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Genome-wide analyses highlight wheat Cinnamoyl-CoA reductases' potential against abiotic and biotic stresses

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Research

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ABSTRACT

Lignin is a crucial component of the cell wall, the first line of plant defense against abiotic and biotic stresses. Cinnamoyl-CoA reductase (CCR) catalyzes the first committed step for lignin monolignol synthesis. Potential roles of *CCRs* in stress responses are still elusive in bread wheat. Here, 114 wheat *CCRs* were identified from wheat genome and were categorized into seven groups. We investigated the phylogenetic relationship, conserved protein motifs, chromosome localization, and gene structure to gain insight and show the homologous relationships among these *CCRs*. Using open transcriptome data, we identified 11 ubiquitous, and 103 tissue specific expressed *CCRs* genes in wheat. Gene expression increased in groups 2, 6, and 7 at the early infection stage and group 1 at the late *Puccinia striiformis f. sp. tritici* infection stage. *Fusarium graminearum* infection significantly induced the group 3 *CCR* genes. Finally, three *CCR* genes significantly increased at the late stage of drought treatment with significantly more lignin contents. This study provided genome-wide identification and overall transcriptome insights of wheat *CCRs*, highlighting their potential role in wheat stress resistance.

Keywords: Wheat; lignin; *Cinnamoyl-CoA reductase*; biotic stress; abiotic stress; drought

1. INTRODUCTION

Wheat (*Triticum aestivum L.*) roughly provides one -fifth of the proteins and calories for human beings [1]. Now, over 700 million tons of wheat are produced annually worldwide (http:// www.fao.org/faostat/en/). However, the yield and quality of wheat are under severe threat from extreme environmental stresses, both biotic and abiotic.

The abiotic stresses, including cold, heat, drought, and salinity, all significantly affect the yield of wheat and weaken global food security [2-4]. The total chlorophyll concentration decreased under low temperature, and the maximum quantum yield of the PSII (*Fv*/*Fm*) was significantly lower than control plants [5]. At high temperatures, the decrease of detoxifying enzymes leads to the accumulation of reactive oxygen species, which accompanied the thylakoid membrane lipid composition and cell organelle damages in wheat leaf cells to reduce the photosynthetic rate [6]. The drought stress reduced water potential and chlorophyll contents in flag leaves, decreased the number of cells in the endosperm, reduced the grain-filling rate and grain weight of wheat [7].

Wheat is widely grown in the world with different environments and faces the threat of a wide range of pests and diseases, including foliar and stem diseases, seed transmitted diseases, and soilborne diseases [8]. Between 2000 and 2012, stripe rust expanded in 35 (55%) of the 64 countries, which constituted 76.8% of the world's wheat crop in 2012 [9]. About 5.47 million tonnes of wheat were estimated to be lost to the pathogen each year, equivalent to a loss of US$979 million per year [10]. *Fusarium graminearum* (sexual stage: *Gibberella zeae*) causes the devastating head blight or scab disease on wheat and causes significant crop and quality losses with the trichothecene mycotoxins (e.g., deoxynivalenol), which constitutes a significant threat to human and animal health [11]. Change of lignin content provide the first line of defense in plants against the pathogenic stress [12].

Lignin, one of the major compositions in the cell wall, is a phenolic polymer derived mainly from hydroxycinnamyl alcohols and is ubiquitously present in plants [13]. Lignin plays a critical role in the morphological formation of plants and works as the structural basis for structural rigidity for tracheophytes to stand upright and strengthens water-conducting tracheary elements to withstand the negative pressure generated during transpiration [14]. Lignin reinforces the cell walls, facilitates water transport, and acts as a physical barrier to pathogens [15]. Both lignin content and syringyl lignin correlated to tobacco (*Nicotiana tabacum* L.)'s resistance against *Phytophthora parasitica var. nicotianae* and *Pseudomonas solanacearum* [16]. 4-coumaroyl CoA is the central metabolite of the phenylpropanoids pathway and works as either the direct precursor for flavonoid or para-hydroxy-phenyl lignin biosynthesis or is fed into the production of increasingly methoxylated guaiacyl (G)- and syringyl (S)-monolignols [17].

Cinnamoyl-CoA reductase (CCR) catalyzes the first committed step for lignin monolignol synthesis [18]. Together with cinnamyl alcohol dehydrogenase (CAD), CCR converts p-coumaroyl-CoA, feruloyl-CoA, and sinapoyl-CoA into p-coumaryl,

coniferyl, and sinapyl alcohols, the monolignol precursors for H, G, and S lignin [19]. Based on the expression profiles, *CCR* contains two subfamilies: one devoted to developmental lignification and the other involved in synthesizing defense-related compounds [20]. In rice (*Oryza sativa* L.), a sphingolipid elicitor induced *OsCCR1*, whose encoded protein was bound and activated by OsRac1, one of the Rac/Rop family small GTPases to accumulated lignin and ROS [21]. The expressions of *OsCCR17* and *OsCCR21* were induced in response to biotic and abiotic stresses, such as *Magnaporthe grisea* and *Xanthomonas oryzae pv. oryzae* (*Xoo*) infections, UV-irradiation, and high salinity, suggesting a role of these genes in rice defense-related processes [22]. Under Polyethylene glycol (PEG) stress, CCR1 increased in foxtail millet (*Setaria italica* L.) during phase I but decreased in phase II and III under PEG stress. Simultaneously, a higher amount of cinnamic acid accumulated in germinating seeds under PEG than compared to control treatment, suggesting phenylpropanoid's involvement in this process [23]. In the developing seedlings of *Leucaena leucocephala*, an increase in lignification was observed in mannitol-treated stems and the corresponding CCR protein accumulated at a level higher than control [24]. Compared with the extensive studies of CCR's biological functions in response to biotic and abiotic stresses in model plants such as *Arabidopsis* and rice, little progress was made to elucidate the biological function of *CCR* genes in wheat.

