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Systematic analysis of CCCH zinc finger family in *Brassica napus* showed that *BnRR-TZFs* are involved in stress resistance

Boyi Pi^{1,2†}, Jiao Pan^{1,2†}, Mu Xiao^{1,2}, Xinchang Hu^{2,3}, Lei Zhang^{1,2}, Min Chen^{1,2}, Boyu Liu^{1,2}, Ying Ruan^{1,2} and Yong Huang^{1,2*}

Abstract

Background: CCCH zinc finger family is one of the largest transcription factor families related to multiple biotic and abiotic stresses. *Brassica napus* L., an allotetraploid oilseed crop formed by natural hybridization between two diploid progenitors, *Brassica rapa* and *Brassica oleracea*. A systematic identification of rapeseed CCCH family genes is missing and their functional characterization is still in infancy.

Results: In this study, 155 CCCH genes, 81 from its parent *B. rapa* and 74 from *B. oleracea*, were identified and divided into 15 subfamilies in *B. napus*. Organization and syntenic analysis explained the distribution and collinearity relationship of CCCH genes, the selection pressure and evolution of duplication gene pairs in *B. napus* genome. 44 diploid duplication gene pairs and 4 triple duplication gene groups were found in *B. napus* of CCCH family and the segmental duplication is attributed to most CCCH gene duplication events in *B. napus*. Nine types of CCCH motifs exist in *B. napus* CCCH family members, and motif C-X_{7/8}-C-X₅-C-X₃-H is the most common and a new conserved CCH motif (C-X₅-C-X₃-H) has been identified. In addition, abundant stress-related cis-elements exist in promoters of 27 subfamily IX (RR-TZF) genes and their expression profiles indicated that RR-TZF genes could be involved in responses to hormone and abiotic stress.

Conclusions: The results provided a foundation to understand the basic characterization and genes evolution of CCCH gene family in *B. napus*, and provided potential targets for genetic engineering in Brassicaceae crops in pursuit of stress-tolerant traits.

Keywords: CCCH-type transcription factor, Tandem CCCH zinc finger, Evolution, Abiotic stress, *Brassica napus*

Introduction

Plant transcription factors (TFs) play an important role in the regulation of plant growth, development, and environmental stress responses. A large number of transcriptional factors function in abiotic stress, such as drought, saline-alkali, extreme temperature and other stresses.

Transcription factors bZIP [1, 2], WRKY [3], AP2/ERF [4], MYB [5] bHLH [6], NAC [7], GRAS [8], HD-ZIP [9] and ZFP [10] are known more in response to abiotic stresses. Zinc Finger Protein (ZFP) family is one of the largest transcription factor families, which contains nine different types, including C2H2, C3H, C3HC4, C2HC5, C4HC3, C2HC, C4, C6, and C8 according to the number of their conserved Cys (C) and His (H) motifs [11]. Among them, the CCCH-type transcription factors, one of the most widespread ZFPs in eukaryotes, consist of

*Correspondence: yonghuang@hunau.edu.cn

[†]Boyi Pi and Jiao Pan contributed equally to this work.

² Key Laboratory of Crop Epigenetic Regulation and Development in Hunan Province, Changsha 410128, China

Full list of author information is available at the end of the article

three cysteines and a histidine coordinated by zinc ion [12].

The typical CCCH Zinc Finger proteins always harbor 1–6 CCCH repeated motifs, and C-X_{7/8}-C-X₅-C-X₃-H is the most ubiquitous motif. CCCH Zinc Finger proteins are divided into two types: the Tandem CCCH-type Zinc Finger (TZF) and the non-TZF proteins based on the number and distribution of CCCH motifs. TZF proteins only contain two tandem CCCH-type zinc finger motifs whereas non-TZF proteins have one or more than two CCCH-type zinc finger motifs [13]. Both the non-TZF and the TZF genes play important roles in many biological processes including the development process, biotic and abiotic stresses [13]. For example, non-TZF gene *AtC3H17*, as a nuclear transcriptional activator, promoted seed germination, seedling development and caused early-flowering through activated transcription of *OLEO1*, *OLEO2* and *CRU3* by binding onto their promoters in *Arabidopsis* [14] and enhanced the resistance of osmotic, oxidative and salt stresses by positively regulating the ABA-dependent stress-response pathway [13]. *IbC3H18* could be induced by NaCl, polyethylene glycol (PEG), H₂O₂ and abscisic acid (ABA) and interact with *IbPR5* to enhance salt and drought tolerance [15]. Plant TZF proteins are evolutionarily conserved regulators in growth and responses to hormones and stresses [16]. *OsC3H10*, a TZF gene in rice, was demonstrated to participate in the regulation of the drought tolerance pathway by elevating the expression of stress-related genes [17]. A plant-unique arginine-rich (RR) region located in the front of C-X_{7/8}-C-X₅-C-X₃-H-X₁₆-C-X₅-C-X₄-C-X₃-H (TZF) motif [18]. Both the RR and TZF domains are essential to RNA binding in *Arabidopsis* [18]. RR-TZF proteins subfamily is one of the largest CCCH subfamilies in plant species, 11 of 68 in *Arabidopsis* [19], 16 of 91 in poplar [20], 17 of 103 in *Brassica rapa* [21], 16 of 103 in switchgrass [22], 25 of 89 in banana [23] and 12 of 119 in *Moso Bamboo* [24]. Plant RR-TZF proteins can further be divided into two groups: the RR-TZF containing RR and TZF domain, and the ANK-RR-TZF containing an extra ANK (Ankyrin) domain. *Arabidopsis* RR-TZF members *AtTZF1*, *AtTZF2*, *AtTZF3*, *AtTZF4*, *AtTZF5* and *AtTZF6* (*AtTZF1–6*) belong to ANK-RR-TZF and their functions are more diversified. *AtTZF1–3* functions in ABA-mediated drought tolerance and JA-induced senescence while *AtTZF4–6* are negative regulators of seed germination [25]. *AtTZF7*, *AtTZF9*, *AtTZF10*, *AtTZF11* could positively regulate vegetative growth and be involved in abiotic stress tolerance responses [16]. Ankyrin (ANK) is ubiquitous in eukaryotes, prokaryotes and viruses and ANK family members are involved in light signal regulation, embryonic development, leaf morphogenesis, lateral root formation and so on [26].

