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Alcohol dehydrogenase (ADH) genes family in wheat (Triticum aestivum): Genome-wide identification, characterization, phylogenetic relationship and expression patterns

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Abstract

Background

Alcohol dehydrogenase (ADH) plays important roles in plant survival under anaerobic conditions. Although some research has been carried out the functions of ADH in other plants, that of wheat TaADH family genes in response to abiotic stress are unclear.

Results

A total of 22 ADH genes were obtained from 14 chromosomes of the wheat genome by systematic screening. Multiple sequence alignment and evolutionary relationship show that these genes contain the characteristics of GroES-like domain and Zinc-binding domain, and these belong to Medium-chain -ADH type and can be divided into three subfamilies. There are 17 pairs of fragment replication genes among TaADH family members in the wheat genome, while there are 9 pairs of collinear gene pairs from ADH family members between wheat and rice genome. We speculate that these fragment repetition events may be the main reason for the amplification of TaADH family genes. Ka/Ks analysis indicated that there were 64 repetitive gene pairs, and the Ka/Ks value of these gene pairs was less than 1, which indicated that these sequences of *TaADH* gene were relatively conservative and did not change greatly in the process of evolution. Promoter element analysis showed that almost all of the upstream promoters of these genes contained the responsive anaerobic inducible element. Tissue localized expression and expression patterns also demonstrated that the *TaADH* genes responded to abiotic stress and may play an important role in waterlogging stress during the seed germination stage.

Conclusions

The results of this study may be helpful to further study the function of *TaADH* genes and determine the candidate gene for wheat stress resistance breeding.

Background

Alcohol dehydrogenase (ADH, EC1.1.1.1) is a zinc-binding enzyme, as a dimer, dependent on NAD (P) cofactor to transform between alcohol and acetaldehyde. It was first reported that this enzyme has high activity under anoxic conditions. It mainly acts in the last step of glycolysis or fermentation under anaerobic conditions, converting acetaldehyde to alcohol [1]. ADH activity was reported for the first time from the grains and anthers of the maize [2], and the expression and activity of this gene were widespread in the "normal" state or under various pressures. The ADH gene family is actually a relatively large family, which is mainly divided into several major superfamilies, namely, short-chain dehydrogenases/reductases (SDR)-ADH (containing about 250 amino acid residues), medium-chain dehydrogenases/reductases (MDR)-ADH (containing about 350 amino acid residues) and long-chain dehydrogenases/reductases (LDR)-ADH gene superfamily (about 385–900 amino acid residues) [3, 4]. The ADHs of mammals, plants and yeast belongs to MDRs, whose active sites usually contain zinc ligands. Most plant ADHs are dependent on Zn-MDRs. There are many reports about the genes of this family in recent years, and some studies mainly focus on the relationship between ADHs and aroma synthesis during fruit development [5–8], these are only part of its function, while the other functions of ADHs remain to be explained.

As ADH activity is considered to be a necessary condition for plant survival under anaerobic conditions, it has been the focus on the study the response of the ADH gene in the plant to anaerobic stress. In *Arabidopsis thaliana*, it was found that when the seedlings grew on the plate, the *AtADH* gene was constitutively expressed in the root tissue (including lateral roots), but not in the green aerial tissue, and this expression pattern was similar to the expression pattern of ADH1 in maize [9]. However, so far, there are no systematic reports on the number of ADH genes in *Arabidopsis thaliana* and maize genomes. Three members of the *HvADH* family are found in barley. When the plant is attacked by pathogens, *HvADH-1*, *HvADH-2*, and *HvADH-3* were induced to express under anaerobic stress, in which the expression of *HvADH-1* and ADH activity was very high [10]. Their expression levels are not only related to oxygen stress, dehydration, low temperature, abscisic acid, and other stresses but also involved in the seedling stage, pollen, and fruit development. A total of 12 ADH genes and one formaldehyde dehydrogenease (FDH) gene were found in melon (*Cucumis melo* L.) genome [11]. Ten of these genes belong to the medium-chain ADH subfamily, and CmFDH1 belongs to the ClassIII ADH, and CmADH12 belongs to the long-chain ADH subfamily. By analyzing the promoter elements of these genes, it is found that CmADHs is involved in the response of hormones such as ABA, IAA, and ethylene. Qin et al. [8] found eight PbrADH genes in the pear genome with intron 8–9. These Pbr-ADH proteins have many conserved domains, such as the GroES-like domain (35–164 amino acids) and the zinc-binding domain (206–340 amino acids), which belong to the typical ADH family.

Wheat is an important food crop widely planted in the world, and it is ranked first in the harvest area among the three major food crops (rice, wheat, and corn). China is the largest wheat-producing and -consuming country in the world, and what is of great significance to China's food security and farmers' increasing incomes. According to the World Food and Agriculture Organization (FAO), approximately 10% of the world's land area is affected by waterlogging to varying degrees [12]. In the Mediterranean region, the germination and growth stage of winter wheat is vulnerable to waterlogging because approximately 40% of the annual rainfall occurs during the sowing period of winter wheat [13]. The wheat-growing areas in south-central China also face the same problems [14]. For example, some areas often encounter continuous rainy days during the wheat-planting period. In addition, uneven terrain or poor drainage systems of farmland easily cause water logging or waterlogging in the soil, which often leads to a lack of oxygen supply to the soil and the inhibition of seed germination, thereby reducing the germination rate of wheat [15] and resulting in wheat reductions. Wheat seeds are under anaerobic stress after waterlogging, and *ADH* genes will be rapidly induced and expressed. Studies on waterlogging tolerance in wheat at different stages have been reported in the early stage [16], but the expression pattern of the *ADH* genes of wheat in response to waterlogging stress is not clear. With the release of the wheat genome, it provides a simple method for identifying new members of ADH family genes. In this study, we identified 22 ADH family genes in wheat through sequence alignment among the wheat whole genome (IWGSC RefSeq v1.0; IWGSC2018) and Arabidopsis, rice and melon genomes, which greatly expanded the previously reported (NCBI) of

six genes in wheat. By comparison and phylogenetic analysis with other species, it was found that these ADH family genes belong to the medium-chain ADH subgroup. In this study, the structure, promoter, tissue-specific expression, and expression of 22 *TaADH* genes in responses to waterlogging stress of two wheat varieties with different sensitivity, Bainong 607 and Zhoumai 22, were investigated. The information obtained from this study will greatly promote our understanding of the gene function of the ADH family.

Results

Genome-wide identification

A total of 22 TaADH genes in the wheat genome were further identified by BLAST with 26 ADH genes reported in muskmelon as bait and wheat genome database using ADH domains (PF00107.26, PF08240.12, and PF13602.6) through HMMER software. According to the location distribution of these genes on chromosomes, they were named *TaADH1-TaADH22* (Table 1, Table S1). Except for chromosomes 2 and 3, *TaADH* genes were distributed on all chromosomes, including 6 genes on chromosome 4 and one *TaADH* gene on chromosomes 5, 6, and 7, respectively. The number of the intron for all 22 *TaADH* genes ranged from 7 to 9, while that of the exons ranged from 8 to 10. The length of amino acid in *TaADH* genes ranged from 347 to 415. The range of pI was from 5.68 to 8.2, and the molecular weight was among 34.4-44.2KDa. Through the subcellular localization prediction of these genes, it was found that they were all located in the cytoplasm.