Here we used the current genomic information and analyzed the *CCR* gene family in wheat. We investigated the location, gene structure, *cis*-elements in the promoters, and *CCR* family genes' expression during wheat development and response to different stresses. Several *CCR* genes showed a strong induction upon pathogen infection, indicating their potential involvement in disease resistance. Moreover, we identified several *CCR* genes responsible for tolerance to osmotic stress simulated by PEG treatment. These results provided useful information for the functional study of the *CCR* gene in wheat against environmental stresses.

2. MATERIALS AND METHODS

2.1. Genome-wide identification of CCR family genes in wheat

The genome data of wheat was downloaded from the Ensemble Plants database (http://plants.ensembl.org/Triticum_aestivum/Info/Index) [25]. The genes annotated as *CCR* were retrieved by searching the keyword in genome annotation and hidden Markov model (HMM) profile using HMMER3.0 following previous work [26]. The cDNA sequence, coding sequence (CD), peptide sequence, and genomic DNA (gDNA) sequence of all *CCR* were downloaded from Ensemble Plants. The resulting candidate sequences were submitted to the NCBI, Pfam (Protein family, http://xfam.org/), and SMART (Simple Modular Architecture Research Tool, http://smart.emblheidelberg. de/) websites for validation and removing redundant sequences. The equivalent electric point (pI) and molecular weight (Mw) of each protein were obtained from the ExPASy website (http://web.expasy.org/compute_pi/). Each gene's subcellular location was predicted using Target1.1 Server, ChloroP1.1 Server, and SignalP5.0 [27]. The gene structure map was drawn based on the CDs and gDNA sequences of each gene to the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) to for intron-exon

pattern and the number of introns for each gene.

2.2. Phylogenetic and conserved domain analyses

The amino acid sequences of 114 genes were aligned in the MEGA X software for sequence alignment [28]. The sequences were aligned in ClustalW to generate a mas file. A phylogenetic tree was constructed using the maximum likelihood estimation method in the MEGA X software. The boots-strap repetition number was set 1,000 times in the Poisson model to obtain the phylogenetic tree [28].

The amino acid sequences of wheat CCRs were aligned in the MEME website (http://meme-suite.org/) to predict the conserved domains by using the classic motif discovery mode. Novel conserved domains were filtered according to indicators such as E-value and sites for their detailed signature matches [29].

2.3 Chromosomal localization and the homologous *CCRs* Circos map

Each wheat chromosome's length and the start and end position of each *CCR* gene were from http://plants.ensembl.org/Triticum_aestivum/Info/Index. The information of *CCR* homoeologue pairs was extracted from the above website. The above homoeologue pairs information was uploaded into the Micro-Synteny View mode on the Tbtools website (https://github.com/CJ-Chen/TBtools/releases). The chromosomal localization of *CCRs* was created in the Advanced Circos tool on the Tbtools website.

2.4. Promoter element analysis

The 2.5 kb untranslated region (UTR) sequence upstream of the initiation codon (ATG) of each *CCR* was retrieved from the Ensemble Plants database to predict *cis*-acting regulatory elements (*cis*-elements). *Cis*-acting elements in the promoter sequences were predicted following previous work in the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[30]. The number of stress-related *cis*-acting elements predicted with each *CCR* gene was sorted into a table to draw the promoter element analysis map in the TB tools software (http://www.tbtools.com/).

2.5. Tissue and stress responsible expression characteristics of *CCRs*

Expression data of *CCRs* were downloaded from the wheat eFP website (http://bar.utoronto.ca/efp_wheat/cgi-bin/efpWeb.cgi) [31]. The expression information for each *CCR* in 51 tissue samples from spring wheat (*Triticum aestivum*) cv. Azhumaya [31] was transformed into a logarithm of the original TPM value into a combined table to draw a heat map in Graphpad Prism 8 (https://www.graphpad.com/scientific-software /prism/). RNA-Seq data of abiotic and biotic stresses were downloaded from the wheat expression website (http://www.wheat-expression.com/). Expression data of 114 *CCRs* were extracted in the form of original TPM values and then converted to logarithmic values to draw heat maps in Graphpad Prism 8 (https://www.graphpad.com/). The images were colored in Adobe Illustrator (https://www.adobe.com/). The paired *t*-test was used to calculate further the significance between the control sample and the treated sample in each experiment.

