Comparative analysis of cytokinin response factors in Brassica diploids and amphidiploids and insights into the evolution of Brassica species

Lijun Kong, Kun Zhao, Yingying Gao, Liming Miao, Chaoquan Chen, Hang Deng, Zhenning Liu, and Xiaolin Yu

Abstract

Background: Cytokinin is a classical phytohormone that plays important roles in numerous plant growth and development processes. In plants, cytokinin signals are transduced by a two-component system, which involves many genes, including cytokinin response factors (CRFs). Although CRFs take vital part in the growth of Arabidopsis thaliana and Solanum lycopersicum, little information of the CRFs in the Brassica U-triangle species has been known yet.

Results: We identified and compared 141 CRFs in the diploids and amphidiploids of Brassica species, including B. rapa, B. oleracea, B. nigra, B. napus, and B. juncea. For all the 141 CRFs, the sequence and structure analysis, physiological and biochemical characteristics analysis were performed. Meanwhile, the Ka/Ks ratios of orthologous and paralogous gene pairs were calculated, which indicated the natural selective pressure upon the overall length or a certain part of the CRFs. The expression profiles of CRFs in different tissues and under various stresses were analyzed in B. oleracea, B. nigra, and B. napus. The similarities and differences in gene sequences and expression profiles among the homologous genes of these species were discussed. In addition, AtCRF11 and its ortholog BrCRF11a were identified to be related to primary root growth in Arabidopsis.

Conclusion: This study performed a genome-wide comparative analysis of the CRFs in the diploids and amphidiploids of the Brassica U-triangle species. Many similarities and differences in gene sequences and expression profiles existed among the CRF homologous genes of these species. In the bioinformatics analysis, we found the close relativity of the CRF homologous genes in the Brassica A and C genomes and the distinctiveness of those in the B genome, and the CRF homologous genes in B subgenome were considerably influenced by the A subgenome of B. juncea. In addition, we identified a new function of the Clade V CRFs related to root growth, which also clarified the functional conservation between Arabidopsis and B. rapa. These results not only offer useful information on the functional analysis of CRFs but also provide new insights into the evolution of Brassica species.

Keywords: Cytokinin response factors, Brassica, Comparative genomics, Evolution, Functional analysis
Background
Cytokinin is a major phytohormone that plays a key role in plant growth and development processes, such as leaf senescence [1, 2], root and shoot development [1, 3], and chloroplast development [4], as well as in biotic and abiotic stress responses [5–7]. In plants, cytokinin is perceived and responded through a multistep phosphorelay pathway, which is similar to the two-component system in bacteria [8, 9]. In Arabidopsis thaliana, cytokinin is recognized by sensor histidine kinases, which autophosphorylate the conserved His residue located at the kinase domain. Then, His-containing phosphotransfer proteins (HPs) transduce a signal. After a multistep His→Asp→His→Asp phosphorelay, the signal is transferred to Arabidopsis response regulators (ARRs), which comprise type-A ARRs, type-B ARRs, and type-C ARRs [9, 10]. Type-A ARRs are rapidly upregulated by cytokinin treatment, whereas type-B ARRs contain transactivating domains that regulate the transcription of cytokinin-activated targets, such as type-A ARRs [11, 12]. Cytokinin response factors (CRFs), a subset of AP2 transcription factors (TFs), are newly identified components in the cytokinin signaling pathway. They likely operate downstream of the HPs and include common or specific targets with type-B ARRs [13].