Table 1
Properties and locations of the predicted TaADH proteins in *T. aestivum*.

Gene ID	NCBI	Gene name	Ta_Chr	Start	End	exon	intron	CDs length (bp)	Number of amino acid	pI	Molecular weight Mw/KDa	Subc local
TraesCS1A02G370100.1		*TaADH1*	1A	547391832	547395833	9	8	1143	380	6.08	41.6	Cyto
TraesCS1A02G370200.1		*TaADH2*	1A	547410788	547417410	9	8	1137	378	5.87	40.8	Cyto
TraesCS1B02G389200.1		*TaADH3*	1B	622706402	622708971	9	8	1469	379	6.28	41.1	Cyto
TraesCS1D02G376300.1	ADH3D	*TaADH4*	1D	452625185	452627671	9	8	1484	379	6.03	41.1	Cyto
TraesCS4A02G202100.2	ADH1A	*TaADH5*	4A	491715851	491719316	10	9	1140	379	6.15	41.0	Cyto
TraesCS4A02G202200.1	ADH2A	*TaADH6*	4A	491914927	491917719	9	8	1430	379	5.81	40.9	Cyto
TraesCS4A02G202300.1	ADH2D	*TaADH7*	4A	492029965	492032871	9	8	1718	379	5.97	41.0	Cyto
TraesCS4B02G106300.1	ADH1D	*TaADH8*	4B	115556136	115560148	10	9	1962	379	6.03	41.0	Cyto
TraesCS4B02G106400.1		*TaADH9*	4B	115845355	115848000	9	8	1348	376	5.91	40.5	Cyto
TraesCS4B02G106500.1		*TaADH10*	4B	115879177	115881956	9	8	1611	379	5.9	34.4	Cyto
TraesCS4D02G103000.1	ADH1A	*TaADH11*	4D	81918232	81921969	10	9	1839	379	6.15	41.0	Cyto
TraesCS4D02G103100.1		*TaADH12*	4D	81971499	81974375	9	8	1467	379	5.92	40.9	Cyto
TraesCS4D02G103300.1		*TaADH13*	4D	81984987	81987448	8	7	1044	347	6.56	37.6	Cyto
TraesCS5A02G193900.1		*TaADH14*	5A	397249660	397251898	9	8	1359	365	5.68	39.7	Cyto
TraesCS5B02G189200.1		*TaADH15*	5B	341062699	341068539	9	8	1140	379	5.83	40.9	Cyto
TraesCS5D02G196300.2		*TaADH16*	5D	299832208	299835185	8	7	1751	379	5.68	41.0	Cyto
TraesCS6A02G386600.1		*TaADH17*	6A	603279456	603282956	9	8	1367	381	6.55	40.6	Cyto
TraesCS6B02G425700.1		*TaADH18*	6B	694401891	694405637	9	8	1529	381	6.37	40.7	Cyto
TraesCS6D02G371200.1		*TaADH19*	6D	456554723	456558968	9	8	1629	381	6.37	40.7	Cyto
TraesCS7A02G322200.1		*TaADH20*	7A	466247186	466249779	8	7	1248	415	8.2	44.0	Cyto
TraesCS7B02G223100.1		*TaADH21*	7B	419035483	419038096	8	7	1248	415	8.18	44.2	Cyto
TraesCS7D02G319100.1		*TaADH22*	7D	407849007	407852341	8	7	2011	415	8.2	44.1	Cyto

Alignment and evolutionary analysis

By comparing protein sequences of these 22 *TaADH* genes, it was found that most of the residues of these protein sequences were the same. Pfam scanning of the sequences showed that all of these sequences contained the characteristic motifs of ADH (GroES-like domain and Zinc-binding domain) (Fig. 1A), in which the residues of the GroES-like domain were within 35–164 amino acid, and the amino acid residues of the Zinc-binding domain were within 206–340 amino acid. However, the location of amino acid residues of the Zinc-binding domain in *TaADH20-TaADH22* was different from those of other genes (marked with a blue box). It inferred that these genes belonged to the ADH family. To examine their evolutionary relationships in wheat and the other plant species: *Arabidopsis thaliana* (7), *Cucumis melo* (13), *Cucumis sativus* (12), *Glycine max* (3), *Hordeum vulgare* (1), *Lycopersicon esculentum* (7), *Oryza sativa* (1) and

Vitis vinifera (8), a phylogenetic tree was constructed by multiple sequence alignment of 22 TaADH proteins using the adjacent linkage of (NJ) method (Fig. 1B). The predicted *ADH* genes were classified into three groups, namely short-chain ADH, medium-chain ADH and long-chain-ADH. 22 *TaADH* genes in wheat belonged to medium-chain ADH type. According to the evolutionary relationship, these genes can be divided into 3 subfamilies: Class I contained the largest number of *TaADH*s (15 genes, *TaADH1-9* and *TaADH*11-15), followed by Class II (4 genes, *TaADH10* and *TaADH17-19*) and III (3 genes, *TaADH20-22*).

Conservative domain analysis

Through the conservative analysis of *TaADH* genes in the wheat genome, it was found that the exon of *TaADH* genes in Class I had 9, and the distribution of intron number was similar; the *TaADH* genes of Class II had 9 exons, and the position of intron number distribution was similar; the *TaADH* genes of Class had 8 exons (Fig. 2A). In order to further clarify the protein structure of TaADHs family members in wheat, we identified the conserved motif (Fig. 2B and Fig. S1) using MEME software, and found that the number of motifs in Class I TaADHs protein was 12 (such as Motif 1-7-4-9-2-8-5-11-6-10-3-14). However, the TaADHs protein of Class II has 11 motifs (such as Motif 1-7-4-9-2-8-5-11-6-10-3), and that of Class was different from other motif composition patterns (such as Motif 1-4-7-12-5-13-3). In order to further analyze the functional domains of these proteins, we analyzed the functional structure of these genes (Fig. 2C). The members of wheat TaADH family have highly conserved functional domains, in which Class I TaADHs protein was mainly alcohol_DH_plant, Class II TaADHs protein was mainly alcohol_DH_class_ domain, while Class III TaADHs protein was Zn_ADH10. Generally speaking, these TaADH family members contained the typical structural domain of alcohol_DH.

Chromosomes distribution and synteny analysis

From the distribution of TaADHs on the wheat chromosomes (Fig. 3A and 3B), it was found that 22 TaADHs family members were mainly distributed on 15 chromosomes, of which there were 3 genes on chromosomes 4A, 4B and 4D, respectively. These genes have tandem replication events. To explore the collinear relationship of the TaADHs in the wheat genome and between the wheat genome and rice genome, collinear analysis was carried out by MCScanX method (Fig. 3A and 3B). We found that there were 17 fragment replication events among members of the TaADHs family in the wheat genome, including three homologous gene pairs on chromosomes 1, 6 and 7, and fragment replication in *TaADH15* and *TaADH16* of chromosomes 5B and 5D. However, there were 7 fragment replication events (*TaADH5-TaADH8*, *TaADH6-TaADH9*, *TaADH7-TaADH10*, *TaADH5-TaADH11*, *TaADH6-TaADH12*, *TaADH8-TaADH11*, *TaADH9-TaADH12*) in chromosomes 4A, 4B and 4D, which were related to the tandem replication events on chromosomes 4A, 4B and 4D. A total of 9 pairs of syntenic paralogs were found in wheat and rice genomes (Fig. 3B), in which of *TaADH6* corresponds to *LOC_Os11g10510.1*; *TaADH8* and *TaADH11* correspond to *LOC_Os11g10480.1*; *TaADH17*, *TaADH18*, and *TaADH19* correspond to *LOC_Os02g57040.1*; *TaADH20*, *TaADH21*, and *TaADH22* correspond to *LOC_Os08g01760.1*.

