Genome-wide identification and expression analysis of the TaRRA gene family in wheat (Triticum aestivum L.)

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Cytokinin is an important endogenous hormone in plants performing a wide spectrum of biological roles. The type-A response regulators (RRAs) are primary cytokinin response genes, which are important components of the cytokinin signaling pathway and are involved in the regulation of plant growth and development. By analysis of the whole genome sequence of wheat, we identified 20 genes encoding RRAs which were clustered into eight homologous groups. The gene structure, conserved motifs, chromosomal location, and cis-acting regulatory elements of the TaRRAs were analyzed. Quantitative real-time polymerase chain reaction (qRT-PCR) results showed that the expression levels of most of the TaRRAs increased rapidly on exogenous cytokinin application. Moreover, the TaRRA family members displayed different expression profiles under the stress treatments of drought, salt, cold, and heat. This study provides valuable insights into the RRA gene family in wheat and promotes the potential application of these genes in wheat genetic improvement.

KEYWORDS
type-A response regulators, gene family, wheat (Triticum aestivum L.), expression pattern, cytokinin, abiotic stress

Introduction

Cytokinin is a vital phytohormone responsible for regulating numerous aspects of plant growth and development, including cell division and differentiation, apical dominance, leaf senescence, photomorphogenesis, fertility, and seed development (Werner and Schmülling, 2009; Hwang et al., 2012; Schaller et al., 2015; Wybouw and De Rybel, 2019). In addition, cytokinin plays an important role in plant response to many environmental stresses such as drought, salt, cold, and heat (Ha et al., 2012; Pavlíček et al., 2018; Liu et al., 2020). Cytokinin signal transduction is mediated by a multistep two-component system (TCS) involving a His-Asp-His-Asp phosphorelay from histidine.
kinase receptors (HKs) and histidine-containing phosphotransfer proteins (HPs) to downstream response regulators (RRs) (Argueso et al., 2010; El-Shokw et al., 2013; Kieber and Schaller, 2018).

The response regulators are classified into four distinct groups based on phylogenetic and conserved domain analysis, namely, type-A response regulators (RRAs), type-B response regulators (RRBs), type-C response regulators (RCs), and circadian clock-related pseudo response regulators (PRRs) (Heyl et al., 2013), all with a conserved N-terminal receiver domain and a variable-length C-terminal. The C-terminal of RRAs is short and has yet to be assigned any specific function. RRBs have a large C-terminal extension containing Myb-like DNA binding, nuclear localization, and transcription activation domains (Sakai et al., 2000; Hosoda et al., 2002). The C-terminal of RRCs is also short; however, they are not grouped in the same class as RRAs. PRRs lack the highly conserved Asp residue required for phosphorylation and have a C-terminal including a CCT domain (CO, CO-LIKE, TOC1) (Makino et al., 2000; Schaller et al., 2008). RRAs are transcription factors that mediate the transcriptional response to cytokinin (Argyros et al., 2008). RRAs are transcriptionally induced in response to cytokinin via direct activation by RRBs and are responsible for repressing cytokinin signaling via a negative feedback loop (Hwang et al., 2012).

Many reports have established that RRAs play a critical role in plant growth and development. In Arabidopsis, 10 RRAs have been reported (ARR3-ARR9 and ARR15-ARR17) (D’Agostino et al., 2000), of which ARR3 and ARR4 act redundantly in the determination of the circadian rhythm (Salomé et al., 2006). ARR4 interacts with phytochrome B, modulating red light signaling by stabilizing the active Pfr form of phytochrome B (Sweere et al., 2001; Mira-Rodado et al., 2007). ARR7 negatively influences meristem size through regulation of its expression by MYC2 (Zhai et al., 2010). ARR16 has been reported to be involved in Arabidopsis seedling development via regulation of its expression by MYC2 (Srivastava et al., 2019).