2.6. Drought stress treatment analysis

Spring wheat (*Triticum aestivum* L.) cultivar Fielder was used in this study. The wheat seeds were sterilized with 75% ethanol for 5 minutes, then with 1% hydrochlorous acid for 15 minutes, and finally washed three times with ddH_2O. The treated seeds were spread on a crepe paper rinsed with tap water, kept in a refrigerator at 4°C overnight, and removed out to room temperature for 24h. Germinated seeds were sown in a black culture box filled with tap water. The culture box is enclosed in a growth chamber at 25°C with 80% humidity with a 16-hour light/8-hour dark-light cycle. Five seedlings at a leaf and a heart stage were treated with 20% PEG6000 for 1, 6, 12, 24 and 48 hours. The seedlings grown without PEG6000 were used as controls. The RNA was extracted from roots, stems, and leaves with Trizol reagent for reverse transcript Hieff™ qPCR SYBR™ Green Master Mix (Shanghai Yeasen Biotechnology, Shanghai, China) in CFX 96-Realtime system (BIORAD, Singapore) according to the user manual. The corresponding primers were designed, and quantitative analyses were performed by qRT-PCR [32].

2.7. Determination of Lignin Content

In the lignin determination experiment, the first leaf of 4 wheat seedlings was collected and mixed as one biological replicate. Each sample has four biological replicates, each with five-technique repeats. Samples were frozen in the liquid nitrogen and ground into a fine powder in a tissue grinder at 55 Hz for 30 s for six times. The samples were washed with methanol three times following 1×PBS, 1% Triton X-100, 1 M NaCl, ddH_2O, and acetone, three times each to remove soluble metabolites. The samples were kept overnight in a fume hood to let the acetone volatilize completely for alcohol-insoluble and protein-free cell walls (AIR) [33].

2.0±0.2 mg of each AIR sample was weighed into 2 mL centrifuge tubes in five biological repeats. The samples were mixed with 125μL 25% acetyl bromide (diluted with glacial acetic acid), treated at 70 °C for 35 min in a metal bath, and cooled to room temperature in a refrigerator at 4 °C. Then, 400 μL 2 M NaOH and 70 μL 0.5 M fresh hydroxylamine hydrochloride were sequentially added into the samples and tapped to mix well. The samples were adjusted to 2 ml by adding glacial acetic acid and centrifuged at 15,000rpm for 15 minutes. 200 μL of supernatant was carefully transferred into a 96-well UV detection plate to measure the absorbance with a microplate reader at a wavelength of 280 nm [34]. The lignin content measured by this method (the proportion coefficient of Gramineae is 17.75):

Lignin content (%) = [absorbance*constant volume (mL)]/[17.75*0.539*100% masses (mg)]

3. RESULTS

3.1. Phylogenetic relationship and conserved motifs of wheat CCRs

CCR family genes encoded in the wheat cultivar Chinese Spring (*Triticum aestivum* L.; ABD genome) were mined by searching publicly available databases and a hidden Markov model (HMM) search based on Pfam homologs. In total, 114 non-redundant *CCRs* were identified in the latest genome data of wheat. The putative wheat CCR proteins have a relatively narrow range in length,

from 123 to 427 amino acids. Wheat CCR proteins have theoretical isoelectric points (pI) ranging from 5 to 9.5. A subcellular localization analysis revealed that 65 CCRs localized in the cytoplasm and 37 in the Golgi apparatus, while remaining in the chloroplast (5), mitochondrion (5), vacuole (4), plasma membrane (2, pI < 6), cell wall (2, pI > 8.7), and peroxisome (1) (Table S1).

To further evaluate the phylogenetic relationships, all the identified wheat CCRs were aligned to construct the wheat *CCR* gene family's phylogenetic tree. In the phylogenetic analysis, wheat CCRs clustered into seven groups, with 6 to 25 genes in each subfamily (Fig. 1a). Chromosomal mapping revealed that group 7 *CCRs* were located on group 5 homeologoue chromosome, while group 6 *CCRs* group 7 homeologoue chromosomes of wheat (Table S1). Group 4 had an evolutionary relationship relatively distant from the other six groups, and most of them located on group 6 homeologoue chromosomes. More variations in the amino acid sequences in this group suggested that they might have diverged earlier in evolution or recently evolved with diverse biological functions.

We further analyzed conserved motifs in the CCRs based on their peptide sequences (Table S2). Three motifs were close to the enzyme's catalytic center and reported to be critical for the biological activity of CCR (Fig. 1b) [35]. The NWYCY domain works as the catalytic center, and the "WY" amino acids were also conserved in other species (Fig. 1b). The VTGA motif is associated with NAD(P)' s binding and essential for CCR to catalyze the reaction (Fig. 1b). The RXXXXK motif is also recognized as a conserved motif notable for the binding specificity NADP (Fig. 1b). Besides the reported motifs, three new motifs were defined in wheat CCRs, which were also presented in rice CCRs (Fig. 1c). Further experimental support is needed to validate their potential roles in the biological function.