CCCH zinc finger proteins might be involved in organism development and stress response through post-transcriptional regulation. Human ZFP36 (Tristetraprolin, TTP) is the prototype of the mammalian TZF that consists of two tandem CCCH motifs inserted with 18 amino acids [18]. TTP or *Arabidopsis* TZF1 promotes the degradation of mRNA by inhibiting the assembly of target mRNA polyA through combining to a specific site of the 3'-UTR region (UUAUUUAUU) of target genes [18, 27]. Likewise, ZFP36L2 is another TZF protein in animal kingdom. And it was known as a very unstable mRNA binding protein that controls maternal fertility and physiological function during early embryonic development [28, 29].

Brassica napus, one of the most important oil crops of Brassicaceae, is an allotetraploid hybrid of *Brassica rapa* (A-subgenome, AA, *n* = 10) and *Brassica oleracea* (C-subgenome, CC, *n* = 9) [30]. The function of CCCH genes in *B. napus* is little known except that overexpression of CCCH-type transcription factor *BnZFP1* increased in oleic acid and oil levels in *B. napus* by positive regulation of its target gene diacylglycerol O-acyltransferase 1 (*DGAT1*) [15]. In fact, the roles of rapeseed CCCH genes in development and abiotic stress are known much little. In this study, CCCH genes of *B. napus* on whole genome level were identified and their expression in response to abiotic stresses were investigated, and these results provided a foundation for further research in CCCH genes.

Results

Identification and chromosome localization of CCCH genes in *B. napus*

One hundred and fifty-five CCCH genes were identified by Blastp tools in *B. napus* database. The subgenome A possesses more CCCH genes than the subgenome C. 81 CCCH genes evolve from the subgenome A and 74 CCCH genes evolve from the subgenome C (Additional file 1). The 135 of 155 CCCH genes are located in ChrA01-A10, ChrC01-C09 (Figs. 1 and 2). Besides, the chromosome localization of the other 20 CCCH genes in *B. napus* is unknown. Among A-subgenome, 74 CCCH genes locate in ChrA1-A10 chromosome while 7 CCCH genes are unconfirmed. ChrA09 (33.9M), the longest chromosome in the A genome, carries 9 CCCH genes. And ChrA03 (29.8M) carries the largest number of 14 CCCH genes. Second to ChrA03, ChrA07 (24.0M) contains 13 CCCH genes. Among C-subgenome, 61 CCCH genes locate in Chr1-Chr9, whereas 13 CCCH genes are still on the scaffold. ChrC03 (60.6M), the longest chromosome in A and C, has 12 CCCH genes.

Gene collinearity and duplication of CCCH in *B. napus*

Most CCCH orthologous genes in *B. rapa* and *B. oleracea* remain as homeologous gene pairs in *B. napus*. There are 24 collinearity CCCH gene pairs only in subgenome A, 16 collinearity CCCH gene pairs only in subgenome C, and 92 collinearity CCCH gene pairs between subgenome A and subgenome C in *B. napus* (Fig. 2, Additional file 2). Comparative analysis with the parent genomes revealed that the *B. napus* genome retained 98.6% CCCH genes of *B. oleracea* (74 of 75) in comparison to only 78.6% CCCH genes of *B. rapa* (81 of 103) (Additional file 1).

The organism gene duplication occurred through segmental, or tandem or whole genome [31]. Tandem and segmental duplication occurred when two or three closely related *BnC3H* genes were located on the same or different chromosomes [32]. Reference on the collinearity of the CCCH family (Fig. 2, Additional file 2) and the criteria of Yang [33], the duplication events have occurred among 108 genes which were disseminated in 10 A-chromosomes and 9 C-chromosomes (Additional file 3). Among them, forty-four diploid duplication gene pairs, four triploid duplication gene groups and two quadruple duplication gene groups were found (Additional file 3). The results showed that most CCCH duplication gene pairs are segmental duplication except three tandem duplications pairs, *BnC3H59/BnC3H60*, *BnC3H60/BnC3H61* and *BnC3H59/BnC3H61* in *B. napus* CCCH family (Additional file 3). There are eight gene pairs have been identified between ChrA03 and ChrC03, which have the highest frequency diploid duplication. The most diploid duplication and quadruple duplication genes groups

occurred between ChrA05 and ChrC05, ChrA04 and ChrC04, respectively (Additional file 3, Fig. 1). It might suggest that duplication events also happened between A- and C- subgenomes in process of *B. napus* formation.