Evolutionary analysis

The non-synonymous substitution rate (Ka), synonymous substitution rate (Ks), and Ka/Ks for 64 duplicated pairs were calculated to reveal the selection pressure of wheat TaADH family genes in the process of evolution (Fig. 4A, Table S2). It was found that the Ka/Ks of these duplicated pairs were less than 1, which tended to a pure selection, indicating that the sequence similarity of *TaADH* genes was very high and relatively conservative in the process of evolution. The evolution time of the duplicated events of *TaADH* genes can be divided into three evolution periods (Fig. 4B, Table S2), of which 30 copies of TaADH duplication genes occurred about 11.19 to 16.42 million years (Mya), 12 copies of TaADH duplicated gene pairs occurred about 7.73 to 9.56 Mya, and the other 22 copies of TaADH duplicated gene pairs occurred about less than 6 Mya, the time period mostly before the wheat polyploidization event. It showed that although these genes sequences were conserved, they were different in evolutionary time.

The cis-regulatory elements analysis of *TaADH* genes in wheat

To further identify the cis-regulatory elements located upstream of the *TaADH* genes, we selected the 2K bp promoter region upstream of the CDS of *TaADH* genes and used TBtools software to predict and visualize the cis-acting elements (Fig. 5). There were a variety of cis-acting elements in the upstream promoters of these genes, which are responsive to 11 kinds of stress (hormone response, anaerobic response, defense, and stress response, drought induction, light response, low-temperature response, etc.). Except for *TaADH13*, the upstream promoters of other genes contain elements (ARE), that respond to anaerobic induction, and *TaADH6* and *TaADH9* contained as many as six cis-regulatory elements of ARE. We also found that the upstream promoter of *TaADH4* contains 8 cis-regulatory elements (ABRE) responsive to abscisic acid. The upstream promoter of *TaADH3* contains as many as 14 cis-regulatory elements (TGACG-motif and CGTCA-motif) responsive to Me-jasmonic acid.

Tissue-specific expression patterns of *TaADH* genes in different tissues and organs

In RNA-seq data of different tissues and organs in *T. aestivum* from, FPKM values of transcript accumulation of 22 *TaADH* genes were obtained from publicly available expression data sets, and then the corresponding heatmaps of relative expression levels were generated using Heatmap tool. The transcription levels in various *T. aestivum* tissues, including the roots, leaf, stem, spike, grain, and seeding were examined (Fig. 6). We found that except for *TaADH2*, there was no expression of *TaADH6-7* in leaves, but the expression of *TaADH6* was the highest in grain and that of *TaADH7* was the highest in stems. The expression pattern of *TaADH4* was similar to that of *TaADH6*, and the expression level was the highest in grains. The *TaADH1* and *TaADH9* expressions were only detected in grains and roots of wheat, but not in other parts of wheat, while the expression pattern of *TaADH15* was opposite to that of *TaADH1* and *TaADH9*. Other genes (*TaADH3*, *TaADH5*, *TaADH8*, *TaADH10-11*, *TaADH14*, *TaADH16-22*) were expressed in all parts of wheat. Among them, the expression of *TaADH5*, *TaADH8*, *TaADH11*, and *TaADH17-22* was the highest in wheat grains.

The expression of *TaADH* genes in wheat seed under waterlogging treatment

To further analyze the response of two wheat seed with different waterlogging tolerance to waterlogging stress during the germination stage, we analyzed the relative expression levels of 22 members of wheat TaADH family (Fig. 7). The results showed that the expression levels of seven *TaADH* genes (*TaADH1/2*, *TaADH13*, *TaADH17*, *TaADH18*, *TaADH19*, *TaADH20*) in the seeds of the intolerant variety Zhoumai 22 were significantly up-regulated at 24 hours after waterlogging treatment compared with the control treatment, but there was no significant difference in the expression levels of *TaADH1/2*, *TaADH17*, *TaADH18*, *TaADH19*, and *TaADH20* genes compared with the control treatment 72 hours after germination, only the expression level of *TaADH13* gene showed an upward trend. Compared with the control treatment, the expression levels of 14 genes (*TaADH1/2*, *TaADH3-6*, *TaADH8-13*, *TaADH19*, and *TaADH20*) in the seeds of Bainong 607 were significantly up-regulated at 24 hours after waterlogging treatment, while the expression levels of *TaADH1/2*, *TaADH3*, and *TaADH9* genes were significantly up-regulated at 72 hours after germination compared with the control treatment, while the expression levels of *TaADH5*, *TaADH6*, *TaADH14*, and *TaADH16* genes decreased. The results showed that the difference between waterlogging-tolerant and non-waterlogging-tolerant varieties after waterlogging treatment was closely related to the early and rapid expression of *TaADH* genes.

Discussion

In recent years, with global warming, extreme weather occurs more frequently, in which flood disaster is one of the abiotic stresses faced by plants, and hypoxia will first occur in the flooded environment. Waterlogging-tolerant plants can often stimulate alcohol fermentation and reduce lactic acid fermentation to prevent acidifying cytoplasm. To avoid cytoplasmic acidosis, ethanol production is necessary for plants to survive under anaerobic conditions [17]. The role of alcohol dehydrogenase has been reported in many species [18–20]. With the development of sequencing technology, the genome sequences of many species can be analyzed, which promotes the identification of plant gene families at the genome-wide level. The *ADH* genes have been detected in various plants, including tomato [5], rice [21], barley [22], melon [7], and pear [8]. With the publication of the wheat genome, it is possible to systematically study the function of wheat TaADH family members.

Structural characterization of TaADHs

A total of 22 TaADH genes were identified in the wheat genome database, including 6 TaADH genes published by NCBI. In this study, we found that there are 8–10 exons and 7–9 introns of TaADH family members in wheat, which is consistent with the 8–9 introns of ADH genes reported by Strommer [23]. For example, the ADH2/3 in barley and the ADH in wheat contain 8 introns. While ADH1 and ADH3 in *Arabidopsis thaliana* contain 6 and 4 introns, respectively, and ADH in Chinese cabbage contains 5 introns [23]. The increase in the number of introns in common wheat (*Triticum aestivum* L.) indicated that the *TaADHs* family was expanding significantly in the evolution from lower plants to higher plants, and the expansion of the number of ADH members could enhance the ability of organisms to adapt to more complex environmental changes.