Thus far, 12 CRFs have been identified in A. thaliana [13, 14], 11 CRFs in Solanum lycopersicum [15], and 21 CRFs in Brassica rapa [16]. As components involved in cytokinin signal transduction, numbers of CRFs are closely related to plant development and stress responses [17]. For example, CRF6 negatively regulates leaf senescence [18], whereas the overexpression lines of CRF1, CRF3, or CRF5 display leaf senescence in early stages in A. thaliana [19]. AtCRF3 and AtCRF5 can promote the growth of lateral roots [19]. CRFs, such as AtCRF2, AtCRF3, and AtCRF6, affect female reproductive organ development by interfering with the development of the placenta and ovules [20]. In addition, numbers of CRF genes in several species are related to abiotic stress responses. In A. thaliana, AtCRF4 can be induced by cold stress [21], and AtCRF6 is related to oxidative stress and salt stress [18, 22]. In S. lycopersicum, SICRF1 can be induced by cold and drought stresses, SICRF2 and SICRF3 are involved in drought and oxidative stresses, and SICRF5 is related to flooding, drought, oxidative, and cold stresses [23, 24]. In B. rapa, BrCRF1, BrCRF2, and BrCRF19 are upregulated by drought stress, whereas BrCRF5 and BrCRF21 are induced by salt stress [16]. Notably, AtCRF2 can positively regulate salicylic acid-mediated plant immunity in A. thaliana, and plants overexpressing AtCRF5 exhibit pathogen resistance. All these phenomena suggest that CRFs play a role in biotic stress responses [25, 26].

Brassica consists of numerous species with a remarkable morphological diversity [27], which is a result of the long-term evolution. There are three diploids, namely, B. rapa (AA, 2n = 20), B. nigra (BB, 2n = 16), and B. oleracea (CC, 2n = 18). As a result of natural hybridization between the diploids, there are three amphidiploids, namely, B. napus (AACC, 2n = 38), B. juncea (AABB, 2n = 36), and B. carinata (BCCC, 2n = 34). The relationship between these six species have been illustrated through the Brassica U-triangle [28]. This group of species provides an excellent model for studies on species evolution by homologous recombination and polyploidization. The relationship among Brassica species has been characterized by many methods [29], such as genomic in situ hybridization (GISH) with fluorescence in situ hybridization (FISH) [30, 31], ribosomal DNA probes [32, 33], sequence-characterized amplified region markers [34] or repetitive sequence elements [35, 36], and simple-sequence repeat (SSR) markers [37, 38]. Meanwhile, relevant analysis of certain genes or gene families were also performed in order to elucidate the evolution of Brassica species [39]. Many genomes of the Brassica species have been sequenced [40–43], thereby providing a basis for evolutionary analysis and smoothing the process of functional gene mining.

In this study, we identified all of the CRF genes in B. rapa, B. nigra, B. oleracea, B. napus, and B. juncea and analyzed their phylogeny, sequence properties, and selective pressure to reveal the evolution among these species. We also examined the expression patterns of these genes in different tissues and organs under various treatments and obtained some useful information for future functional analysis of CRFs. The functions of CRFs are substantially determined by the similarities and differences among their sequences [44, 45]. The similarities and differences among the sequences of CRFs in these species also provided some clues of their functions. This research not only analyzed the functions of CRFs but also offered insights into the evolution of Brassica species, which would make significant impact on functional genomics and breeding improvement in Brassica crops.

Methods
Identification of CRFs in Brassica diploid species and their amphidiploids

The protein sequences of known CRFs in A. thaliana were downloaded from TAIR and then used as seed sequences to search NCBI (https://www.ncbi.nlm.nih.gov/) for B. rapa, B. oleracea, and B. napus and the Brassica Database [46] for B. rapa, B. oleracea, B. nigra, B. juncea, and B. napus. BlastP search with an expected value of 100 was applied. The protein sequences of the identified CRFs were examined with SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1) [47] and ClustalX [48]. Protein sequences that did not contain the AP2
domain or CRF domain were excluded from the further analysis. These protein sequences, CDS, and genome sequences of the CRFs were downloaded from the Brassica Database [46].

Nomenclature and characterization analysis of CRFs
We identified orthologous genes by phylogenetic approach, and did blast to confirm their relations. All of the newly identified CRFs and BrCRFs were named on the basis of their orthologous genes in A. thaliana [14, 16], and the paralogous genes in each species were distinguished by English letters. CRF-related information, such as locus, chromosome position, ORF length, and deduced polypeptide length, was searched from B. rapa version 1.5 genome [42], B. oleracea version 1.1 genome [41], B. napus version 4.1 genome [40], B. nigra version 1.1 genome, and B. juncea version 1.5 genome [43]. The isoelectric point (pI) and molecular weight of the CRFs were predicted using Compute pi/Mw (http://web.expasy.org/compute_pi/) [49–51].