Accumulating evidence indicates that RRAs are involved in abiotic stress responses. Dehydration stress transiently induces the expression of ARR5, ARR7, and ARR15, but reduces the expression of ARR8 and ARR17 (Kang et al., 2012). Also, the phosphorylation of ARR5 Ser residues by SnRK2s enhances its stability and plant drought tolerance (Huang et al., 2018). The expression of a variety of RRAs, including ARR5, ARR6, ARR7, and ARR15, are induced by cold (Jeon et al., 2010). However, ARR-OE plants (ARR5-OE, ARR7-OE, and ARR15-OE) and arr mutants (arr5, arr6, and arr7) show similar enhanced freezing tolerance, indicating that RRAs play a complex role in regulating cold stress response (Jeon et al., 2010; Shi et al., 2012). In rice, the expression of type-A OsRR6 is induced by salt, dehydration, and low-temperature treatments (Jain et al., 2006), and overexpression of OsRR6 enhances seedling drought and salinity tolerance (Bhaskar et al., 2021); whereas, OsRR9 and OsRR10 negatively regulate rice salinity tolerance (Wang et al., 2019). Recently, ZmRR1, a maize type-A RR, has been demonstrated to positively regulate maize chilling tolerance by modulating the expression of ZmDREB1s and ZmCesA. The phosphorylation of ZmRR1 Ser residues by ZmMPK8 accelerates its degradation, thereby reducing the chilling tolerance (Zeng et al., 2021).

RRAs have been widely studied in Arabidopsis and rice, however, limited information is available for RRAs in wheat. The completion of the wheat whole genome sequence and further improvements of the wheat genome database have immensely contributed to decoding the wheat genome at the molecular level. In the present study, we systematically performed a genome-wide analysis of the wheat RRA gene family and investigated their gene structures, conserved motifs, chromosomal locations, cis-acting regulatory elements, and expression patterns in response to cytokinin treatment and various stresses. This work provides valuable information on the wheat RRA gene family and lays a foundation for further functional analysis of this gene family.

Materials and methods

Identification of TaRRAs genes in wheat

Whole-genome and protein sequence data of wheat (IWGSC Assembly GCA_900519105.1) and the hidden Markov model (HMM) file for the response regulator receiver domain (PF00072) were downloaded from the EnsemblPlants database1 and Pfam database,2 respectively. A wheat-specific HMM file was established by the alignment of the response regulator receiver domain HMM file with the wheat protein sequences (E-value < 1e−20). The wheat-specific HMM file was then used as bait to search against the local reference genome database to identify candidate wheat RRs (E-value < 0.01). Redundant genes were removed, and the longest representative transcripts were selected for further analysis. The identified proteins were then submitted to Pfam (see text footnote 2), SMART,3 and NCBI conserved domains search tool4 to further check the receiver domain as well as other conserved domains. The protein sequence of RRs in wheat, Arabidopsis, and rice was used to carry out multiple sequence

1 https://plants.ensembl.org/index.html
2 http://pfam.xfam.org
3 http://smart.embl.de/
4 https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
alignment using ClustalX 2.1 software (Larkin et al., 2007). The phylogenetic tree was established using MEGA 7.0 based on the neighbor-joining (NJ) method with 1,000 bootstrap replicates (Kumar et al., 2016), and RRs were named according to the standard nomenclature for the plant TCS elements (Heyl et al., 2013).

**Characterization of TaRRAs**

Information about the TaRRA gene family, such as chromosomal localization, number of exons, and cDNA and protein length, was obtained from the EnsemblPlants. The protein sequence of TaRRAs was analyzed in the Expasy server to obtain the theoretical isoelectric point (PI) and molecular weight (MW).

**Gene structure and motif analysis of TaRRAs**

The gene structure of TaRRAs was constructed by the gene structure display server (GSDS 2.0) using the coding sequence (CDS) and corresponding genomic sequence retrieved from the EnsemblPlants database. Conserved motifs of TaRRAs were predicted using the Multiple Em for Motif Elicitation (MEME 5.4.1), with the following parameters: maximum number of 10 motifs and optimum motif widths of 6-50 residues.

**Collinearity relationship of TaRRAs**

The wheat genomic sequence and genome annotation files downloaded from the EnsemblPlants database were used to generate a graph of chromosomal location and collinearity relationship of TaRRAs by TBtools software (Chen et al., 2020). The synteny relationship of RRs between wheat and rice was constructed using the Dual Systeny Plot for MCscanX.