The plant ADH family usually divides into short-chain-ADH and medium-chain-ADH. Kitaoka et al. [21] found that there was only one short-chain alcohol dehydrogenase / reductase SDRs (*OsMAS/SDR110C-MS1*) in rice, which belonged to the short-chain-ADH type, while the other long-chain-ADHs were rarely reported [24, 25]. 22 TaADH family members identified in this study belonged to medium-chain-ADH, and these genes have a highly conserved functional domain (GroES-like domain and zinc-binding domain) (Fig. 1A). This was similar to the structure of pear PbrADHs reported by Qin et al. [8]. ADH is a member of the medium-length dehydrogenase/reductase (MDR) protein superfamily. There is a typical domain of GroES-like in MDR, which is determined by chaperonin-10, based on its similarity to GroES molecular chaperone [26]. Association of Zn co-factors with a primitive MDR may have occurred in the early days of atmospheric oxygen, when Zn is likely to have been a preferred co-factor due to its valence stability [27]. From the perspective of evolution, Vv-ADH2 in grape [28], CmADH1 in melon [29] and At-ADH1 in *Arabidopsis thaliana* [30] all clustered in a class of medium-chain zinc-bound ADHs (Fig. 1B). Therefore, 22 *TaADH* genes in wheat belonged to the typical ADHs family.

Phylogenetic analysis and evolution of TaADHs

Gene replication is an important evolutionary process of gene family expansion, and the replicated genetic material provides an opportunity for functional differentiation. Functional differences caused by gene duplication are considered to be an important factor in species formation and environmental adaptability [31]. Therefore, gene replication analysis can help us better understand the evolution of genes and species. Two repetitive genes located on the same chromosome are called tandem replication. They exist on different chromosomes but come from the same subgenome, which is called fragment replication [32]. There are 4 gene tandem replication and 17 fragment replication events among members of the TaADHs family in the wheat genome (Fig. 3A). Most of the fragment replication events occurred in homologous chromosomes, such as chromosomes 1, 4, 6, and 7. In addition, there were gene collinearity between chromosomes 4, 6 and 7 in the wheat genome and chromosomes 2, 8 and 11 in the rice genome, which indicated that wheat polyploidy was the main reason for the expansion of TaADH family members, which also confirmed the conservatism of TaADH family members on individual chromosomes.

Ka, Ks, and Ka/Ks can well explain the history of a gene or gene region facing selection pressure [33]. In this study, we found that there were 64 duplicate gene pairs in the TaADHs family, and their Ka/ KS values were all less than 1 (Fig. 4B), which means that all duplicate gene pairs are purified and selected. The Ks value was used to estimate the occurrence time of repetitive events. The results showed that 64 repetitive events of TaADH family genes occurred between 1.55 and 16.42 Mya, including 30 repetitive gene pairs with evolution time of 11.19–16.42 Mya and 12 repetitive gene pairs with evolution time of 7.73–9.56 Mya, all of which were earlier than the first time of genomic replication in wheat [34]. The second time is that the subgenome of common wheat can be traced back to 300000 years ago, including *Triticum urartu* (A sub-genome) and the hybridization of the Aegilops speltoides to form tetraploid wheat (*T. dicoccoides*, A and B sub-genome). About 8, 000 years ago, tetraploid wheat, also known as the *Aegilopstauschii* (D subgenome, produced hexaploid wheat (*T. aestivum*, A, B, and D subgenomes) [35]. It showed that most of the *TaADH* family genes in wheat have undergone strict purification and screening in the process of evolution, and retained the original function through fragment replication events.

Expression profile analysis of TaADHs

Gene promoters are important factors in regulating gene expression patterns. They regulate gene expression at the transcriptional and post-transcriptional levels [36]. Cis-acting elements in a specific promoter region participate in tissue-specific expression patterns under various environmental conditions. In the corresponding upstream region for a gene, there was a positive correlation between the number of cis-acting elements and multiple stimuli [37]. Through the prediction of cis-acting elements of wheat TaADH family members, it was found that these genes were selected by a variety of biotic and abiotic factors (Fig. 5). Alcohol dehydrogenases catalyze the reversible conversion of aldehydes to the corresponding alcohols. They are involved in the stress response of plants and are mainly responsible for the production of ethanol in an anaerobic environment. At the same time, it is also widely involved in other stress, activator, and abscisic acid responses [38]. ADH has a good protective effect on hypoxia stress after flooding [39], as well as seed development and pollen aerobic metabolism [40]. There are many isozyme genes of ADH in the seed. Studies on tracking the activity of ADH during seed development have proved that ADH isozyme genes are active at different times. When seeds were under hypoxia stress at the germination stage, ADH activity might play a very important role in germination [23]. Three ADH genes (*HvADH-1*, *HvADH-2*, and *HvADH-3*) in barley were found, in which the activity of HvADH-1 during aerobic growth could be detected, while the expression of *HvADH-1* and *HvADH-2* could be induced by hypoxia, while the expression level of *HvADH-3* under hypoxia was significantly lower than that of the other two genes [41]. Proels et al. [42]found that *HvADH-1* was the only constitutively expressed in barley seedlings. However, ADH-3 activity could not be detected in barley leaves under any conditions.

In this study, it was also found that *TaADH3*, *TaADH5*, *TaADH8*, *TaADH10-11*, *TaADH14*, and *TaADH16-22* were expressed in all parts of wheat. This suggested that these genes might also have constitutive expression. Manangkil et al. [22]confirmed that the expression of *ADH1* and *ALDH2a* in rice seedlings increased rapidly under submergence stress, but decreased rapidly when submergence was removed, indicating that higher express levels of *AHD1* and *ALDH2a* might be one of the reasons why rice is more tolerant to submergence than other plants. When wheat seeds were exposed to anaerobic environment (waterlogging treatment), TaADHs was expressed differently with different exposure time. No matter the waterlogging tolerant variety Bainong 607 or the intolerant variety Zhoumai 22, the expression levels of *TaADH1/2*, *TaADH13*, *TaADH19*, and *TaADH20* genes at 24 hours after waterlogging treatment were significantly higher than those of the control treatment (Fig. 7). From the tissue expression analysis, it was found that the relative expression of these genes was the highest in grains, especially the specific expression of *TaADH1* and *TaADH13* in wheat grains. This suggested that these genes might be induced the rapid expression under anaerobic stress. However, after 72 hours of germination, the relative expression levels of *TaADH1/2*, *TaADH3* and *TaADH9* genes in Bainong 607 seeds were significantly up-regulated compared with the control treatment. However, only the expression level of the *TaADH13* gene increased at 72 hours after germination of Zhoumai 22 seed (Fig. 7). This showed that *TaADH1/2*, *TaADH3*, and *TaADH9* played an important role in responding to waterlogging stress and served as an important basis for screening waterlogging-tolerant wheat varieties.