Motif recognition, gene structure, and phylogenetic analysis
The motifs of CRFs were identified with MEME (http://meme-suite.org/tools/meme) [52]. The protein sequences of the identified CRFs were used for multiple-sequence alignment by ClustalW [53] with a gap open penalty of 10 and a gap extension penalty of 0.2, and unrooted phylogenetic trees were generated with the neighbor-joining method with a 1000-replicate bootstrap and other default parameters in MEGA version 6 [54]. The sequence logoes were created by WedLogo 3 (http://weblogo.threeplusone.com/). Finally, the phylogenetic tree of all CRFs was decorated in Itol (http://itol.embl.de/) [55]. The gene structures of CRFs were analyzed and drawn with Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) [56].

Chromosomal mapping and synteny analysis
Chromosomal location maps were drawn with MapChart in accordance with the positions of initiation codons and decorated in PhotoShop CS5 for the transcriptional orientation marks and bars. Synteny analysis was performed on GEvo (https://genomevolution.org/CoGe/GEvo.pl) [57] by using 100 kb sequence before and after each gene respectively, and non-CDS sequences were masked. Figures were decorated in PhotoShop CS5 to mark the gene names.

Analysis of evolutionary selection pressure
The synonymous (Ks) and nonsynonymous substitution (Ka) rates of orthologous and paralogous genes were calculated with MEGA version 6 [54] by using a Compute Pairway Distance plate. Ka/Ks ratios were calculated and subtotaled in Microsoft Excel 2007, and box plots were drawn in E Chart (http://www.ehbio.com/ImageGP/index.php/Home/Index/ Boxplot.html). Ka/Ks values of orthologous genes between Brassica species and A. thaliana with a sliding window of 20 codons were calculated and drafted in MATLAB R2017b [58].

Analysis of the putative promoter regions of CRFs
The upstream sequences (1500 bp) of the initiation codons of BrCRFs, Br1CRFs, Br2CRFs, BnaCRFs, and BjuCRFs were chosen as the putative promoter regions of CRFs and used to identify the cis-elements related to hormones and stresses. The cis-regulatory elements along the putative promoter sequences were identified by using PLACE (https://sogo.dna.afrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace) [59] and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [60].

Plant growth and treatments
B. oleracea cv. Sanxiong was grown in the experimental farm of the Zhejiang Academy of Agricultural Sciences, whereas B. napus line 166–13 and B. nigra line 1611–01 were cultivated in the experimental farm of Zhejiang University. Roots, floral stems, leaves, flowers, siliques, sepals, petals, stamens, and pistils were sampled to analyze tissue- and organ-specific expression.

The three materials were also cultivated under a 14 h light/10 h dark photoperiod at 24 °C/22 °C for about 3 weeks before treatments. For the exogenous hormone treatments, 100 μM 6-BA, NAA, and abscisic acid (ABA) were sprayed onto the three materials, and controls were sprayed with double distilled water only. All of the materials were sampled at 0, 0.5, and 1 h after treatment. In the salt treatment, a nutrient solution with 200 mM NaCl was used as a treatment, and a normal nutrient solution was utilized as control. The materials were sampled at 0, 4, 8, and 16 h after treatment. The second true leaves in all of the treatment groups were sampled to minimize differences. All of the samples were frozen in liquid nitrogen immediately and then stored at −75 °C.

RNA extraction and qRT-PCR analysis
For B. napus and B. nigra samples, TRIzol reagent (Invitrogen, Germany) was used to extract total RNA in accordance with the manufacturer’s instructions, and the first cDNA strand was synthesized using a TaKaRa reverse transcription system (Japan) in accordance with the manufacturer’s protocol. For B. oleracea samples, RNA extraction kits (Omega, USA) were utilized in accordance with the manufacturer’s instructions. qRT-PCR was performed with Primers for qRT-PCR (Additional file 1: Table S1) were designed by Primer version 5.0. A qRT-PCR mixture was 15 μL in volume and composed of 7.5 μL of SYBR Green Master Mix reagent (Toyobo, Japan), 0.6 μL of specific primer, 2 μL...
of cDNA, and 5.9 μL of ddH₂O. qRT-PCR was run using a StepOne real-time PCR machine (BioRAD, USA) programmed to heat for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 45 s at 55–58 °C. The specificity of the reactions was verified through melting curve analysis. GAPDH [61] and 25S [62] were used as internal controls. Comparative ΔΔCT method was applied to analyze the relative expression levels of CRFs. Hierarchical clustering and heatmap representation of the expression pattern of CRFs were drawn in Heml [63]. Three biological replicates were included for each sample.