The selection mode of the coding sequences can be predicted through the Ka/Ks ratio. In *B. napus*, the Ka/Ks ratio of segmentally duplicated CCCH gene pairs were < 1 (the majority Ka/Ks ratio < 0.5), and it suggested that duplicated *BnC3H* gene pairs were under purifying negative selection. Additionally, the duplication events might occur less than 10 MYA in *B. napus* (Additional file 3).

Phylogenetic relationship analysis of CCCH family

To further explore the diversity and conservation of BnC3H proteins, the 155 CCCH full-length protein sequences were used to construct a phylogenetic tree by the Maximum Likelihood (ML) method (Fig. 3). 134 CCCH proteins were divided into 15 subfamilies, and 21 CCCH proteins were not confirmed. Subfamily I is the largest clade with 34 CCCH proteins, followed by the subfamily IX RR-TZF with 27 CCCH proteins. Besides, the subfamily V and subfamily X only has one CCCH member. Compared with *Arabidopsis* and rice, the subfamily VI is pretty special in that the three BnC3H proteins are divided into two groups.

Gene structure and protein structure of CCCH zinc finger in *B. napus*

To pinpole the evolution trajectory and study the function diversity of *B. napus* CCCH genes, the gene structure of *BnC3Hs* was analyzed. It was found that the exons

and introns of *BnC3H* genes varied from 1 to 18, but the gene structure of *CCCH* in each family was relatively conservative except subfamily XI genes structure diversify variety (Fig. 4). The number of exons of subfamily I is relatively conservative than others, ranging from 5 to 8. In terms of the structure of subfamily IX, they behaved in two types of the structure of genes. 11 of them only have one exon, no introns, and the rest of them all have

2–5 exons except *BnC3H137* and *BnC3H81*. This family can be classified into two groups. The longest genes were in subfamily XV and 4 members of them possess 11–13 exons. *BnC3H18* possesses the largest number of exons (18) in subfamily XI. (Fig. 4).

Domains are the building blocks of proteins. During evolution, domains produce novel structures and functions of proteins [34]. The results showed that

there were great differences in the structure of CCCH proteins. Nine different types of CCCH motifs were found in 155 CCCH proteins of *B. napus* (Fig. 5; Additional file 4). Each CCCH protein contained at least 1–6 CCCH motifs. The number and type of BnC3H proteins in each subgroup are relatively conservative. C-X₇₋₈-C-X₅-C-X₃-H motif is the most common and extensive

CCCH motif in *B. napus*, and it mainly occurred in the subfamily I, II, III, IV, VII, VIII and XV. Most proteins of subfamily I have five conserved C-X₇₋₈-C-X₅-C-X₃-H motifs except BnC3H1 and BnC3H70 with six CCCH motifs. Subfamily IX CCCH proteins contain two conserved CCCH motifs divided by 18 amino acids (C-X₇₋₈-C-X₅-C-X₃-H-X₁₆₋₁₈-C-X₅-C-X₄-C-X₃-H).

Fig. 4 The gene structure of *CCCH* genes in *B. napus*. The exons and introns of *BnC3H* genes varied from 1 to 18, but the gene structure of *C3H* in each family was relatively conservative except subfamily XI. The gene structure of 155 *CCCH* genes was constructed by Gene Structure Display Server (<http://gsds.gao-lab.org/index.php>)

The protein length of subfamily XV is the longest in *B. napus* *CCCH* family, and the four members are above 1000 amino acids length with a conserved C-X₇-C-X₅-C-X₃-H motif. Interestingly, six special C-X₅-C-X₃-H motifs consisted of two cysteines (C) and one histidine (H) was found in *B. napus* subfamily VI. In addition to *CCCH* motifs, RING, WD40, KH, ANK and RRM domains also appeared conservatively in subfamily III, IV, VII, IX and XI (Fig. 5).

Conserved structure of subfamily IX in *B. napus*

RR-TZF family plays an important role in plant growth, development and stress response [19]. To identify the *RR-TZF* family genes to respond to stress in *B. napus*, the promoter elements and RR-TZF domain composition of subfamily IX were analyzed.

The promoter elements of the *RR-TZFs* were predicted on the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The results showed that all the *BnRR-TZF* promoters possessed typical CAAT and TATA boxes which are the core cis-acting element

Fig. 5 Conserved domain of CCCH proteins in *B. napus*. Nine different types of CCCH motifs were found in 155 BnC3H proteins. Each C3H protein contained 1–6 CCCH motifs in *B. napus*. The conserved domain of CCCH proteins was detected by SMART and NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), and the Expect Value was set at 10. The site of domains information was constructed on IBS1.0.3 software

in promoter and enhancer regions. Except for the basic promoter elements, a large number of cis-elements related to abiotic stress were widely found. They could be grouped into three types, hormone-responsive elements, stress-responsive elements and light-responsive elements (Additional file 5, Fig. 6). For example, the ABA response element (ABRE) cis-elements related to the ABA response exist in almost all *RR-TZF* promoters except *BnC3H26*, *BnC3H35* and *BnC3H118*. Except for *BnC3H25*, *BnC3H66*, *BnC3H81*, *BnC3H88*, *BnC3H116*, *BnC3H137*, *BnC3H138* and *BnC3H155*, the promoters of other genes contain 1 to 5 elements: CGTCA-motif and TGACG-motif that related to jasmonic acid response, GARE-motif and P-box that associated with GA-induced plant growth regulation [35], while TGA-element and AuxRR-core that related to abiotic stress induced by a hormone, and MYB is a binding site of drought-induced genes, related to drought or drought stress caused by other abiotic stresses [36]. The results showed that a large number of promoter cis-elements of *BnRR-TZF* family

genes were related to hormone and drought-induced abiotic stress (Fig. 6). Therefore, *BnRR-TZF* genes may be involved in hormone and drought-induced abiotic stress.