Conclusions

A total of 22 TaADH genes were identified and analyzed at the wheat genome. These genes were distributed on 15 chromosomes, of which there were 3 homologous genes on chromosomes 4A, 4B, and 4D, respectively, and there were tandem replication events in these genes. According to Pfam domain compositions, it was found that all these protein sequences contained the characteristic motifs of ADH (GroES-like domain and Zinc-binding domain). Through phylogenetic tree analysis with other species, it was found that 22 TaADH genes in wheat belonged to medium-chain ADH type and were grouped into 3 subfamilies. Collinearity analysis within the wheat genome and between the wheat genome and rice genome showed that there were 17 fragment replication events among members of the TaADH family, while the number of collinear ADH gene pairs of wheat and rice was 9 pairs. According to the Ka/Ks and evolutionary years, it was found that there were 64 repetitive gene pairs with Ka/Ks less than 1, which tended to purification selection, indicating that strong purifying selection had taken place in the process of evolution of TaADH genes. Cis-acting elements and tissue expression analysis showed that these genes were responsive to 11 kinds of abiotic stress. *TaADH3*, *TaADH5*, *TaADH8*, *TaADH10-11*, *TaADH14*, and *TaADH16-22* were expressed in all parts of wheat, belonging to constitutive expression, and other genes were specifically expressed. By comparing the expression profiles of waterlogging-tolerant wheat Bainong 607 and waterlogging-intolerant wheat Zhoumai 22 at different germination stages after waterlogging treatment, it was found that *TaADH1/2*, *TaADH3* and *TaADH9* played an important role in responding to waterlogging stress, and could be used as an important basis for screening waterlogging-tolerant wheat varieties. These results will provide valuable information regarding further functional elucidation of *TaADH* genes in wheat.

Methods

Wide-genome identification of *TaADH* genes in wheat

The whole-genome data was downloaded from the wheat genome database (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/) [43] and was used to identify *ADHs* candidate genes in wheat with two methods. First of all, 26 *ADH* genes reported in melon were used as the inquiry to search for the homologous gene of *ADHs* through the BLAST function of BLAST2.3.0 + tool provided by the National Center for Biotechnology Information (NCBI). *ADH* genes of melon were downloaded from the reported melon genome database. Secondly, we download the ADH domains (PF00107.26, PF08240.12, and PF13602.6) from the PFAM website (http://pfam.xfam.org/) to further identify the domains of these candidate genes using HMMER software. Finally, ExPASy (http://prosite.expasy.org/)) was used to verify the integrity of the ADH proteins domain, and motif analysis was performed for ADH proteins using the Batch-CDD. The maximum number of motifs was set to 15. Each ADHs gene is renamed according to its exact location on the chromosome (from top to bottom each chromosome). The protein molecular weight and isoelectric point of the candidate genes were calculated by ExPASy website (https://web.expasy.org/compute_pi/). Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi) was used to predict the subcellular location of the *TaADH* genes [44].

Sequences alignment and evolutionary analysis of *TaADH* genes in wheat

The ADH protein sequences were downloaded from the genomes of Arabidopsis thaliana, Chinese spring wheat, rice, and muskmelon respectively. The protein sequences of these species were compared by ClustalW2 (http://www.genome.jp/tools-bin/CLUSTORW) software, and the phylogenetic tree was constructed

by the Neighbor-Joing (NJ) method in MEGA7.0 (Test of phylogeny: Bootstrap method, Bootstrap replications: 1000) [45].

Gene structure and motif distribution of *TaADH* genes in wheat

The introns and exons of the ADH genes were generated by using the Gene Structure Display Server 2. 0 (GSDS2.0, http://gsds.cbi.pku.edu.cn/). The protein sequence of ADHs was analyzed by online software Multiple EM for Motif Elicitation (MEME, http://meme-suite.org/tools/meme [46]. The number of motifs is set to 15. Visualization of the results were performed by the TBtools (https://github.com/CJ-Chen/TBtools) [47].

Gene duplications event and Ka/Ks of *TaADH* genes in wheat

In order to identify gene duplications event, all CDS sequences of wheat *ADH* genes were subjected to BLAST searches against each other (identity > 80%, e value < $1e^{-10}$) by using the local Blast program. Gene alignment coverage was then acquired by pair-wise alignment using the previously calculated method: Gene alignment coverage = (alignment length-mismatch length) / the length of larger genes. When the gene alignment coverage is more than 0.75, it is considered to be a duplicate gene pair. In addition, in the 100 kb region, two genes separated by five or fewer genes are considered as tandemly duplicated genes, while replication genes between different chromosomes are defined as fragment repeat genes. The values of Ka/ Ks between Ka and Ks and between paired genes were calculated by DnaSP software (http://www.ub.edu/dnasp/). For the timing of duplication events, the formula: $T = Ks/2\lambda \times 10^{-6}$ Mya was used to calculate divergence time (T) in millions of years (Mya), where $\lambda = 6.5 \times 10^{-9}$ represented the rate of replacement of each locus per herb plant year [48].

Chromosome mapping and collinearity analysis of *TaADH* genes in wheat

The chromosome locations of the *TaADH* genes from the Chinese spring wheat genome (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/) were determined using the TBtools [47]. To confirm gene homology, the protein sequences of *TaADH* genes in wheat were blasted against each other by BLASTP (E value < 10^{-20}, identity > 75%). The chromosome distribution and collinearity of these *ADHs* genes were visualized by Circos [49].

Promoter cis-acting element analysis of *TaADH* genes in wheat

To analyze the promoter elements of *TaADH* genes, the promoter sequences (2000 bp before the start codon) of all *TaADH* genes were extracted from the Chinese spring wheat database. The cis-acting elements of these genes were predicted by online tool PLANTCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [50] and visualized by TBtools (https://github.com/CJ-Chen/TBtools) [47].

Expression pattern of *TaADH* genes in wheat

To analyze the expression profiles of *TaADH* genes in wheat, RNA-Seq data (http://www.wheat-expression.com/) of different parts of Chinese spring wheat from the reported database were downloaded, and the FPKM data of all *TaADH* genes were extracted [51]. Heatmap (https://github.com/CJ-Chen/TBtools) were drawn with TBtools software [47].

Analysis of expression of *TaADH* genes under waterlogging stress

Experimental treatment: waterlogging treatment was 72 h at 20 ℃ constant temperatures in the dark for fifty plump seed placed in the glass Petri dishes. At the end of waterlogging treatment, the seeds were transferred and germinated in a Petri dish containing one layer filter paper with 10 mL of sterilized deionized water. Control treatment: a control (CK), consisting of not water-logged seed, was added for each variety. Germination was conducted on the seed as same as waterlogging treatment. The experimental and control treatments were given 5 repetitions, each containing 50 seeds. All seeds were grown in a growth chamber at 25 ℃ and 75% relative humidity (16 h light/8 h dark cycles). In this study, two wheat (*Triticum aestivum* L.) varieties, 'Zhoumai 22' (ZM22, waterlogging-intolerant) and 'Bainong 607' (BN607, waterlogging-tolerant), were selected as the materials. ZM22 is a commercial winter wheat cultivar in Henan Province, China. The BN207 and BN607 seeds used in this study were cultivated by Prof. Xingqi Ou from the School of Life Science and Technology, Henan Institute of Science and Technology, Xinxiang, China. All the seeds were provided by the School of Life Science and Technology, Henan Institute of Science and Technology.

To explore the response of wheat seeds to waterlogging stress, samples of embryo and endosperm of germinated seeds from ZM22 and BN607 were collected at 24 h and 72 h of germination under the waterlogging and control treatments. Total RNA was removed with RNase-free DNase I (Takara, Tokyo, Japan) to avoid genomic DNA contamination. First-strand cDNAs were synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. The qRT-PCR assays were performed with the Primer Script RT Reagent Kit (Takara, Dalian, China). The *18S* (AJ272181.1) was used as a reference gene. Data were analyzed with Opticon monitor software (Bio-Rad). All primers for qRT-PCR were designed using Primer 6.0 software and primer sequences are listed in Table S3. The PCR conditions were as follows: 95 °C for 10 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The qRT-PCRs were performed using an ABI Step One Plus. All the experiments were performed with three biological replicates. One sample constitutes a mixture of three seeds. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [52].