**Arabidopsis mutant and transformation**

Atcrf11 (AT3G25890) mutant was purchased from ABRC (SALK205786C). The full-length coding sequence of BrCRF11a was inserted into the pCAMBIA1301 vector and driven by the CaMV 35S promoter. The constructed vector was transformed into Arabidopsis plants by the floral-dip method with Agrobacterium GV3101 to obtain BrCRF11a-overexpressing transgenic Arabidopsis. The relative expressions of BrCRF11a and Atcrf11 in the three types of Arabidopsis seedlings were analyzed by qRT-PCR. AtTUB4 (TUBULIN BETA CHAIN 4, AT5G44340) was used as internal reference. The primers used here can be found in the Additional file 1: Table S1. All of the Arabidopsis plants (wild-type Col-0, Atcrf11 mutant and BrCRF11a-overexpressing transgenic Arabidopsis p35S::BrCRF11a) were grown on Murashige and Skoog (MS) medium for 5 days before the root lengths of the seedlings were measured.

**Results**

Identification, classification, and phylogenetic analysis of CRFs in the Brassica genomes

A total of 120 new CRFs, which simultaneously contain the AP2 domain and the CRF domain, were identified in Brassica species: 18 CRFs in B. oleracea, 24 CRFs in B. nigra, 38 CRFs in B. juncea, and 40 CRFs in B. napus (Table 1). The results searched from B. rapa version 1.5 genome were consistent with those from former research [16]. All of the 120 genes were named, and the 21 CRFs in B. rapa were renamed on the basis of their orthologs in A. thaliana. The paralogs were distinguished by English letters (Additional file 2: Table S2).

In addition, the basic information of the genes was searched. The length of the gene sequences ranged from 441 bp to 1151 bp. In all of the species, the shortest gene sequence was that of CRF7, and the longest gene sequence was that of CRF3, except B. juncea in which the longest was CRF12. For the protein length (147–364 amino acids) and molecular weight (16.1–41.4 kDa), the shortest was found in CRF7, and the longest was observed in CRF3 in all of the species herein. The pl of the proteins ranged from 4.56 to 10.00. The protein with the lowest pl was detected in CRF3 in all of the species except B. juncea, whose CRF10 was the protein with the lowest pl. The protein with the highest pl was identified in CRF7 in all of the species except B. nigra, whose CRF8 was the protein with the highest pl.

A phylogenetic tree was constructed using the protein sequences of the genes (Fig. 1). This result showed that the orthologs among genomes and the paralogs in one species were closely related. The genes can be divided into five clades: Clade I (CRF1s and CRF2s), Clade II (CRF3s and CRF4s), Clade III (CRF5s and CRF6s), Clade IV (CRF7s and CRF8s), and Clade V (CRF9–12 s). We found that Clades I, II, III, and V were closely related with one another, whereas Clade IV was distantly related with them.

**Gene structure and conserved motif analysis of CRFs in Brassica species**

The phylogenetic trees of CRFs in B. nigra (Fig. 2a), B. oleracea (Fig. 2b), B. napus (Fig. 2c), and B. juncea (Fig. 2d)