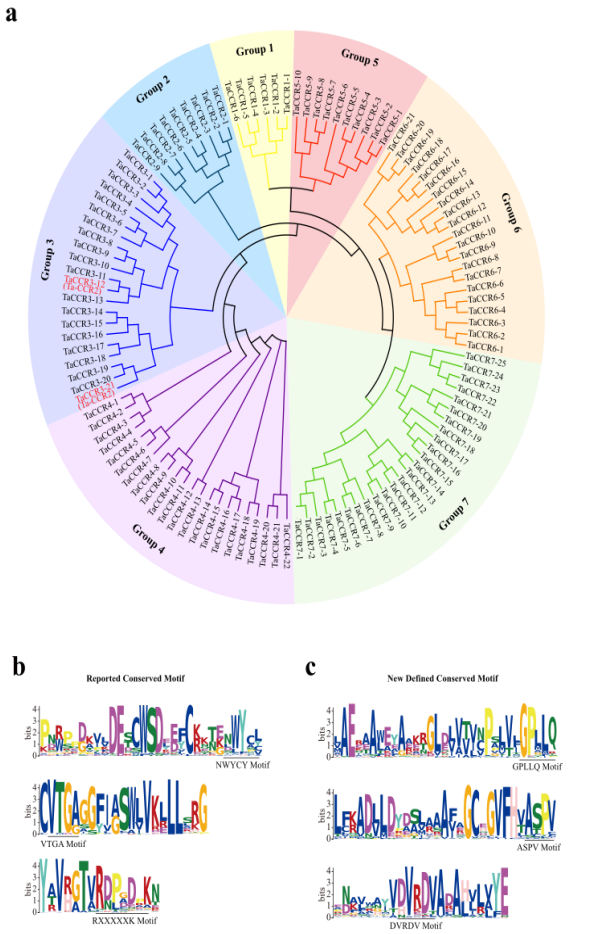

Fig. 1. Phylogenetic and conserved motif analysis of wheat CCR proteins. a, Phylogenetic relationship among wheat CCRs. b, Sequence constitution of reported NWYCY, VGTA, and RXXXXXK motifs in wheat CCRs. c Sequence constitution of three novel motifs in wheat CCRs.

3.2. Gene Structure analysis of wheat *CCRs*

Chromosome localization analysis showed that the 114 wheat *CCRs* distributed unevenly on the 21 chromosomes, with group 5 homeologoue chromosomes containing the most *CCRs* (Fig. 2). Two clusters of C*CRs* existed on chromosome 5 (5A, 5B and 5D) in large numbers as tandem repeats, and they had high homology in the phylogenetic tree (Table S1). These data suggested that the above genes could favor wheat's environmental adaptability.