Abbreviations

ZM22: Zhoumai 22; BN607:Bainong 607; ADH:Alcohol Dehydrogenase; mRNA:Messenger RNA; qRT-PCR:Quantitative Real-time PCR; FPKM:Fragments Per Kilobase Per Million Mapped Reads; NCBI:National Center for Biotechnology Information; MW:Molecular weight; pI:Isoelectric points; Ka:Non-synonymous substitution rate, Ks:Synonymous substitution rate; SDR-ADH:Short-chain dehydrogenases/reductases; MDR-ADH:Medium-chain dehydrogenases/reductases; LDR-ADH:Long-chain dehydrogenases/reductases; FDH:Formaldehyde dehydrogenease.

Declarations

Availability of data and materials

All data generated or analyzed during this study were included in this published article and the additional files.

Consent for publication

Not applicable

Competing interests

No potential conflict of interest was reported by the authors.

Ethics approval and consent to participate

Two wheat cultivars, 'Zhoumai 22' (ZM22) and 'Bainong 607' (BN607), were used in the present study. ZM22 originated from a cross of three breeding lines, namely Zhoumai 12, Wenmai 6 and Zhoumai 13. The BN607 seeds used in this study were cultivated by Prof. Xingqi Ou from the School of Life Science and Technology, Henan Institute of Science and Technology, Xinxiang, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

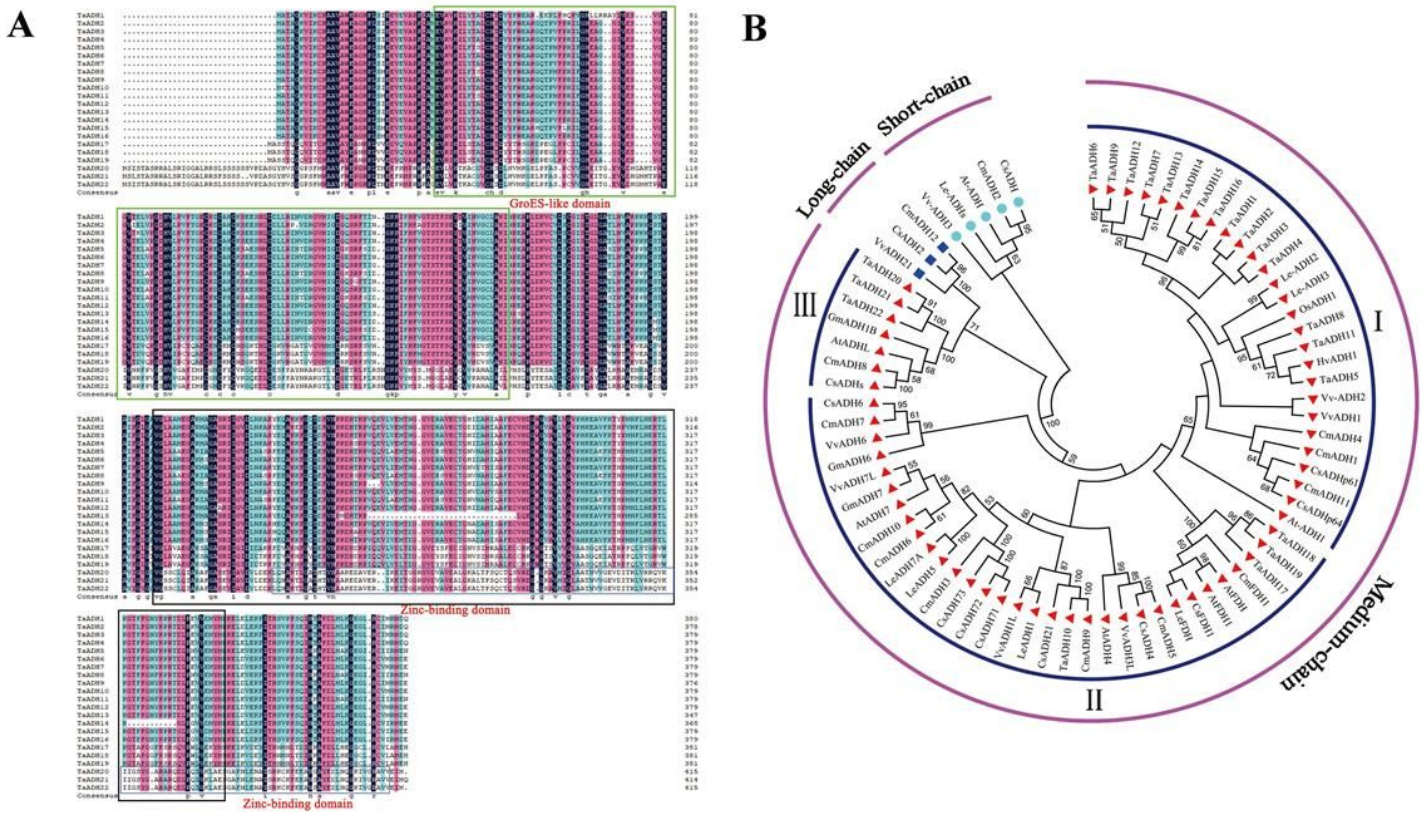

Figure 1

Sequences alignment and phylogenetic tree of TaADH proteins in wheat with other species. A. amino acid sequence alignment among TaADHs. Sequences were aligned using the DNAMAN5.0 Program. GroES-like domain and a structural zinc-binding domain were identified. B. phylogenetic tree among TaADHs and other ADHs. At: Arabidopsis thaliana, Cm: Cucumis melo, Cs: Cucumis sativus, Hv: Hordeum vulgare, Os: Oryza sativa, Gm: Glycine max, Le: Lycopersicon esculentum, Ta: Triticum aestivum, Vv: Vitis vinifera.