It is similar to Arabidopsis that *RR-TZF* proteins of *B. napus* can be divided into two groups (Fig. 7A), group I ANK-*RR-TZF* including 16 members, and group II *RR-TZF* including 11 members (Fig. 7B). ANK (Ankyrin) protein would be involved in responding to various biotic and abiotic stresses and regulating the growth and development of plants. *B. napus* *RR-TZF* proteins contain two conserved motifs, C-X₇₋₈-C-X₅-C-X₃-H and C-X₅-C-X₄-C-X₃-H spaced by 16 amino acids (*TZF*) and an arginine-rich motif (*RR*) which contains a conserved C-X₅-H-X₄-C-X₃-H motif in front of the *TZF* domain (Fig. 7B). In animals, TTP was translocated from the nucleus mediated by a Leucine-rich Nuclear Export Signal (NES). 117 NES sequences were identified from 27 members of subfamily IX. The result suggests that all subfamily IX proteins of *B. napus* may be

Fig. 6 Type and number of cis-elements of *CCCH* genes of subfamily-IX in *B. napus*. A large number of cis-elements related to abiotic stress were widely found in *BnRR-TZF* subfamilies. The 1500 bp upstream region of *BnRR-TZF* genes is downloaded on a website (<https://www.genoscope.cns.fr/brassicapapus/>)

nucleocytoplasmic shuttle proteins involved in signal transduction (Fig. 7C).

Stress response of subfamily IX in *B. napus*

A total of 27 *RR-TZF* genes in subfamily IX, 14 of them belong to subgenome A and 13 of them belong to subgenome C. To study the response of subfamily IX genes in abiotic stresses ABA and drought, the expression of *RR-TZF* genes under ABA or PEG conditions was verified by qRT-PCR at four different time points. The results showed that 22 of 27 *BnRR-TZF* genes were able to respond to ABA or PEG stress (Fig. 8). Among them, the expression of 9 *CCCH* genes (*BnC3H3*, *BnC3H6*, *BnC3H21*, *BnC3H56*, *BnC3H88*, *BnC3H127*, *BnC3H144*, *BnC3H148* and *BnC3H155*) was quickly elevated by ABA and PEG treatment and maintained higher than that of the control at all examined time points. In subfamily IX, ten diploid duplication gene pairs and two triploid duplication gene groups were found. And all gene pairs occurred segmental duplication. Among duplication gene pairs, a part of gene pairs may be

functionally conserved so they have similar expression patterns. For example, *BnC3H56* and *BnC3H131*, a pair of diploid duplication gene pairs homologous to *AtTZF2* (*AtC3H20*), were notably up-regulated by ABA and PEG [37, 38] (Figs. 7 and 8). A part of *BnRR-TZF* genes only response to ABA or PEG treatment. *BnC3H25*, *BnC3H26* and *BnC3H98* were the only ABA-induced genes. Also, some gene pairs have different structures, which may contribute to functional diversity. A group of diploid duplication gene pairs homologous to *AtTZF8* (*AtC3H56*), *BnC3H17* and *BnC3H98*, their expression pattern was different. *BnC3H98* possesses one more ANK motif than *BnC3H17*, but it just response to ABA at 3 h whereas *BnC3H17* responds both to ABA and PEG treatment (Figs. 5 and 8). Additionally, 4 genes (*BnC3H15*, *BnC3H116*, *BnC3H118* and *BnC3H141*) was only response to PEG treatment. *AtTZF10* (*AtC3H29*) was induced by salt to enhance mRNA accumulation and had nothing to ABA [39]. *BnC3H15*, *BnC3H35* and *BnC3H118*, a group of triploid duplication gene pairs, were homologous to *AtC3H29*, they have similar protein

Fig. 7 Protein sequence analysis of subfamily-IX in *B. napus*. **A**, Phylogenetic tree of subfamily-IX proteins in *Arabidopsis-B. napus* (Statistical Method: Neighbor-joining; Model/Method: p-distance; Gaps/Missing Data Treatment: Complete deletion; No. of Bootstrap Replications: 1000). **B**, Multiple sequence alignment of subfamily-IX proteins in *Arabidopsis-B. napus*. **C**, Amino acid sequence alignment of putative NES sequences of subfamily IX in *B. napus*

structures, but functions diversity. Our results showed that *BnC3H15* and *BnC3H118* responded fast to PEG, but not to ABA whereas *BnC3H35* had no response to ABA and PEG (Fig. 8).