**Cis-acting elements analysis of TaRRAs**

The promoter region, 1,500 bp upstream of the initiation code (ATG), of all of the TaRRAs, was obtained from the EnsemblPlants database and the cis-acting regulatory elements were predicted by PlantCARE.

**Gene expression analysis of TaRRAs**

The expression data in various tissues were downloaded from the WheatOmics 1.0 (Ma et al., 2021). The transcripts per million (TPM) values were used to create a heat map by using Heatmap (see text footnote 8).

**Results**

**Identification and classification of TaRRA genes in wheat**

The wheat-specific HMM file for the response regulator receiver domain was aligned with the whole protein sequences in wheat, and 151 non-redundant TaRR genes in wheat were identified after receiver domain confirmation. An unrooted phylogenetic tree was generated by using the conserved receiver domain and incorporating the well-established family members from Arabidopsis and rice for the subfamily classification of TaRRs (Figure 1; Heyl et al., 2013). To confirm the subfamily classification, the protein sequences of all TaRRs were further analyzed for conserved domains, including the receiver domain, Myb-like DNA binding domain of RRbs, and CCT domain of PRRs. Among the 151 TaRR genes, there were 20 TaRRAs, 71 TaRRBs, 43 TaRRCs, and 17 TaPRRs (Supplementary Table 2), and each type of TaRR gene in wheat was found to be more abundant than the corresponding type of RR genes in Arabidopsis and rice (Table 1).

Further phylogenetic analysis using the full-length protein sequence of type-A RRs in wheat clustered the 20 TaRRAs into eight homologous groups (Figure 2A), which were named TaRRA1 to TaRRA8. TaRRA1, 2, 3, 6, and 7 had three orthologous genes (TaRRA1-A/B/D, TaRRA2-A/B/D, TaRRA3-A/B/D, TaRRA6-A/B/D, and TaRRA7-A/B/D), while TaRRA4 and 8 contained two orthologous genes (TaRRA4-B/D and TaRRA8-A/D) and TaRRA5 possessed only one gene copy (TaRRA5-B). All the proteins encoded by
TaRRA genes varied from 108 to 269 amino acids with predicted molecular weights (MW) ranging from 12.22 to 28.89 kDa and the isoelectric points (pI) ranging from 5.05 to 8.48 (Table 2).

Gene structure and conserved motifs of TaRRAs

To gain further insights into the TaRRA gene members, we surveyed the gene structure and conserved motifs of each TaRRA. Although the lengths of genomic DNA varied from 859 to 3,709 bp in different TaRRAs, orthologous genes at each TaRRA locus usually had similar genomic DNA lengths. For instance, genomic DNA length of TaRRA7-A, TaRRA7-B, and TaRRA7-D was 888, 859, and 932 bp, respectively, while genomic DNA length of TaRRA4-B and TaRRA4-D was 3,709 and 3,604 bp, respectively (Figure 2B). Additionally, the number of exons, ranging from 2 to 6 in different TaRRAs, was generally the same in orthologous genes at each TaRRA locus. For example, TaRRA3-A, TaRRA3-B, and TaRRA3-D all harbored 2 exons, while TaRRA1-A, TaRRA1-B, and TaRRA1-D all contained 6 exons (Figure 2B and Table 2).

Using the MEME tool to predict the conserved protein motifs of the TaRRA family, 10 conserved motifs were identified (Figure 2C and Supplementary Table 3). Except for TaRRA5-B
TABLE 1 Summary of the RR superfamily in wheat, Arabidopsis, and rice.