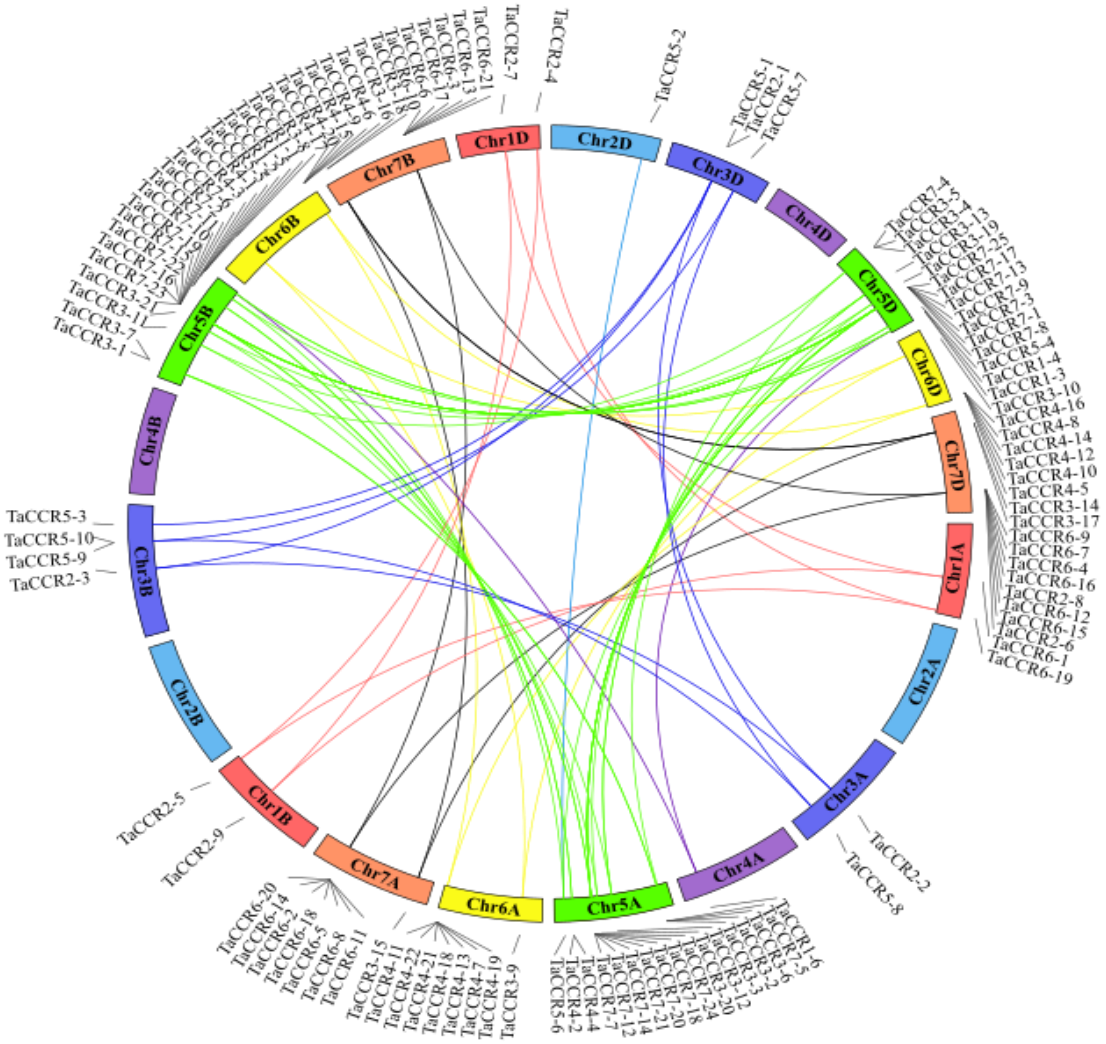

Fig. 2 Chromosomal localization and the homologous *CCRs* in wheat sub-genomes. Duplicated *CCR* genes in each homo-group are linked using lines with the corresponding color of the wheat chromosomes. The corresponding Traes ID for each *CCR* gene is listed in Table S1.

Cis-acting elements in the promoter sequences are the potential binding sites of the transcription factors that initiate transcription, which play a crucial role in regulating spatial and temporal gene expressions. We extracted the 2.5 kb upstream region of the translational start site of the 114 wheat *CCRs* to screen *cis*-regulatory elements. The *CCR* promoters contain ten potential *cis*-acting elements, including TC rich repeats (defense and stress responses), LTR (low-temperature response), TCA-element (salicylic acid, SA, response), ABRE (abscisic acid, ABA, response), CGTCA (methyl jasmonic acid, MeJA, response), TGACG (MeJA response), P-box (gibberellin, GA, response), GARE (GA response), TATC-box (GA response), MBS (MYB binding site in drought response), MBSI (MYB binding site in phenylpropanoid metabolism) and WUN (wound-responsible element) (Table S3). Based on a visual presentation of the *cis*-regulatory elements in CCRs' promoter regions, ABA and MeJA responsible elements were presented in 97.5% and 95.6% of total *CCR* promoters, suggesting their high-abundance and wide-distributions (Table S3). The SA inducible TCA-element existed in 41.2% of the *CCR* promoters, consistent with the reported involvement of lignin biosynthesis in pathogenic responses. The drought and low-temperature inducible elements, MBS and LTR, were observed in 57.8% and 55.2% of *CCR* promoters, suggesting that the *CCR* family genes could play a role in wheat response to environmental stresses.

Gene structure (exon/intron) analysis provides clues to potential gene function, organization, evolution, and divergence patterns. Thus, we mapped the exon/intron organization of wheat *CCRs*. We found that 104 wheat *CCRs* have 3 to 5 introns (Fig. 3), which were highly conserved compared to the reported introns numbers in JAZ (0-7), ZIP (1-10), and MAPKK (1-7). For the *CCRs* with 1 and 2 introns, six out of 7 were among 13.64 to 21.37 in Mw, and are very likely truncated CCRs. The three truncated *CCRs* in group 5, *TaCCR5-1*, *TaCCR5-2* and *TaCCR5-3*, formed a small cluster, indicating that they have a common origin. However, the other truncated genes in group4, *TaCCR4-19*, *TaCCR4-15* and *TaCCR4-21*, have close homologs different from each other, suggesting that they were likely to be truncated as individual events during evolution.

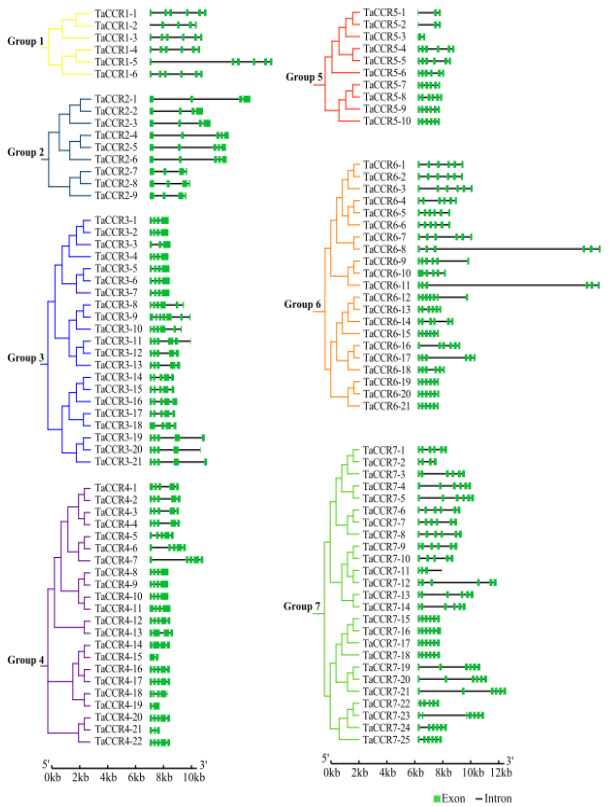

Fig. 3. Schematic gene structures of wheat *CCR* genes. The exons and introns are represented by green boxes and black horizontal lines, respectively.

3.3. The gene expression patterns of *CCRs* during wheat development

The phylogenetic relationship and motif patterns suggested that CCRs in different groups exhibit vast disparities in their binding motifs and potential affinities with substrates. The expression abundances among different tissues provide information for them to accommodate different physiological processes. We investigated the expression levels of wheat *CCRs* in over 50 wheat tissues at different developmental stages. Based on the gene expression patterns, groups 1-3, 5-7 had at least one pair of highly expressed homologs in all the tissues at the whole or most developmental stages (Fig. 4), which could play a dominant role amongst their group. Surprisingly, all the genes in group 4 had a relatively lower expression level, suggesting that they could have a minor biological function during wheat development, consistent with the significant divergence from other groups in the phylogenetic analysis.