In *B. napus*, most of the RR-TZF genes that responded to ABA and PEG showed extreme differences around treatment 3-5 h. Some genes showed significant changes in expression around 1 h under ABA and PEG treatment. While over time, transcripts of most genes were gradually stabilized and some even showed a downward trend, with only the highest expression at a certain point in time. There is another part of genes that are not induced by ABA and PEG, which may not be involved in the stress response to ABA and PEG induction (Fig. 8).

Discussion

CCCH is a large transcription factor family in *B. napus*

CCCH families have been identified in several plant species. *B. napus* is an allotetraploid species that experienced extensive genome duplication and merging events [30]. A total of 155 putative CCCH protein-encoding genes in *B. napus* were identified (Additional file 1). The CCCH proteins in *B. napus* are much more than some species based on other reports (Additional file 4), 68 in *Arabidopsis* (2n=10), 67 in rice (2n=24) [19], 68 in maize (2n=20) [40], 91 in poplar (2n=38) [12], 103 in switchgrass (2n=18) [22], 89 in banana (3n=33) [23], but less than the sum of its parents *B. rapa* (103) and *B. oleracea* (75) [21]. The genome size of *B. napus* (AACC,

Fig. 8 Expression profiles of CCCH genes of subfamily-IX in response to ABA and PEG treatment. The relative expression of *BnRR-TZFs* in *B.napus* under 100 μ M ABA, 25% PEG (drought) conditions. The data are representative of three independent experiments ($n=3$, mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, t-test)

2n=38) is 1132 Mb [41], which is less than the sum of its diploid ancestors *B. rapa* (AA, 2n=20, 485 Mb) [42] and *B. oleracea* (CC, 2n=18, 648 Mb) [43]. Previous study shows that most orthologous gene pairs in *B. rapa* and *B. oleracea* remain as homeologous pairs in *B. napus*, whereas the occurrence of gene deletion in its parents

probably predated the allopolyploidization of *B. napus* [44]. That's probably why the number of BnC3H proteins was less than the sum of its parents.

B. rapa provides A-subgenome for *B. napus*. Transcription factors of *B. rapa*, respond to important environmental factors (salt, cold, osmotic stress, light, wounding,

pathogen defense, cadmium and zinc ions) and plant hormones (jasmonic acid, auxin, salicylic acid, ethylene, brassinosteroid, cytokinin, and abscisic acid) are over-retained [42]. Genome polyploidization may have extended to gene families and serve as a basis to cope with extreme environments [36]. Whole genome duplication (WGD) and polyploidy events might have contributed to the CCCH number increased in the *Brassica* species [30, 45]. Whole-genome sequences showed that *B. rapa* transcription factors underwent diploidization and triploidization [42]. *B. napus* CCCH transcription factors might be over-retained as well as deletion (Fig. 2; Additional file 1).

The evolution and conservation of CCCH proteins in *B. napus*

Gene structure, domain organization and phylogenetic tree showed that CCCH is relatively conserved in plants. Similar to the model plants *Arabidopsis* and rice [19] and its parent *B. rapa* [21], introns/exons of *BnC3H* genes change in a wide range, from 1–18, but much conservation in the same subfamily (Fig. 4). Among duplicated gene pairs, paralogues also showed many similarities in gene structures and domain organization (Figs. 4 and 5, Additional file 3). The similarities indicate similar functions [23].

CCCH motifs were normal in plant species. The C-X₈-C-X₅-C-X₃-H and C-X₇-C-X₅-C-X₃-H types of motifs are predominant motifs in the CCCH protein family of *B. napus*, and the ratio is 64% and 24%, respectively (Fig. 5, Additional file 4). As Zhuang [46] indicated that over-expression *PdC3H17* can enhance the ability to remove reactive oxygen species (ROS), thereby enhancing salt tolerance depends on its CCCH domains. Thus, these CCCH motifs existed both in monocots and dicots and might play vital functions as a transcriptional binding site in abiotic stress. Compared with the dicotyledon model plant *Arabidopsis*, the C-X₁₇-C-X₅-C-X₃-H motif was found in *B. napus*, but C-X_{7/8}-C-X₆-C-X₃-H and C-X₉-C-X₅-C-X₃-H motifs were disappeared. And compared with monocotyledon model plant rice, the C-X₁₇-C-X₅-C-X₃-H motif was also found, but C-X₁₅-C-X₅-C-X₃-H and C-X₈-C-X₅-C-X₄-H were disappeared [19]. Compared with *B. rapa*, one parent of *B. napus*, there are six CCCH motifs (C-X_{7/8}-C-X₆-C-X₃-H, C-X_{12/14}-C-X₅-C-X₃-H, C-X₈-C-X₅-C-X₂-H and C-X₉-C-X₅-C-X₃-H) were not found, but the C-X₆-C-X₆-C-X₃-H motif was discovered [21]. Except CCCH motifs, the TIR domain, a toll/interleukin receptor involved in relative processes of innate immunity pathways [47] and signal transduction [48], was found in subfamily XIV in *B. napus* but not in *Arabidopsis* and *B. rapa*. It suggests that subfamily XIV *BnC3H* might be neofunctionalization during the evolution process and

play roles in innate immunity and signal transduction. Besides, RING [49], RRM [50], ANK [51], WD40 [52] and KH domain [53] are detected (Fig. 5). These motifs are related to protein-protein or protein-DNA or RNA binding in plants [54].