| Classification | Wheat | Arabidopsis | Rice |
|----------------|-------|-------------|------|
| RRA            | 20    | 10          | 13   |
| RRB            | 71    | 14          | 16   |
| RRC            | 43    | 2           | 2    |
| PRR            | 17    | 5           | 5    |
| RR (for potential new clades) | 0 | 1 | 0 |
| Total          | 151   | 32          | 36   |

and TaRRA7-A, all TaRRAs contained motif 1, motif 2, motif 3, and motif 4. Additionally, TaRRA1-A, TaRRA1-B, and TaRRA1-D also possessed motif 5 and motif 8 (both unique in this group). TaRRA3-A, TaRRA3-B, and TaRRA3-D also had motif 6, while TaRRA4-B and TaRRA4-D had an added motif 9. TaRRA6-A, TaRRA6-B, and TaRRA6-D additionally contained motif 6, motif 7 (unique in this group), and motif 9. Likewise, TaRRA8-A and TaRRA8-D had motif 10 (unique in this group). In addition, all TaRRAs contained the highly conserved Lys and two Asp residues (D-D-K) in the receiver domain, except for TaRRA5-B and TaRRA7-A, which lacked the Lys and the first Asp respectively (Supplementary Figure 1). It is worth mentioning that all the 20 TaRRAs contained the predicted Asp phosphorylation site (the second Asp in the conserved D-D-K motif), which was embedded in a conserved TDY sequence. The above results indicate that orthologous TaRRA genes in the A, B, and D wheat subgenomes were usually similar in gene structure and encoding protein motifs, suggesting that TaRRA genes were conserved during evolution.

### Chromosomal distribution and synteny analysis of TaRRAs

Chromosomal localization analysis showed that the 20 TaRRA genes were unevenly distributed on 12 of the 21 wheat chromosomes (Figure 3), with the number of TaRRA genes on each chromosome ranging from 1 (3B, 3D, 4B, 5A, 5B, 5D, and 6B) to 3 (2A, 2B, and 2D). Chromosomal group II harbored 9 (45.0%) TaRRA genes, the largest number, followed by chromosomal groups VI, V, III, and IV, which contained 5 (25.0%), 3 (15.0%), 2 (10.0%), and 1 (5.0%) TaRRA genes, respectively (Figure 3). Whereas, there was no TaRRA
gene located in the rest two chromosomal groups (I and VII). Collinear relationship displayed the homology between TaRRAs (Figure 3), which was consistent with the phylogenetic analysis (Figure 2).

Syntenic analysis between the wheat and rice was conducted and 30 orthologous RRA gene pairs were found (Figure 4 and Supplementary Table 4), with a close similarity to the phylogenetic analysis (Figure 1), indicating that these syntenic gene pairs were relatively conserved during the evolution of gramineous species.

**Prediction of Cis-acting regulatory elements in the promoter of TaRRAs**

The cis-acting elements in the promoter region play important roles in gene transcription regulation. The RRAs are cytokinin response genes that are targets of RRB transcription factors in Arabidopsis (To et al., 2004). Therefore, the 1,500 bp DNA sequence upstream of TaRRAs was analyzed for BA-dependent (+BA) and BA-independent (-BA) RRB binding cis-acting elements (Figure 5A and Supplementary Table 5; Xie et al., 2018). Both the BA-dependent and BA-independent RRB binding cis-acting elements were distributed widely throughout all of the TaRRA genes, suggesting that transcription of TaRRAs was probably regulated in part by RRBS.

To fully understand the potential role of TaRRAs, the 1,500 bp promoter region of TaRRAs was further analyzed in the PlantCARE database for more cis-acting elements. A total of 43 types of cis-acting elements with known functions were identified in the TaRRAs promoter region, which were divided into three different categories, i.e., growth and development response elements, biotic/abiotic stress response elements, and phytohormone response elements (Figure 5B and Supplementary Tables 6, 7). Among the growth and development response cis-elements, CAAT-box (common cis-acting element in promoter and enhancer regions) and TATA-box (core promoter element around -30 of transcription start) were highly enriched in all the TaRRA promoters. In addition, O2-site involved in zein metabolism regulation, RY-element involved in seed-specific regulation, and CAT-box related to meristem expression were identified in some of the TaRRA promoters. Among the biotic/abiotic stress response cis-elements, the proportion of light-response elements was large, including 19 cis-regulatory factors, such as G-box, Sp1, and TCCCG-motif. Additionally, ARE and GC-motif involved in anaerobic induction and LTR and MBS elements involved in low-temperature and drought responsiveness, respectively, were found in several TaRRA promoters. In the phytohormone response category, ABRE in ABA response and CGTCA-motif and TGACG-motif in MeJA response were distributed in most of the TaRRA promoters. Moreover, GARE-motif, P-box, and TATC-box implicated in gibberellin response, AuxRR-core and TGA-element in auxin response, and TCA-element in salicylic acid response were also distributed in several TaRRA promoters. The presence of different numbers and types of cis-acting