| Table 1 Numbers of CRFs in Brassica species | CRF1 | CRF2 | CRF3 | CRF4 | CRF5 | CRF6 | CRF7 | CRF8 | CRF9 | CRF10 | CRF11 | CRF12 | Total |
|-------------------------------------------|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|
| Species                                   |      |      |      |      |      |      |      |      |      |       |       |       |       |
| A. thaliana                               | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1     | 1     | 1     | 12    |
| B. rapa                                   | 1    | 2    | 3    | 3    | 1    | 1    | 2    | 2    | 0    | 3     | 2     | 1     | 21    |
| B. oleracea                               | 1    | 1    | 3    | 3    | 1    | 2    | 2    | 2    | 2    | 0     | 2     | 1     | 18    |
| B. nigra                                  | 1    | 2    | 3    | 3    | 2    | 3    | 2    | 2    | 0    | 4     | 2     | 0     | 24    |
| B. juncea                                 | Aa 1 | 1    | 2    | 3    | 0    | 2    | 2    | 2    | 1    | 0     | 3     | 2     | 19    |
|                                           | Bb 1 | 1    | 1    | 2    | 0    | 0    | 2    | 1    | 3    | 0     | 2     | 1     | 14    |
|                                           | Ua 0 | 1    | 1    | 1    | 1    | 0    | 0    | 0    | 0    | 1     | 0     | 0     | 5     |
| B. napus                                  | Aa 1 | 1    | 2    | 2    | 3    | 1    | 2    | 2    | 0    | 4     | 1     | 0     | 20    |
|                                           | Cb 1 | 1    | 2    | 3    | 3    | 1    | 2    | 2    | 2    | 0     | 2     | 1     | 18    |
| Total                                     | 8    | 14   | 21   | 17   | 10   | 15   | 14   | 15   | 1    | 22    | 11    | 5     | 153   |

*a means subgenome of the allotetraploid; "U" means unknown
were constructed separately. Five clades remained in each species, and this finding was consistent with the previous phylogenetic analysis (Fig. 1). Notably, all of the species here did not possess any other orthologs of AtCRF9. Gene structure analysis showed that most CRFs had only one exon except BnaCRF1a, BnaCRF1b, BnaCRF2a, BnaCRF3a, and BjuCRF12a, which contained two exons and one intron (Fig. 2). Conserved motif analysis and multiple alignment were performed using the CRFs' protein sequences. The sequences could be divided into three types based on sequence similarity: Type A included proteins in Clades I, II, and III (CRF1–6 s), Type B comprised the proteins in Clade IV (CRF7s and CRF8s), and Type C consisted of proteins in Clade V (CRF9–12 s). All of the proteins had one AP2 domain (Motif 1, 2) and one CRF domain (Motif 3, 4). The TEH region (Motif 6) only existed in the N-terminal region of Type A proteins, and all of the Type A proteins contained a putative mitogen-activated protein kinase (MAPK) phosphorylation site (Motif 5) on the C-terminal region. Type B proteins were shorter and did not have motifs other than the AP2 and CRF domains. The length of the Type C proteins was similar to that of the Type A proteins. However, the former did not contain a TEH region and an MAPK motif. Instead, they possessed motifs with consensus sequences [FNF × ×L × IPD] and [GPSxLPD × DF × DV] (Fig. 2 and Additional file 3: Figure S1).

**Chromosomal distribution comparison and synteny analysis of CRFs among Brassica species**

A refined analysis of synteny blocks was performed by GEvo website (Additional file 4: Figure S2) and provided some clear hints to the origin of genes in allotetraploids (Table 2 and Additional file 5: Table S3). The gene origins of most of the genes in the allotetraploids could be found in the diploids, whereas few of them were newly generated. Moreover, some genes in the diploids did not have corresponding genes in the allotetraploids. In addition, some genes (BnaCRF2d, BnaCRF6c, BjuCRF6c

---

**Fig. 1** Phylogenetic tree based on CRF homologous protein sequences in *Brassica* plants. The CRFs are divided into five clades and marked by different colors. The equidistant rings indicate the scale of relative divergence. The bootstrap values were calculated from 1000 replicates and the values less than 50% were hided. The figure was obtained by MEGA version 6 and Itol (http://itol.embl.de/)
and BjuCRF12b) were highly similar to their paralogous genes (Additional file 4: Figure S2).

Chromosomal mapping of CRFs in B. rapa, B. oleracea, B. nigra, B. juncea, and B. napus was performed separately on basis of genomic information (Fig. 3 and Additional file 6: Figure S3). Results showed that the CRFs were distributed throughout almost all the chromosomes in every genome, except A04 in B. rapa; B05 in B. nigra; C04 and C05 in B. oleracea; A04, A10, and C09 in B. napus; and A04 and B01 in B. juncea.