Putative cis-elements and motif indicating stress response of RR-TZF in *B. napus*

Transcription factors activated by biotic and abiotic stresses initiated the expression of corresponding genes by binding to related elements. Abundant hormone-responsive, stress-responsive and light-responsive elements exist in all promoters of RR-TZF homologous genes of *B. napus* (Additional file 5, Fig. 6). Overexpression of *OsC3H10* that carries three DREs and two ABREs (ABA response element) in its promoter improved drought tolerance in rice by regulating drought-induced *OsDREB2* transcription factors through ABA-independent pathway [17]. *Arabidopsis AtTZF1*, *AtTZF2*, and *AtTZF3* equipped with ABRE (ABA response element), SARE (SA response element), TCA-element (MeJA response element) in their promoters respond to ABA, drought, oxygen, and salt stress [37, 55]. Homologous to *AtTZF2*, *BnC3H56* and *BnC3H131* have ABRE, ARE, MYB, CGTCA and TGACG motifs in their promoter (Additional file 5). The expression of ANK-RR-TZF subfamily genes in *Arabidopsis* (*AtTZF1–6*) might have evolved from the pre-existing pathways that regulate ABA-mediated responses to salt stress during the germination process [56]. The MYB binding sites function as cis-acting elements in the dehydration-induced expression of *RD22* in *Arabidopsis* [57]. Thus, *BnC3H88* and *BnC3H138* might enhance drought stress through MYB binding site located in its promoter by an ABA-dependent pathway. Comparing with *Arabidopsis*, a variety of cis-elements were detected in subfamily IX of *B. napus* CCCH family, and it showed that differentiation event might have occurred in BnRR-TZF to a certain extent during the CCCH gene family evolution process. It suggests that RR-TZF genes may play a crucial role in response to hormone-induced and abiotic stresses.

Tandem CCCH Zinc Finger proteins (TZFs) are conserved from yeast to metazoans [16]. In animals, the structure of CCCH type TZF domain has been determined from the TIS11D [58] and the AU-rich element from the 3'-UTR of TNF- α transcript as a binding partner of the TZF domain [59]. Different from yeast and metazoans, plant TZF motif was conserved preceded by arginine-rich (RR) domains. Similar to *Arabidopsis*, subfamily IX of *B. napus* CCCH gene family were divided into two groups, group I characterized with RR-TZF domain and extra two or three ANK domains, group II characterized with RR-TZF domain (Fig. 7). The Nuclear

Export Signal (NES) of subfamily IX protein infers that the *BnRR-TZF* might be involved in signal transduction [19] (Fig. 7). The function of all members of *RR-TZFs* related to biotic and abiotic stresses in *Arabidopsis* was summed up. Because of the conservation of TZF domain in evolution, plant *RR-TZF* domain might have a similar mechanism to animals TZF on RNA targeting and transcriptional regulation in stress response.

RR-TZF genes involving stress response in *B. napus*

Arabidopsis RR-TZF genes are involved in ABA, salt, cold, H₂O₂, osmotic, and sugar depletion stresses [16]. The expression patterns of abiotic stress responsive show that more than half of *B. napus RR-TZF* can be induced by ABA and PEG (Fig. 8). *AtC3H23(AtTZF1)*, *AtC3H20 (AtTZF2)* and *AtC3H49 (AtTZF3)* located in cytoplasm and expressed in vegetative tissues and flowers, and overexpression *AtTZF1*, *AtTZF2*, or *AtTZF3* caused ABA hypersensitivity, enhanced drought tolerance and reduced transpiration [37, 38]. *BnC3H45*, *BnC3H56*, *BnC3H127* and *BnC3H131*, homologous genes of *AtTZF1*, *AtTZF2* or *AtTZF3*, responded to the induction of ABA and PEG stress. In particular *BnC3H127*, always kept a high expression level under ABA and PEG stress. *AtTZF4*, *AtTZF5*, *AtTZF6* were much important to regulate seed germination in *Arabidopsis* [25, 60]. And part of their homologous genes in *B. napus BnC3H3*, *BnC3H66*, *BnC3H88*, *BnC3H138* and *BnC3H155* were also responded ABA and PEG (Fig. 8). Double mutant *tzf10 tzf11* is more sensitive to salt stress and drought stress in *Arabidopsis* [61]. Homologous genes of *AtC3H29 (AtTZF10)* and *AtC3H47 (AtTZF11)* in *B. napus BnC3H15*, *BnC3H21*, *BnC3H118*, *BnC3H35*, *BnC3H19* and *BnC3H148* might undergo function diversification. *BnC3H15/BnC3H21/BnC3H118/BnC3H148* responded to PEG, while *BnC3H21* and *BnC3H148* responded to ABA. These four *RR-TZF* genes belong to the ANK-*RR-TZF* subfamily, and they have similar gene and protein structures. Furthermore, *BnC3H118* lacked ABA-related elements (Fig. 6). *BnC3H15*, a homologous gene to *AtTZF10*, has an ABRE like *BnC3H21* and *BnC3H148*, but only responds to PEG but not ABA. The results of gene expression (Fig. 8) and the stress-responsive cis-elements in *BnRR-TZFs* promoters (Fig. 6) suggest that some *BnRR-TZFs* respond to hormones (like ABA, JA, etc.), drought and salty treatments.

