-phosphate dehydrogenase (GAPDH) was used as normalization control. Relative expression levels were calculated with the \(2^{-\Delta\Delta CT} \) method. The primer sequences used for qRT-PCR are listed in Supplementary Table 1.

3. Results

3.1. Identification of Bread Wheat AGO Genes. To identify the candidate AGO genes in bread wheat, we downloaded all annotated protein sequences (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.0/). A total number of 69 genes were identified as TaAGO, and the protein length ranged from 675 to 1210 amino acids (Supplementary Table 2). All of them contained N-
terminal, PAZ, and PIWI domains based on the SMART analyses, suggesting that they are authentic AGO genes (Supplementary Figure 1).

We further performed exon-intron structure analyses and found that the average exon number of TaAGOs was 19.6, which was significantly higher than that of all the annotated wheat genes (4.6; Wilcoxon rank-sum test, $P < 2.2e^{-16}$; Figure 1(a)). Conversely, the average exon length of TaAGOs was dramatically lower than that of all wheat genes (275.8 bp vs. 562.6 bp; Wilcoxon rank-sum test, $P < 2.2e^{-16}$; Figure 1(b)). In addition, protein identity between TaAGOs and their orthologs in rice was significantly higher than that of all wheat genes ($8.4e^{-6}$; Wilcoxon rank-sum test, $P = 0.002$; Figure 1(c)), whereas Ka/Ks was significantly lower ($0.002$; Figure 1(d)). All these results suggest that the evolutionary rate of TaAGOs is lower than that of all wheat genes. Therefore, AGO genes are highly conserved and their biological functions have been retained during evolutionary processes.

3.2. TaAGO Genes Belong to Well-Defined Subfamilies. A maximum likelihood phylogenetic tree was constructed with Arabidopsis, rice, and maize AGO proteins (Figure 2(a)). TaAGOs were divided into 10 subfamilies: AGO1, AGO2, AGO4, AGO5, AGO6, AGO7, AGO9, AGO10, AGO17, and AGO18. Consistent with rice and maize AGOs, no AGO3 and AGO8 orthologs were detected in wheat. In some clades, gene phylogeny roughly followed species phylogeny, with one rice gene closely related to a wheat homoeolog triad (e.g., subclades AGO6 and AGO7). Duplication events made topology more complex in other subclades (e.g., subclades AGO1, AGO5, and AGO10). In addition, compared with those in rice and maize, subfamilies AGO9 were expanded even when the ploidy level was corrected (Figure 2(b)). The expansion mainly resulted from the B subgenome since the gene number in *Triticum urartu* and *Aegilops tauschii* was the same as that in maize and rice but expanded in the B subgenome of *Triticum turgidum* L. ssp. *Durum* and bread wheat (Supplementary Table 3). The expansion of AGO9 may be a
result of adaptive evolution of RdDM in wheat, which helps to repress the amplified transposable element (85% in wheat vs. 35% in rice) [26, 30].

3.3. Chromosomal Location and High Rate of Homoeolog Retention of TaAGOs. The TaAGO genes were dispersed nonuniformly among the chromosomes, with chromosome 5B containing a significantly higher number of genes than expected from the chromosome length ($\chi^2$ test, $P < 0.05$; Supplementary Figure 2). This mainly resulted from segmental duplication (or whole-genome duplication) of the subfamily AGO5. Furthermore, this duplication pattern also led to more TaAGO genes located on chromosome 7B than expected (10.5% vs. 5.3%; Supplementary Figure 2). In addition, bread wheat contained A, B, and D subgenomes, and 41.3% of the high-con-}