Despite those above constitutively expressed genes, some *CCRs* only expressed in a particular tissue at a specific developmental stage (Table S4). The expression of *TaCCR4-17* and *TaCCR4-20* was very high in the root apical meristem at the three-leaf stage. Three genes in group 6, *TaCCR6-19*, *TaCCR6-20* and *TaCCR6-21*, were only expressed in the grains at soft dough to hard dough stages. The above expression pattern suggested that these genes are primarily involved in phenylpropanoid biosynthesis in the grain.

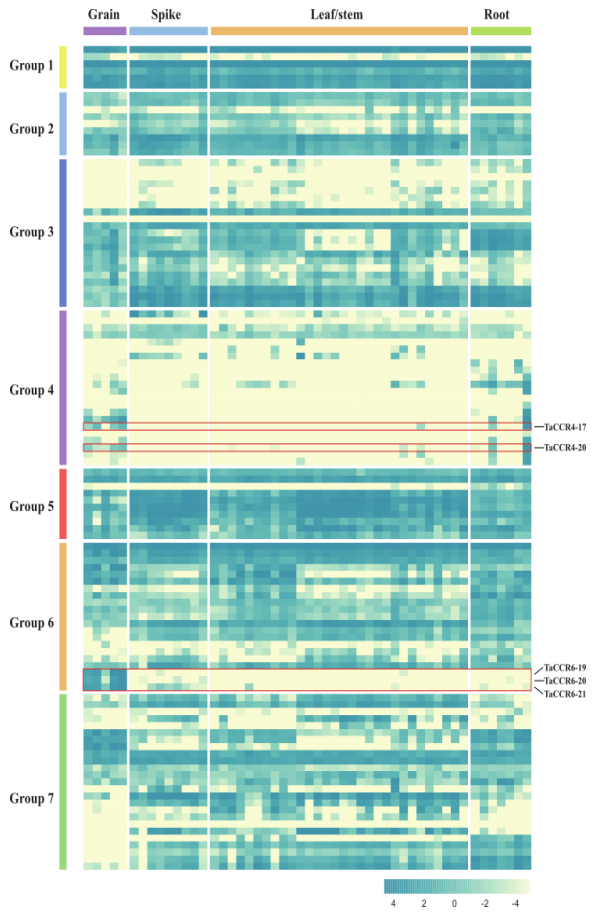

Fig. 4. Heat map of the expression profiles of *CCR*s in different tissues/developmental stages in wheat.

3.4. The gene expression patterns of *CCRs* during wheat stress responses

The phenylpropanoid pathway, including lignin and flavonoids, is often co-upregulated in stress responses [36]. We set to analyze the expression of *CCRs* for candidates involved in defense responses, including harsh environmental stresses on seedlings and divesting fungal diseases, e.g., biotrophic *Pst* on leaf and semi-biotrophic *Fusarium graminearum* on spikelet (Table S5).

We first analyzed the expression of *CCRs* in the wheat-pathogen interacting system. Upon inoculation with *Pst 87/66*, the overall expression level increased to around two folds in 1-day post-inoculation (dpi), declined from 2~5 days, and increased again in the later stage, e.g., 1.5-folds of mock at 11dpi, (Fig. 5a, Table S6). Further analyses showed that groups 2, 6, and 7 contributed to the early induction, while group 1 was dominant in the later induction (Fig. 5b). Upon inoculation with *Fusarium graminearum*, *CCRs* were induced in anthesis-stage spikelets (only using palea, lemma, and rachis) from 24 to 48 hours (Table S6). Group 3 was induced at the highest level by the infection of *Fusarium graminearum* (Fig. 5d). The induction patterns suggested that different *CCR* subgroups could play different roles to biotrophic (*Pst*) and necrotrophic (*Fusarium graminearum*) fungi at different inoculation stages.

Next, we analyzed the expression of *CCRs* during wheat responses to harsh environmental stresses. There was no significant difference from control at the whole genome level in response to drought, heat, and PEG6000 (students' pair-wise *t*-test value, $P > 0.05$) (Fig. 5e). A minor decline of some *CCRs* was also observed in a combined treatment of heat and drought treatment (Fig. 5f). In a recent meta-analysis across plant species, *CCR1* presented as a conserved gene repressed by heat [37]. Therefore, based on these transcriptome data, it would be logical to predict that *CCRs* could play a minor role in wheat response to environmental stresses.