Most of the time, duplication genes have a similar expression pattern which one is from subgenome A and another is from subgenome C [62]. This kind of duplicated genes may be functionally conserved. But some duplicated genes diversify in response to ABA and PEG in *BnRR-TZFs*. During the evolution of *CCCH* gene family in *B. napus*, new neofunctionalization appeared [20].

This phenomenon may have occurred in *CCCH* family in *B. napus*. Different structures may lead to different duplication types and functional differences [63]. In *BnRR-TZF* subfamily, a diploid duplication gene pairs *BnC3H17* and *BnC3H98*, their protein structures were different, they responded differently to ABA and PEG. It may indicate that some functional divergence has occurred to the duplication genes in *BnRR-TZF* family.

RR-TZF proteins trigger mRNA degradation by binding to 3'-UTR of target mRNAs in a sequence-specific manner [16, 64]. But stress-responsive target genes activated by *RR-TZF* proteins have not been confirmed in plants. It is inferred that *BnRR-TZF* genes might respond to ABA and drought stress in a similar way to *Arabidopsis* because of their close relationship, similar cis-acting elements in the promoter region and conservative domain organization (Figs. 3, 5 and 6). Identification of the target genes or mRNAs of *CCCH* proteins, understanding the mechanism of binding and activation between *CCCH* protein and target gene or mRNA are worth further analyzing.

Materials and methods

Characterization and identification of CCCH proteins in *Brassica napus*

To identify *CCCH* proteins of *B. napus*, the genome sequence of *Arabidopsis*, rice, *B. rapa* and *B. oleracea* are cited from references [19, 21], and the *CCCH* genes and proteins sequence of *B. napus* were obtained from the Genome Resources database (<http://www.genoscope.cns.fr/brassicanapus/>) by using the Basic Local Alignment Search Tool algorithms program (BLASTP) with *Arabidopsis CCCH* protein sequences as queries. Further, the candidate sequences were confirmed by SMART website (<http://smart.embl-heidelberg.de/>) and NCBI conserved domain search tools (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

The chromosome location information of *BnC3H* genes was subjected to MapChart 2.2 software to draw the draft [65]. The physicochemical parameters of *BnC3H* proteins were generated by the program ExPASy (<https://web.expasy.org/protparam/>).

Genomic organization and syntenic analysis of CCCH in *B. napus*

To visualize the location and syntenic gene pairs of *BnC3H* in genome, the gene position, gene length, chromosome size, and centromere position were extracted from the gff files of *B. napus* genome (<https://www.genoscope.cns.fr/brassicanapus/data/>). All protein sequences of *B. napus* were compared against themselves, and the

distribution map was drawn by the MCScanX tool on TBtools software (E-value < $1e^{-5}$, number of hit ≤ 5) [66].

Gene duplication, Ka/Ks calculation and selection pressure analysis

The duplicated gene groups were defined as the methods of Yang [33] and tandem duplicated groups were defined as the methods of Sun [67]. The full-length-CDS sequence covering and identify of amino acid were detected by Blastn/Blastp in NCBI [68].

The non-synonymous substitution rate (Ka), synonymous substitution rate (Ks), and the duplication time (T, million years ago, MYA) were calculated by a Simple Ka/Ks Calculator tool on TBtools software [66]. The selection pressure on *BnC3H* duplicated gene groups were detected through Ka/Ks ratio and considered positive, negative or neutral selection when Ka/Ks ratio was >1, <1, or =1, respectively [32].

Analysis of gene structure, domain organization, and phylogenetic relationship

To further understand the structural features of *BnC3H* genes, we deduced the exon-intron organization map by comparing cDNA with their corresponding genomic sequences of *BnC3H*. After genomic and cDNA sequences were downloaded from the *B. napus* database (<http://www.genoscope.cns.fr/brassicnapus/>), the gene structure was constructed by the Gene Structure Display Server (<http://gsds.gao-lab.org/index.php>) [69]. The information of domain organization was identified by SMART and Conserved Domain Search tool on NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), then the sites of domain organization were constructed by IBS 1.0.3 software [70].

To explore the phylogenetic relationship of *BnC3H*, we have constructed a phylogenetic tree including 68 *AtC3H*, 67 *OsC3H*, 103 *BraC3H*, 75 *BolC3H* and 155 *BnC3H* proteins. Multiple sequence alignment of *BnC3H* proteins was carried out using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) programs [71] and the resulting file was subjected to phylogenetic analysis using the MEGA 7.0 program [72]. A tree was constructed based on the full-length protein sequences using the Maximum Likelihood (ML) method with Poisson model, and a Bootstrap test of 1000 replicates for internal branch reliability.

The conserved domain of ANK and RR-TZF in subfamily IX were isolated from CCCH zinc finger proteins by ESPript3.0 website (<http://esprpt.ibcp.fr/ESPript/ESPript/index.php>), the Nuclear Export Signal (NES) sequences were detected with a program as Wang

[19], and the draft files were also created by ESPript3.0 website.

Prediction of BnRR-TZF promoter cis-acting element

To identify the cis-acting element of subfamily IX in *B. napus*, an upstream 1500bp promoter sequence of the *CCCH* gene start codon was extracted to predict their putative cis-element by PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [73].