3.4. Expression Pattern Analysis of TaAGOs during Wheat Development. To characterize the expression pattern of TaAGOs in different tissues, RNA-seq data from seedlings, roots, stems, leaves, stamens, pistils, spikes, and grains were analyzed. The results showed that 69.6% (48/69) of TaAGOs were expressed in at least one developmental stage (TPM > 1; Supplementary Table 5). A total of 27.5% (19/69) of genes were expressed in at least one developmental stage (TPM > 1; Supplementary Table 5). The percentage of triads displayed that similar transcript abundance from the A, B, and D homoeologs (defined as balanced) was between 16.8% and 42.9%, with an average of 33.2% (Supplementary Table 6). Further analyses indicated that histone modification may contribute to the biased expression of TaAGOs. For example, in seedlings, the higher level of the active marker H3K36me3 around the transcripts of TaAGO2b-2A than that around TaAGO2b-2D and TaAGO2b-2B may lead to an A-dominant expression pattern, while the higher levels of H3K36me3 around the transcripts of TaAGO5b-2D than those around TaAGO5b-2A and TaAGO5b-2B may result in a D-dominant expression pattern (Figure 3).

3.5. TaAGO Genes Were Influenced by Heat, Osmotic, and Salt Stress Treatments. To understand if the expression of TaAGOs was affected by abiotic stresses, the transcription levels of TaAGOs were measured after the treatment with heat (42°C), PEG (25%), and NaCl (200 mM) for 6 and 24 hours at anthesis stage of wheat (Figure 4). The results showed that heat stress caused greater effects on the expression of TaAGOs. Almost all of the detected TaAGOs, except TaAGO4a-3D and members of subclade TaAGO6, were significantly suppressed by heat treatment, while 24 h treatment of heat significantly induced the expression of members of TaAGO6. In addition, the expression levels of seven TaAGOs were significantly affected by all the stress treatments, with a significantly downregulated expression of TaAGO1a-6A, TaAGO1b-2A, TaAGO9-1A, and TaAGO17-5B. Furthermore, TaAGO4a might have undergone neofunctionalization after duplication because TaAGO4a-3A and TaAGO4a-3B were significantly suppressed by heat treatments, while its homoeolog, TaAGO4a-3D, was not affected (Figure 4).
4. Discussion

RNA silencing is a conserved mechanism that regulates gene expression at the transcriptional and posttranscriptional levels. This process is mediated by several kinds of proteins including AGOs. The AGO family proteins have expanded via duplication during plant evolution, from three or fewer members in ancient unicellular green algae (e.g., *Chlamydomonas reinhardtii*) to ten or more members in flowering plants (e.g., *Arabidopsis* and rice). In this study, a total number of 69 *TaAGOs* were systematically identified and comprehensively analyzed (Supplementary Table 2). Our results indicated that segmental duplication (or whole-genome duplication) contributed to the expansion in wheat. Consistent with previous results in *Brassica* species, expansion in AGO9 clades was detected compared with that in both rice and maize (Figure 2(b)) [31]. The expansion of AGO9 presumably reflected the expansion of RdDM pathway in wheat.

In mammals, exon conservation increases with exon number and decreases with exon length [32]. In plants, *TaAGOs* have evolutionarily conserved roles. We found that *TaAGOs* showed higher exon numbers but lower exon lengths compared with those of all wheat genes (Figures 1(a) and 1(b)). Consistently, *TaAGOs* showed lower evolutionary rates, as reflected by their higher identity and lower Ka/Ks (Figures 1(c) and 1(d)). The high conservation may allow AGOs to recognize specific small RNA types in different species. For example, AGO7 binds to miR390 and promotes the production of tasiRNA in Arabidopsis, rice, and maize [7, 8, 33]. The expression pattern of many *TaAGOs* is similar to that in other plants that also indicated their conserved function among different plant species. For example, AGO9 participated in female gamete development in both *Arabidopsis* and maize [11, 34]. Consistently, *TaAGO9* displayed a higher expression level in wheat pistils (Supplementary Table 5), implying that *TaAGO9* may be involved in female gamete formation. The evolutionary conservation may underline the biological importance of AGO gene family in wheat.