### Table 2: Information of the TaRRA gene members in wheat.

| Gene name | ID         | Chromosome location             | Exon | cDNA (bp) | Protein (aa) | MW (kDa) | PI |
|-----------|------------|-------------------------------|------|-----------|-------------|----------|----|
| TaRRA1-A  | TraesCS2A02G309700.1 | 2A:532865797-532867753 | 6    | 699       | 232         | 25.49    | 6.74|
| TaRRA1-B  | TraesCS2B02G326800.2 | 2B:467870720-467873343 | 6    | 1,305     | 220         | 24.09    | 8.48|
| TaRRA1-D  | TraesCS2D02G308000.1 | 2D:39498248-394980077    | 6    | 1,276     | 220         | 24.06    | 8.48|
| TaRRA2-A  | TraesCS2A02G377900.1 | 2A:620212372-620213357    | 5    | 728       | 135         | 14.72    | 7.77|
| TaRRA2-B  | TraesCS2B02G394900.1 | 2B:559553430-559554782    | 5    | 768       | 135         | 14.72    | 7.77|
| TaRRA2-D  | TraesCS2D02G374300.1 | 2D:477627271-477628424    | 5    | 735       | 135         | 14.72    | 7.77|
| TaRRA3-A  | TraesCS2A02G551100.1 | 2A:758102501-758103826     | 2    | 1,210     | 176         | 19.12    | 5.90|
| TaRRA3-B  | TraesCS2B02G582300.1 | 2B:769949352-769950767     | 2    | 1,293     | 172         | 18.72    | 5.50|
| TaRRA3-D  | TraesCS2D02G552200.1 | 2D:627582838-627583982     | 2    | 1,061     | 176         | 19.12    | 5.90|
| TaRRA4-A  | TraesCS4B02G586600.1 | 3B:784040014-784043723     | 5    | 1,616     | 240         | 25.90    | 5.33|
| TaRRA4-B  | TraesCS4D02G494600.1 | 3D:587200633-587204237     | 4    | 2,013     | 269         | 28.89    | 5.88|
| TaRRA4-D  | TraesCS4B02G178900.1 | 4B:392001195-392004069     | 6    | 1,248     | 141         | 15.36    | 5.55|
| TaRRA5-A  | TraesCS5A02G131600.2 | 5A:296468274-296469739     | 5    | 717       | 238         | 26.24    | 5.61|
| TaRRA5-B  | TraesCS5B02G132900.1 | 5B:247665062-247666984     | 5    | 1,116     | 232         | 25.36    | 5.14|
| TaRRA5-D  | TraesCS5D02G140200.1 | 5D:223887585-223889509     | 5    | 1,088     | 231         | 25.36    | 5.05|
| TaRRA7-A  | TraesCS6A02G221800.1 | 6A:412848604-412849492     | 4    | 493       | 108         | 12.22    | 6.58|
| TaRRA7-B  | TraesCS6B02G259700.1 | 6B:469707108-469707967     | 5    | 451       | 143         | 15.39    | 7.88|
| TaRRA7-D  | TraesCS6D02G213700.2 | 6D:303981610-303982542     | 5    | 447       | 148         | 15.91    | 5.85|
| TaRRA8-A  | TraesCS6A02G164600.1 | 6A:615462243-615464213     | 3    | 727       | 183         | 20.10    | 6.31|
| TaRRA8-B  | TraesCS6B02G416000.1 | 6B:470632933-470634909     | 3    | 585       | 194         | 21.29    | 8.45|
elements in TaRRA promoters indicates that these genes may be involved in different regulatory mechanisms.