Plant materials and stress treatment

B. napus Xiang You 15 (XY15) was used as plant material. It was bred from Hunan Agricultural University (Changsha, China) and stored in the Key Laboratory of Crop Epigenetic Regulation and Development in Hunan Province. XY15 seeds grew in roseite in the green room at 24°C and 70% humidity with 16h light/8h dark photoperiod. Three-week-old seedlings with 2–3 true leaves were cleaned up and cultivated in 1/2 liquid MS medium in a growth chamber for 3 days to acclimatize before treatment with ABA and PEG. The whole seedlings were harvested and put into 1/2 liquid MS medium with 100 μ M ABA or 25% PEG. The seedlings were sampled to detect *CCCH* gene expression at 1, 3, 5, and 8 h at the process. Seedlings in 1/2 liquid MS medium were used as control at the same time points. Triplicate seedling samples were collected and quickly frozen in liquid nitrogen and then stored at -80°C [74, 75]. Triplicate was confirmed.

Quantitative real-time PCR (qRT-PCR) validation

RNA isolation of *B. napus* were carried out by TRIzol reagent kit (Invitrogen, Carlsbad, CA, US) according to the instructions. The quality of RNA was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. Approximately 1.0 μ g total RNA was reverse-transcribed into cDNA using an RT reagent kit (RevertAid First Strand cDNA Synthesis, ThermoFisher Scientific, USA) [68].

The quantitative real-time PCR was carried out with SYBR-green fluorescence using a CFX 96 Real-Time System (BIO-RAD) with a 20 μ l PCR reaction mixture that included 8.8 μ l of 10 decuple diluted cDNA, 10 μ l of 2 $\times$ FastStart Universal SYBR Green Master (ROX) (Roche, Switzerland), and 10 mM 0.6 μ l of forward and reverse primer as previously. The *BnaA10g22340D* gene was used as a reference gene [76]. All primer was designed by NCBI primer blast tools (Additional file 6). Each sample was run in triplicate for analysis. At the end of the PCR cycles, the melting curve analysis was performed to validate the specific generation of the expected

PCR product. The expression levels of *BnRR-TZF* genes were calculated with $2^{-\Delta\Delta CT}$ method as a previous report [77].

Conclusion

Allotetraploid *B. napus* inherited *CCCH* genes from its diploid parents *B. rapa* and *B. oleracea*, and its genome has undergone multiple duplications and deletions. 155 *CCCH* genes, 81 from subgenome A and 74 from subgenome C were identified. Evolutionary relationship, gene and protein structure analysis in *CCCH* family in *B. napus* showed diversity among subfamilies, but highly conservation within the same subfamily. Subfamily-IX *RR-TZF* genes are involved in ABA or drought stress. The results presented basic information of *BnCCCH* genes and provided a useful resource for gene function and breeding of Brassicace crops.

Abbreviations

ABRE: ABA response element; ABA: Absciscic acid; ANK: Ankyrin; AP2/ERF: APETALA2/Ethylene-Responsive Factor; RR-TZF: Arginine rich-tandem CCCH-type zinc finger; bHLH: Basic Helix–Loop–Helix; bZIP: Basic Leucine Zipper; BLASTP: Basic Local Alignment Search Tool algorithms Program; C3H: CCCH; *DGAT1*: Diacylglycerol O-acyltransferase 1; HD-ZIP: Homeodomain-leucine Zipper; JA: Jasmonic acid; KH: K-Homology; ML: Maximum Likelihood; MYA: Million years ago; MS: Murashige and Skoog; MUSCLE: Multiple Sequence Comparison by Log-Expectation; Ka: Non-synonymous substitution rate; non-TZF: Non-tandem CCCH-type zinc finger; NES: Nuclear Export Signal; PEG: Polyethylene glycol; ROS: Reactive oxygen species; RRM: RNA recognition motif; SARE: SA response element; SMART: Simple Modular Architecture Research Tool; Ks: Synonymous substitution rate; TZF: Tandem CCCH-type zinc finger; MeJA: Methyl Jasmonate; TIR: Toll/interleukin receptor; TFs: Transcription factors; TTP: Tristetraprolin; UTR: Untranslated Region; WD40: WD or beta-transducin repeats; WGD: Whole genome duplication; ZFP: Zinc Finger Protein.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-021-03340-8>.

Additional file 1. (XLS 272 kb)

Additional file 2. (XLS 4288 kb)

Additional file 3. (XLS 128 kb)

Additional file 4. (XLS 32 kb)

Additional file 5. (XLS 53 kb)

Additional file 6. (XLS 31 kb)

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Not applicable.

Authors' contributions

Experiments were designed by HY and RY. PBY and PJ contributed most of the experiments; HXC contributed to the collinear analysis; LBY contributed to plant sample; PBY and XM wrote the original manuscript; ZL and CM collated the reference; HY and RY wrote the final version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are obtained from published articles and their Additional files. The protein sequences of CCCH family genes in *B. napus* were downloaded from <https://www.genoscope.cns.fr/brassicana>. All sequences were identified by using the online protein structure prediction tool SMART (<http://smart.embl-heidelberg.de/>). The supporting data of this article are included in the article and its Supplementary files. In this article, the collection and cultivation of plant material comply with relevant institutional, national, and international guidelines and legislation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, China. ²Key Laboratory of Crop Epigenetic Regulation and Development in Hunan Province, Changsha 410128, China. ³College of Agronomy, Hunan Agricultural University, Changsha 410128, China.

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