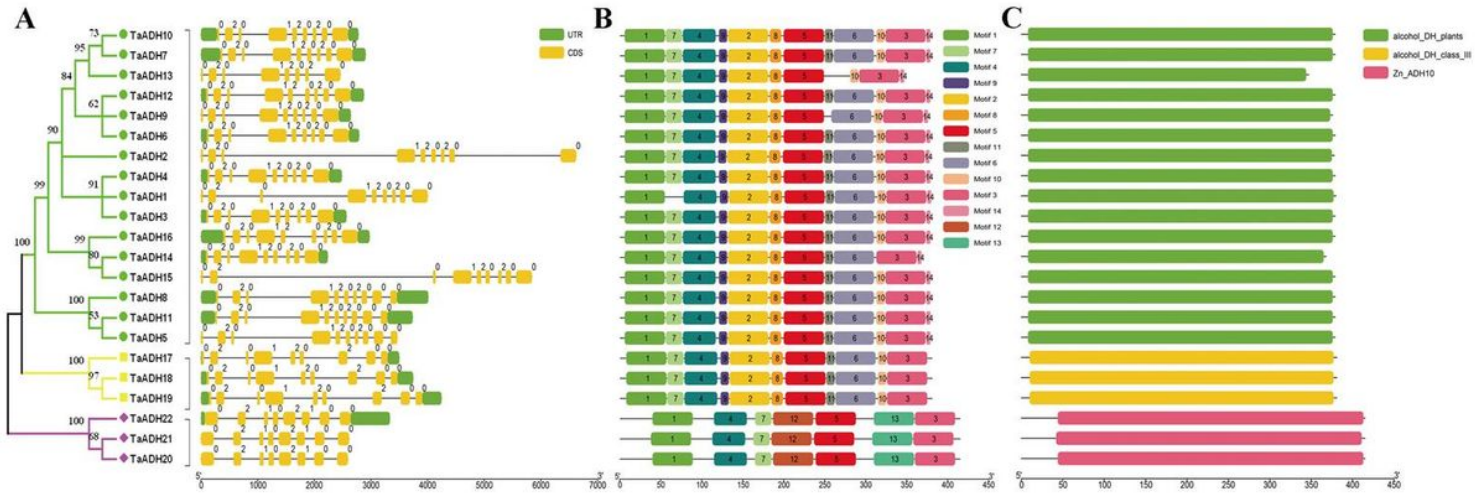

Figure 2

Exon-intron structures and motif compositions of TaADH proteins in wheat. The sequence information for each motif is provided in Fig. S1.

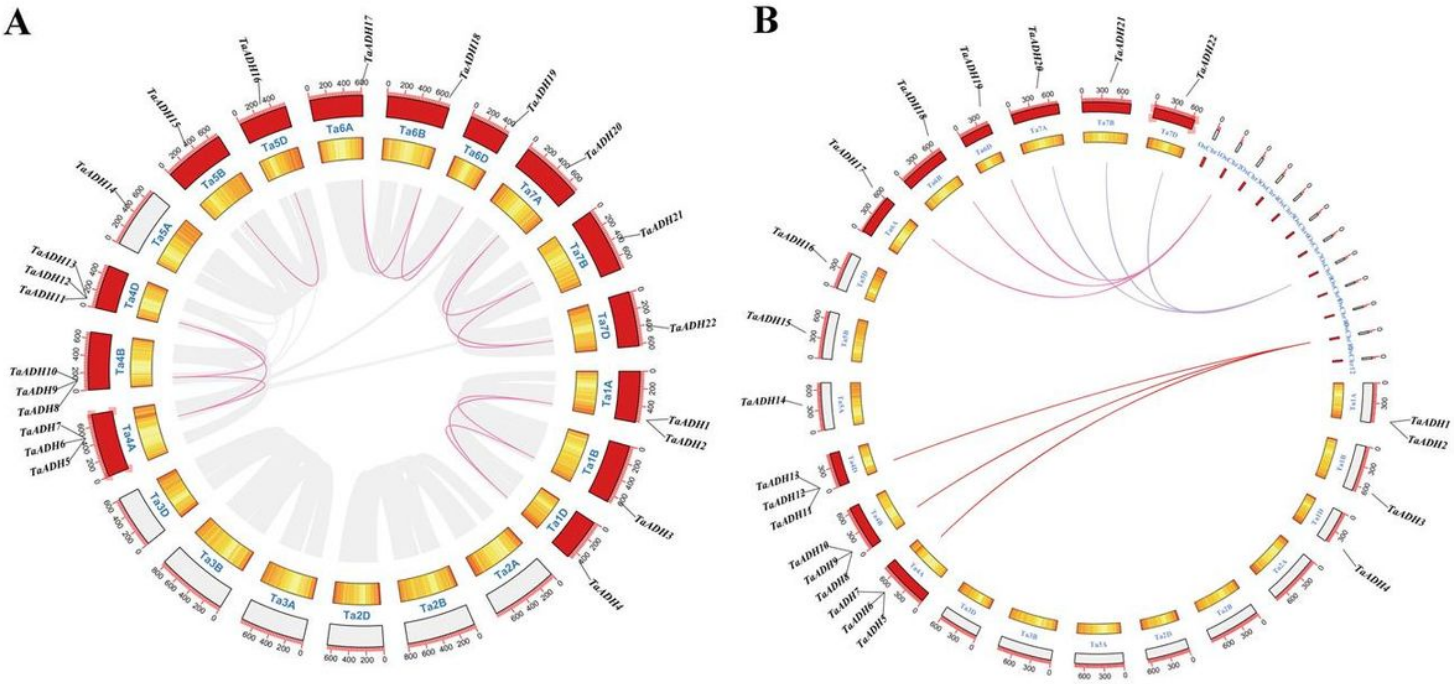

Figure 3

Chromosomes distribution and synteny analysis of TaADH genes in wheat. A. Ta1-7 represented chromosomes in A, B and D sub-genomes in Triticum aestivum. Red lines linked the syntenic orthologous in T. aestivum genomes. B. OsChr1-12 represented chromosomes in Oryza sativa. Red, pink and purple lines linked the syntenic paralogs within genomes of T. aestivum and Oryza sativa.

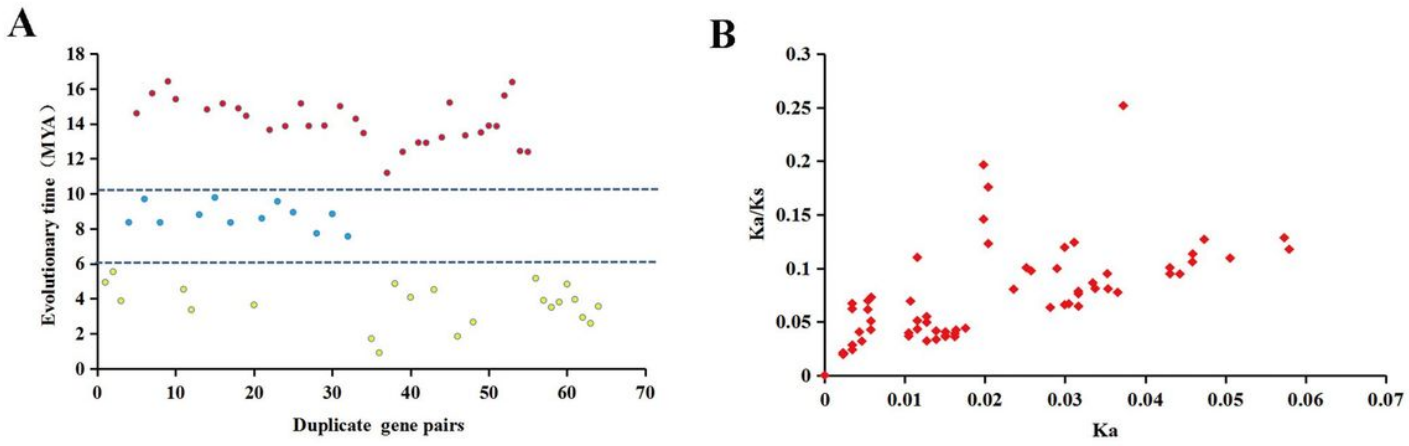

Figure 4

Evolutionary time and Ka/Ks analysis of TaADH genes in the wheat genome. A. Evolutionary time for duplicated TaADH gene pairs; B. Ka/Ks values for duplicated TaADH gene pairs