**Expression pattern of TaRRAs in different tissues and response to cytokinin**

To characterize the expression profiles of the TaRRA gene family, we analyzed the RNA-seq data downloaded from the WheatOmics 1.0 (Ma et al., 2021). Notably, TaRRA2-A/B/D and TaRRA7-A/B/D were hardly detected in any of the tissues tested, while other TaRRAs showed relatively high expression in the root, except for TaRRA1-A/B/D with higher expression in the stem (Figure 6A).

Since most of the RRAs are rapidly induced by exogenous cytokinin in monocots and dicots (D’Agostino et al., 2000; Jain et al., 2006), we investigated the expression profiles of TaRRAs in response to cytokinin treatment by qRT-PCR (Figure 6B). In the root, 10 out of 13 detectable TaRRAs were up-regulated after 1 h BA treatment and displayed an increasing expression trend afterward. Most of the TaRRAs reached maximal induction...
at 12 h, except for TaRRA5-B and TaRRA6-A, which were maximally induced at 6 h, and TaRRA1-B, TaRRA4-D, and TaRRA8-D, which were maximally induced at 24 h. Although all the 13 detectable TaRRAs were cytokinin-induced, their fold-change varied greatly after BA treatment, ranging from 2.6 (TaRRA5-B, 6 h) to 47.3 (TaRRA1-D, 12 h) times compared with the control (0 h). It is worth noting that expression levels of orthologous genes at each TaRRA locus could differ significantly after BA treatment. For instance, TaRRA1-D was up-regulated by 47.3 times at 12 h, whereas TaRRA1-A was up-regulated by 5.5 times. In the leaf, 9 out of 13 detectable TaRRAs displayed an obvious increase in the transcription level after 6 h BA treatment and showed maximal induction at 12 h. However, there was no increase in the transcription levels of TaRRA5-B and TaRRA8-A, and a decrease was observed in the transcription levels of TaRRA1-A and TaRRA8-D. The above results showed that most TaRRAs responded more rapidly and strongly to BA treatment in the root than that in the leaf. However, we could hardly detect the expression of TaRRA2-A/B/D and TaRRA7-A/B/D by qRT-PCR, which was consistent with previous RNA-seq data (Figure 6A).

**Expression pattern of TaRRAs under different stresses**

To further evaluate the potential function of TaRRAs in response to abiotic stress, the TaRRA gene expression patterns were analyzed by qRT-PCR under drought, salt, cold, and heat stress treatments (Figures 7, 8). The results showed that under drought stress, the expression levels of TaRRA1-B, TaRRA3-B, TaRRA3-D, TaRRA4-D, and TaRRA8-A decreased continuously from 0 to 6 h and later increased marginally at 12 and 24 h (Figure 7A). TaRRA8-D was exclusively up-regulated by drought stress, while the expression of the rest of the TaRRAs showed insignificant changes under drought stress. Under salt stress, the expression levels of most TaRRAs were up-regulated at least at one time point (Figure 7B). Notably, TaRRA1-D, TaRRA3-D, TaRRA6-B, and TaRRA6-D were induced at each time point compared with the control (0 h). Inversely, TaRRA5-B was gradually down-regulated by salt stress. Under cold stress, the expression of 8 TaRRAs increased after 6 h treatment, among which the expression of TaRRA1-D, TaRRA4-B, TaRRA6-A, TaRRA6-B, and TaRRA6-D continued to increase till 12 h,
FIGURE 6
Expression analysis of TaRRA genes. (A) Expression profiles of TaRRA genes in various tissues. The RNA-sequence data was obtained from leaf, root, stem, spike, and grain of Chinese Spring (International Wheat Genome Sequencing Consortium [IWGSC], 2014). (B) Quantitative RT-PCR analysis of TaRRA genes in response to cytokinin treatment. Leaves and roots of 10-day-old seedlings were sampled after 50 µM 6-BA treatment for 0, 1, 3, 6, 12, and 24 h. The data are given as means ± SE of three biological replicates.

whereas the expression of TaRRA3-B, TaRRA3-D, and TaRRA8-D was reduced subsequently (Figure 8A). Heat significantly inhibited the expression of most TaRRAs after 3 h treatment, except for that of TaRRA8-A and TaRRA8-D, which increased after 1 h treatment (Figure 8B).