It is well known that the AGO family can protect plants from abiotic stresses. Many plant *AGO* genes showed responsive expression to a variety of abiotic stresses, including ABA, drought, high salinity, heat, and cold stresses [35, 36].
decreased resistance to drought stress [37]. Chilling stress
siAGO1b mutation in ZmAGO18b level, suggesting its role in cold resistance [38]. At the transcript
improved resistance to biotic and abiotic stresses.
level, TaAGOs (TaAGO6-5A and TaAGO6-5D) were
downregulated at 6 hours after heat stress and then signif-
antly upregulated at 24 h, while TaAGO6-5B was upregu-
lated at 6 and 24 h. Similar results were also observed in
cucumber CsaGOs. These results indicate that TaAGOs may
play an important role in response to high-temperature stress
(Figure 4). Moreover, the expression of 12 (75%) TaAGOs was significantly affected by PEG and salt
stress, respectively. And TaAGOs (TaAGO1a-6A, TaAGO1b-
2A, TaAGO1b-U, TaAGO4b-7Bb, TaAGO6-5D, TaAGO9-
1A, and TaAGO17-5B) were significantly affected by all the
stress treatments, suggesting that these TaAGOs might par-
ticipate in multiple abiotic stress responses. Collectively, our
findings provide potential gene-editing targets to improve
wheat performance.

Polyploidization and duplication provide additional
opportunities to adapt to the environment changes through
homoeologous gene expression divergence, such as nonfunc-
tionalization or subfunctionalization [28]. Bread wheat is an
evolutionarily young polyploid. Its hexaploid nature provides
an opportunity to study the fate of TaAGOs after polyploidi-
ization and analyze its contribution to the high adaptability of
wheat. Based on the observation that TaAGO4a-3A and TaA-
GO4a-3D but not TaAGO4a-3D were suppressed by heat
stress treatment, the evolution of TaAGOs could have under-
gone neofunctionalization (Figure 4). Furthermore, since the
highly expressed homoeologs showed a higher active histone
marker, H3K36me3, epigenetic modification may be associ-
ated with the expression divergence of TaAGOs in bread
wheat (Figure 3).

Taken together, TaAGO genes are critical for the develop-
ment and adaptability to diverse environmental conditions
in bread wheat. The results of this study will broaden our
understanding on the structure and function of the AGO
gene family in wheat and help to clarify potential candidate
AGO genes to be used for the future breeding of new wheat
and other cereal crop species with increased grain yield and
improved resistance to biotic and abiotic stresses.

5. Conclusions
The bread wheat genome possessed 69 TaAGO genes. We
discussed the structures and performed a detailed phyloge-
netic analysis of this gene family. TaAGOs displayed slower
evolutionary rate when compared with all wheat genes.
Chromosome localization analysis revealed that segmental
duplication (or whole-genome duplication) contributed to
the expansion of the AGO gene family in wheat. Gene
expression profiles revealed that TaAGOs were responsive
to various abiotic stresses. This study provides basis for fur-
ther functional characterization of these genes.

Data Availability
The data used to support the findings of this study are
included within the supplementary information file(s).

Conflicts of Interest
We declare that we do not have any commercial or associa-
tive interest that represents a conflict of interest with the
work submitted.

Authors’ Contributions
WW and HXZ designed the experiments; YFL, LMW, and
LZZ performed the experiments; YFL and WW analyzed the
data; YFL, WW, and HXZ wrote the manuscript. All
authors have read and approved the final manuscript.

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Supplementary Materials
Supplementary Figure 1: exon-intron structure and domains
of TaAGO genes. Supplementary Figure 2: chromosomal dis-
tribution of TaAGOs in wheat genomes. The TaAGO gene
name was shown in different colors. The outer and inner
track indicated the chromosome and chromosomal segment
(light grey: C; grey: R2a and R2b; dark grey: R1 and R3). Con-
necting lines in the center of the diagram indicated the
homologous genes. Supplementary Table 1: primers used
in this study for qRT-PCR. Supplementary Table 2: list of
all TaAGO genes identified in bread wheat. Supplementary
Table 3: GeneID of subfamily AGO9 in di-
fferent species. Supplementary Table 4: homoeologous groups of
TaAGO genes. Supplementary Table 5: relative expression level
(TPM) of TaAGOs in different tissues. Supplementary Table
6: homolog expression bias for triads in grain, spike, stem,
leaf, root, seedling, stamen, and pistil. (Supplementary
Materials)

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