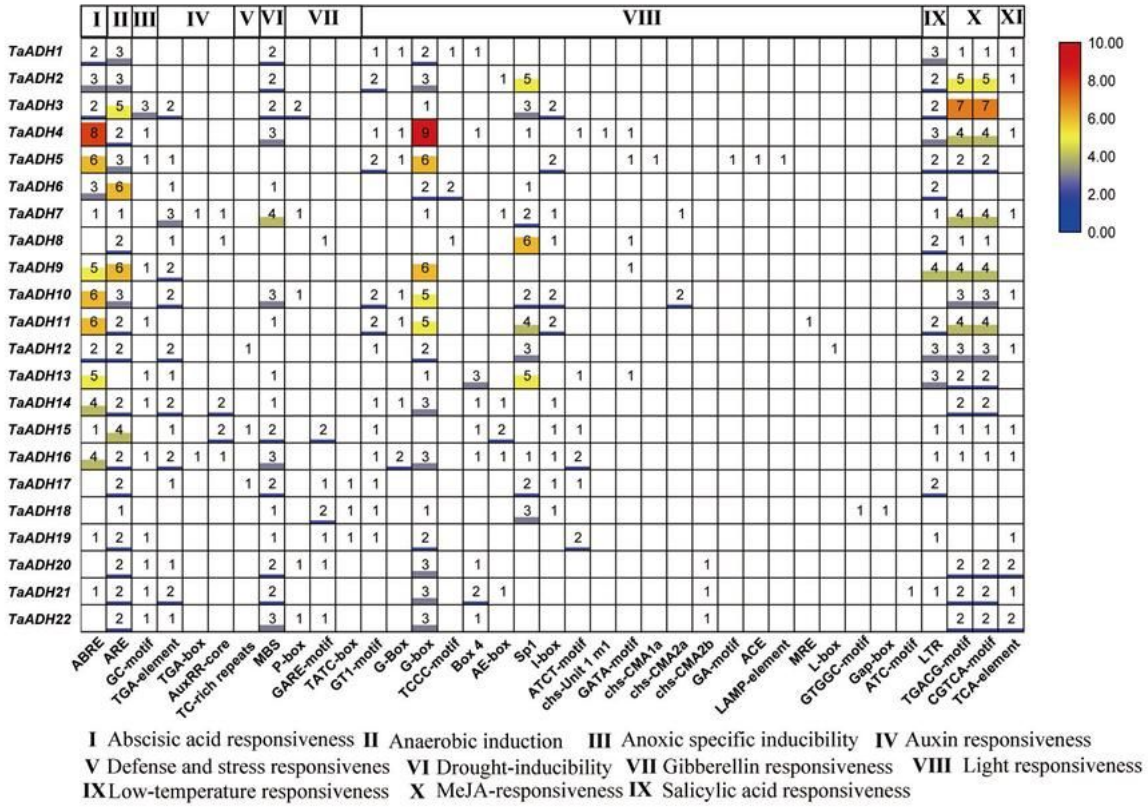

Figure 5

Cis-acting elements distribution and statistical analysis of TaADHs promoters. The color of the square depicts the quantity of the predicted cis-acting elements in the promoter region.

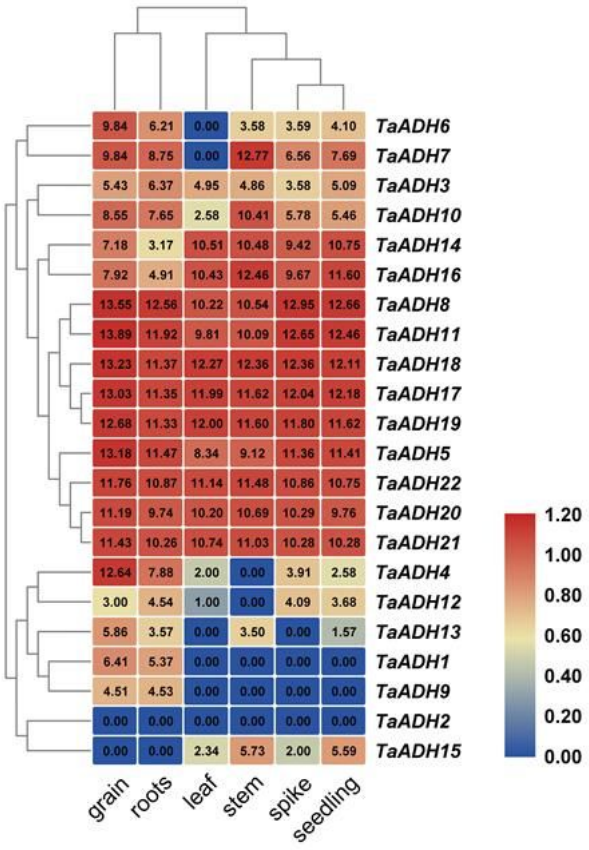

Figure 6

The expression of TaADH genes in wheat various tissues. A heatmap was created in TBtools software and based on the FPKM data (http://www.wheat-expression.com/) of different parts of Chinese spring wheat. The FPKM data was standardized on a base of 10. The color scale represents relative expression levels, with red indicating higher levels of expression and blue indicating lower expression levels.

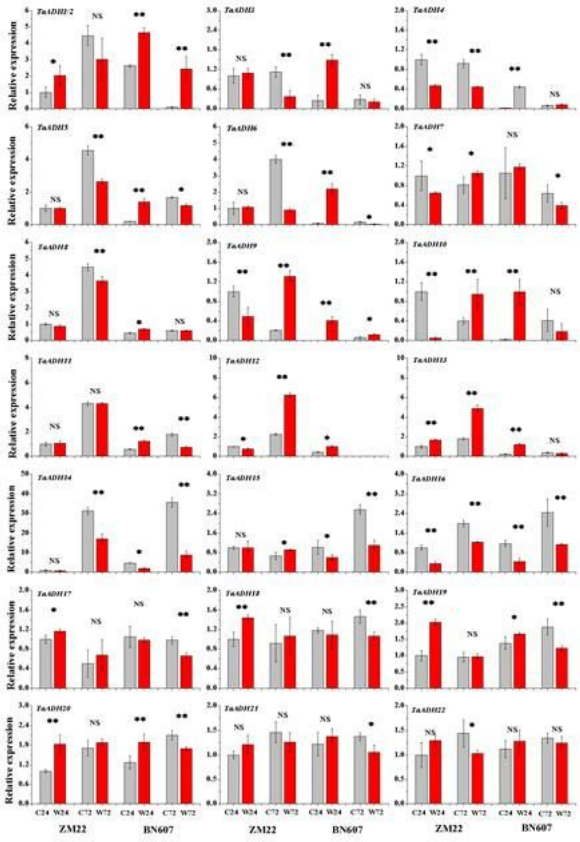

Figure 7

Expression profiles of TaADH genes in wheat seeds of waterlogging-tolerant and intolerant wheat varieties under waterlogging stress. ZM22: Zhoumai 22, BN607: Bainong 607; C24, after 24 h of germination under the control treatment; W24: after 24 h of germination under the waterlogging treatment; C72, after 72 h of germination under the control treatment; W72, after 72 h of germination under the waterlogging treatment; Each point represents the average from three samples. The error bars represent the SDs. ∗ indicates significance at $p<0.05$, ∗∗ indicates significance at $p<0.01$, NS indicates No significance.

Supplementary Files

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

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- TableS3.xlsx
- TableS2.xlsx
- TableS1.xlsx