It is worth noting that some orthologous genes at each TaRRA locus (TaRRA3-B/D and TaRRA6-A/B/D) exhibited similar expression patterns in response to abiotic stress, indicating that they may have similar biological functions under stress conditions. On the contrary, TaRRA8-A and TaRRA8-D showed different expression patterns under drought and cold stress, suggesting that they could play different roles under some abiotic stresses. In general, all the TaRRAs showed significant changes in response to at least one abiotic stress.

Discussion
RRAs are rapidly induced by exogenous cytokinin and are thus considered to be primary cytokinin response genes (D’Agostino et al., 2000; Jain et al., 2006). The RRA gene family, a subfamily of the RR gene family, is relatively small in higher plants, with only 10, 13, 21, and 18 members in Arabidopsis, rice, maize, and soybean, respectively (Mochida et al., 2010; Chu et al., 2011; Heyl et al., 2013). In this study, we performed a systematic genome-wide analysis of the wheat RRA gene family by combining two different approaches. First, we performed phylogenetic analysis by using the conserved receiver domain of 151 TaRRs, and simultaneously adding the well-established family members from Arabidopsis and rice into the analysis (Figure 1; Heyl et al., 2013). Second, we analyzed the conserved domains of all the TaRRs to further confirm whether they contain a Myb-like DNA binding domain or a CCT domain in addition to the receiver domain. We used the protein sequence of the longest transcript of each TaRR for domain analysis to avoid domain missing of the shorter transcript. According to the above method, we identified 20 RRAs, 71 RRBs, 43 RCCs, and 17 PRRs from the wheat reference genome (Table 1). However, in a previous study, 41 RRAs, 2 RRBs, and 2 PRRs have been identified in wheat (Gahlaut et al., 2014). Given that RRAs only contain a receiver domain, while RRBs contain an additional Myb-like DNA binding domain and PRRs have an extra CCT domain, some truncated RRBs and PRRs may be identified as RRAs due to the earlier incomplete wheat reference genome. In recent years, great progress has been made in genome sequencing, assembly, and annotation of wheat (International Wheat Genome Sequencing Consortium [IWGSC], 2018; Alonge et al., 2020; Zhu et al., 2021), which provides a high-quality reference genome for the study of the TaRRA gene family. The 20 TaRRA genes belonging to 8 homologous groups were unequally distributed on 12 wheat chromosomes (Figure 3). As wheat is an allohexaploid (AABBDD), most TaRRA genes had homologs in the A, B, and D subgenomes due to polyploidization, sharing similar gene structures and protein motifs within the homologous group (Figure 2).
FIGURE 7
Quantitative RT-PCR analysis of TaRRA genes under drought and salt stress. Roots of 10-day-old seedlings were sampled after 0, 1, 3, 6, 12, and 24 h drought stress (A) and salt stress (B). The data are given as means ± SE of three biological replicates.
FIGURE 8
Quantitative RT-PCR analysis of TaRRA genes under cold and heat stress. Roots of 10-day-old seedlings were sampled after 0, 1, 3, 6, 12, and 24 h cold stress (A) and heat stress (B). The data are given as means ± SE of three biological replicates.
Most of the RRAs are rapidly induced by exogenous cytokinin in plants (D’Agostino et al., 2000; Asakura et al., 2003; Jain et al., 2006). Consistent with previous results, the transcription levels of most detectable TaRRAs displayed an obvious increase after BA treatment in both the root and leaf (Figure 6B). However, the transcripts of most TaRRAs reached a maximal induction at 12 h cytokinin treatment, while most RRAs in Arabidopsis and rice showed maximal induction within 1 h cytokinin treatment and then gradually declined (D’Agostino et al., 2000; Jain et al., 2006). Although TaRRA1-A, TaRRA5-B, TaRRA8-A, and TaRRA8-D were induced by cytokinin in the root, their transcription levels were unchanged or even decreased in the leaf after cytokinin treatment (Figure 6B), indicating their potential function differentiation between root and leaf. Similarly, there is no significant change in the transcript abundance of a few RRAs in Arabidopsis and rice following cytokinin treatment, including ARR8, ARR9, OsRR3, and OsRR8 (D’Agostino et al., 2000; Jain et al., 2006). The rapid induction of RRAs by exogenous cytokinin has been shown to mediate a feedback mechanism, probably by competing with RRBS for the phosphotransfer from HPs. Phosphorylation of RRAs increases their protein stability (To et al., 2007), whereas phosphorylation of RRBS enables them to bind to DNA, thus initiating transcription of downstream targets (Kim et al., 2006; Zubo et al., 2017). Double and higher-order type-A arr mutants, rather than the single arr mutants, show increasing sensitivity to cytokinin, indicating that RRAs act as negative regulators of cytokinin signaling with partially redundant functions (To et al., 2007).