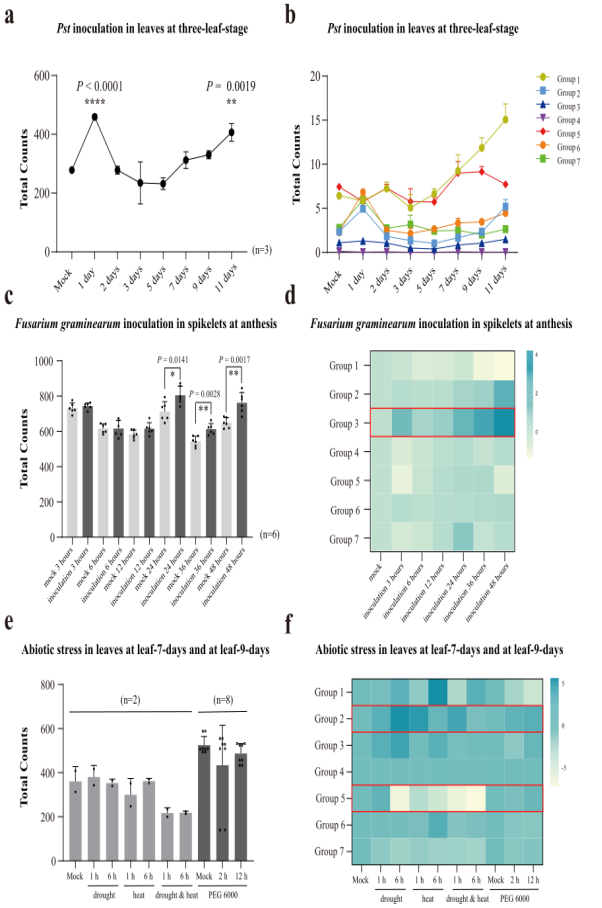

Fig. 5. The expression profiles of *CCRs* in response to abiotic and biotic stresses. a, The overall expression of *CCRs* in response to *Pst* at different inoculation stages. b, The expression of *CCR* subgroups in response to *Pst* at different inoculation stages. c, The overall expression of *CCRs* in spikes inoculated by *Fusarium graminearum* at different stages. d, The expression of *CCR* subgroups in response to *Fusarium graminearum* at different stages. e, The overall expression of *CCRs* in heat and drought treatments. f, The expression of *CCR* subgroups in response to abiotic stresses at different stages. Pair-wise students' *t*-test *P*-value, * < 0.05, ** < 0.01, **** < 0.0001. Bar = ±s.d.

3.5. Lignin content and *CCR* expression changes in wheat drought response

Lignification represents an essential evolution in adaptation to terrestrial environments for the plants and is closely related to plant drought resistance [13]. In the above transcriptome analysis, wheat *CCRs* did not significantly increase, which led to a logical inconsistency between *CCRs'* gene expression and known concepts.

First, we quantified the lignin contents in drought-stressed seedling leaves assimilated by Polyethylene glycol treatment. The short-term drought treatments, e.g., 12 and 24 hours, did not significantly affect the lignin contents of wheat seedling leaves. The variance analysis showed a significant increase in lignin content after a drought treatment for 48 hours (Fig. 6a, Supplementary Fig. 3). The above change of lignin content in the drought-treatment suggested that lignin biosynthesis and the related genes' expression represented metabolism changes later than primary signaling pathways in wheat leaves. We predicted that the treatment duration time of 1 to 6 hours could not be sufficient for *CCRs* to increase.

To test the above hypothesis, we conducted the same drought treatment and analyzed the expression of *CCRs* at different time points. The expression of *TaCCR2-9* increased at 1hour, while *TaCCR5-9* and *TaCCR5-5* decreased at 6hour upon lignin treatment in the roots (Fig. 6b). In the leaves, all the three *CCRs*, *TaCCR2-9*, *TaCCR5-9* and *TaCCR5-5*, significantly increased at 12 H (Fig. 6c).

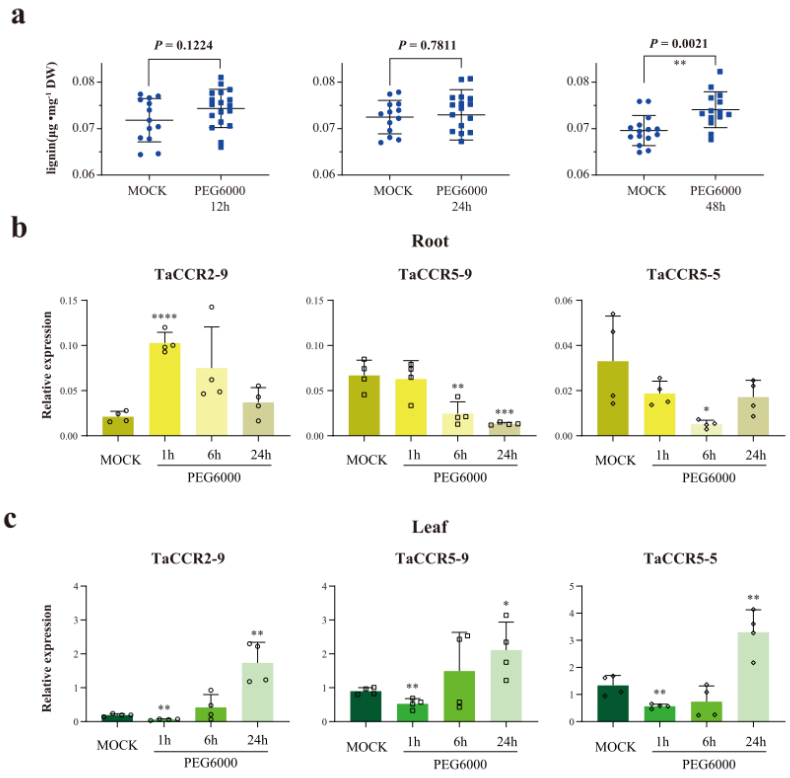

Fig. 6. The change of lignin content and expression profiles of *CCRs* in drought-treated wheat seedlings. a, The change of lignin contents in seedlings. The data represented the average of four biological repeats, each with five-technique repeats. b-c, The relative expression levels of representative *CCRs* in drought treatment in the root (b) and leaf (c). Students' *t*-test *P*-value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. n = 4.