Cytokinin signaling components are widely involved in plant response to abiotic stress. A series of Arabidopsis mutants, including ahk2,3, ahk2,3,5, and arr1,10,12, show significantly increased drought and salt tolerance, indicating that HKs, HPs, and RRAs are negative regulators of these stress responses (Tran et al., 2007; Nishiyama et al., 2013; Nguyen et al., 2016; Abdelrahman et al., 2021). In contrast, plants overexpressing ARR5 exhibit enhanced drought tolerance, suggesting that type-A ARR5 is a positive regulator of drought tolerance (Huang et al., 2018). Functional analysis of the single and double ahk mutants indicates that HKs function partially redundantly as negative regulators of the cold stress adaptation response (Jeon et al., 2010). However, type-B ARR1 is a positive factor in cold signaling, because arr1 shows reduced cold resistance, whereas ARR1 overexpression increases plant cold resistance (Jeon and Kim, 2013). Additionally, RRAs also play important roles in cold stress signaling (Jeon et al., 2010; Shi et al., 2012).

Given that the expression pattern of genes has a correlation with its function, we monitored the expression profiles of TaRRA family members under multiple stresses. Drought stress reduced the expression of TaRRA1-B, TaRRA3-B, TaRRA3-D, TaRRA4-D, and TaRRA8-A, but induced the expression of TaRRA8-D (Figure 7A). Similarly, drought stress reduces the expression of ARR8 and ARR17, whereas induces the expression of ARR5, ARR7, and ARR15 in Arabidopsis (Kang et al., 2012). We identified 8 TaRRAs with significantly up-regulated expression under cold stress (Figure 8A), which is consistent with the cold-induced changes in ARR5, ARR6, ARR7, and ARR15 expression in Arabidopsis (Jeon et al., 2010). Furthermore, overexpression of ARR5, ARR7, and ARR15 enhances the freezing tolerance of plants, while arr5, arr6, and arr7 also lead to higher freezing tolerance (Jeon et al., 2010; Shi et al., 2012). These different results suggest the complexity of the molecular mechanism involved and hence further research is warranted. In addition, a considerable number of TaRRAs were shown to respond to salt and heat stress (Figures 7B, 8B), suggesting that they are promising regulators for salt and heat response in wheat. TaRRA3-B/D, orthologs of drought and salt positive regulator OsRR6, and TaRRA6-A/B/D, orthologs of salt negative regulator OsRR9 and OsRR10 (Supplementary Table 4), showed responses to salt, cold, and heat stress, which may be important candidate genes for genetic improvement of stress tolerance in wheat.

In conclusion, this study provided comprehensive insights into the TaRRA gene family in wheat. The systematical identification and investigation of the TaRRA gene family will inevitably contribute to further elucidation of the biological function and genetic improvement application of TaRRAs in wheat.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

HL and YJZ designed the experiments. LS, LL, JZ, MH, YLZ, YZ, XT, PW, QL, and XC performed the experiments and analyzed the data. LS and LL wrote the manuscript. All authors reviewed and approved the manuscript.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1006409/full#supplementary-material

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