4. DISCUSSION

CCR proteins belong to a conserved gene family in the plant kingdom to confer a vital role during plant growth and development and protect the plant from abiotic and biotic stress by synthesizing lignin to adapt to the terrestrial environment. These enzymes' functions have been widely studied, including model and crop plants, e.g., *Arabidopsis* and rice. However, fewer efforts have been devoted to investigating the function of *CCR* gene family in wheat. In this study, we identified and characterized the *CCR* gene family in wheat at the whole-genome level.

A total of 114 putative wheat *CCRs* belonging to 7 *CCR* subfamilies were obtained through a genome -wide search approach. The number of *CCRs* is far beyond those in rice and *Populus tomentosa*, which have 33 and 11 *CCRs*, respectively [38]. Gene duplication is an essential mechanism for acquiring new genes and creating genetic novelty during evolution in organisms [39]. *CCRs* existed in chromosomes 5 (5A, 5B, and 5D) in higher number (Fig. 2), representing tandem duplication, one of the two crucial gene amplification ways. Moreover, the significant increase of CCR gene number in wheat over model plants also reflects whole-genome duplication due to hexaploid (AABBDD) formed by ancient hybridizations among the ancestral wheat species [40].

The CCR gene family's functional studies were conducted in many plants to investigate the gene structure, protein structure, biochemical characters, and stress responses [41, 42]. Switchgrass (*Panicum virgatum*) contains a diverse gene family of CCR, which are phylogenetically distinct, differentially expressed, and have different biochemical properties [43]. Sorghum CCRs showed evolutionary conservation of the functional domains in a structure and phylogeny analysis but were differentially expressed in a drought treatment [44]. Previous biochemical studies provided useful information for the role of *TaCCR1* and *TaCCR2* in stem development [27, 45]. However, further investigation of *CCRs* at the whole genome level is necessary for a full view image.

The 114 wheat *CCRs* were distributed in 7 distinct subgroups based on their phylogenetic relationships. The majority of the *CCRs* fall into one of the three intron-exon models reported in rice, and each model has a similar intron length among different members in them [46]. Most of the CCRs shared the reported basic conserved motifs, e.g., NWYCY exists in 102 CCRs, as the characteristic motif to recognize CCR in the plants. Besides the reported motifs, three novel conserved motifs present in different subfamilies, indicating that they could contribute to different subfamilies' specific biochemical characters.

Large-scale prediction of promoter sequences and their contributing *cis*-acting elements are crucial for improving our fundamental understanding of gene regulation [47]. Here, wheat *CCR* promoters contain a wide array of *cis*-acting elements ranging from plant hormone (MeJA, ABA, SA, and GA) response to stress responses (cold, drought, and defense). Deleting an MBSIIG MYB *cis*-element in *EgCCR* promoters blocked the DNA-protein complex's formation in EMSA - electrophoretic mobility shift assay experiments and the transcriptional activation of the *EgCCR*

promoter[48]. A correlation was presented between some *cis*-elements in *CCR* promoters and the stress-induced gene expression in this work. Therefore, the corresponding *cis*-elements could involve regulating *CCRs* for the adaption of wheat to environmental stresses.

Lignin is critical for plants' adaption to terrestrial environments during evolution [13]. A positive relationship between lignin and drought could enhance water transport and prevent water loss, therefore promoted drought tolerance in plants [48]. In a drought stress treatment simulated by 10% PEG6000 treatment, *SiMYB56* overexpression in rice accumulated more lignin and had significantly higher root length, plant height, and biomass, which represented a drought resistance phenotype [49]. Here, three *CCRs* significantly increased at 24 hours upon drought treatment, consistent with the increase of lignin content at 48 hours. The above expression changes lead to a conclusion different from the transcriptome data (Fig. 5e) and earlier reports [37], in which samples were collected at 1 to 6 hours. This difference suggested different sampling times for the analyses of master regulators (early) or enzymes catalyzing biochemical reactions (late).

Post-translational modifications (PTM) also have significant impacts on the biological functions of enzymes. An auxin-responsive Kelch-domain-containing F-box protein, OsFBK1, binds OsCCR and leads to its degradation through the 26S proteasome pathway to control lignification in the development of rice anthers and roots [50]. OsRac1, one of the Rac/Rop family of small GTPases, interacts with and activates OsCCR1 to accumulate lignin and increase reactive oxygen species production during defense responses in rice [21]. Therefore, CCRs with known PTMs and the groups with different induction patterns among stress responses should have priority in future functional studies.

5. CONCLUSION

Wheat CCRs formed seven subfamilies with different distributions, gene structures, and functional motifs. The expression variations among different CCRs during development and stresses suggested that certain CCRs' subfamilies could play essential roles in corresponding biological processes. The evidence suggests that lignin biosynthesis displays a significant change in drought stress.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY DATA

https://www.siftdesk.org/articles/images/10714/supplemental-data.pdf

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