By comparing the B. oleracea genome with the C sub-genome of B. napus (Fig. 3b and c), the chromosomal location of most allotetraploid genes are similar to their origin genes. Although some genes, such as BolCRF5, BolCRF7a, BolCRF8a, BolCRF10a, and BnaCRF8c, possess incomplete location information, their corresponding genes offer hints. However, gene rearrangements are also present. For example, BolCRF3c is located in chromosome C07 in B. oleracea, and its corresponding gene BnaCRF3d is in chromosome C02 in B. napus. Moreover, no origin gene of BnaCRF2d is present on the corresponding site in the B. oleracea genome. On one hand, the origin gene may have degenerated after hybridization. On the other hand, the chromosomal sequence around BnaCRF2d is highly similar to that of

![Fig. 2](image-url)  
**Fig. 2** Phylogenetic relationships, gene structures, and motif analysis of CRFs in Brassica species. The CRFs in B. nigra (a), B. oleracea (b), B. napus (c), and B. juncea (d) are shown. In every figure, the phylogenetic relationship is shown on the left, the gene structure in middle, and the motifs on the right. The genes belonging to different clades were marked by red Latin numbers. Motif 1 and Motif 2 formed the AP2 domain. The CRF domain was comprised of Motif 3 and Motif 4. Motif 5 was a putative mitogen-activated protein kinase (MAPK) phosphorylation site. Motif 6 represented the TEH region.
BnaCRF2b and may be a copy of BnaCRF2b derived from segmental duplication or another mechanism. Indeed, BolCRF3b and BolCRF4c possess no corresponding gene in the other genome. The relationship of the corresponding genes between B. rapa and the A subgenomes of B. napus and B. juncea is similar to that between B. oleracea and the C subgenome of B. napus (Fig. 3a, c and Additional file 6: Figure S3B). In the A subgenome of B. napus, BnaCRF4a, BnaCRF6a, and BnaCRF8d are rearranged after hybridization, and the corresponding genes of BrCRF3a, BrCRF4c, BrCRF7a, and BrCRF11b were not found. Especially, BnaCRF12, the corresponding gene of BrCRF12, is located in chromosome C05, which belongs to the C subgenome of B. napus. Similarly, BnaCRF6c is also significantly similar to BnaCRF6b in chromosome C08. In the A subgenome of B. juncea, only the corresponding genes of BrCRF4a, BrCRF4b, and BrCRF4c were not observed. Although the information was incomplete for BjuCRF4, the number of CRF4 genes decreased significantly in B. juncea. By contrast, no origin gene of BjuCRF5b was found in B. rapa; the gene possibly degenerated after hybridization. BjuCRF8a, the corresponding gene of BrCRF8a, was rearranged from the A subgenome to C subgenome. At the same time, BjuCRF6c was highly similar to BjuCRF6b, which was coincident with BnaCRF6c in B. napus. This situation was also noted between BjuCRF12a and BjuCRF12b.

These findings were much more complicated for B. nigra and B. juncea (Additional file 6: Figure S3). All the corresponding genes were rearranged, and no significant regularity was found among these genes. A total of 7 of 24 CRFs in B. nigra have no corresponding genes in B. juncea, and this proportion was higher than that in B. rapa (1 of 21) and in B. oleracea (2 of 18).

**Gene retention ratio and evolutionary selection pressure analysis of CRFs in the Brassica species**

The numbers of CRFs counted by genome (subgenome) and gene name separately are displayed in Table 1. Genome triplication occurred in the Brassica species since their divergence from the A. thaliana lineage at about 13–17 million years ago (MYA) [42]. Thus, the diploids and their amphidiploids reasonably possess 3 and 6 paralogs, respectively, for every corresponding CRF gene in A. thaliana. On this basis, gene retention ratios were calculated by genome (subgenome) and gene name separately (Additional file 7: Figure S4). Although the gene retention ratios in the genomes or subgenomes were almost the same, the gene retention ratios for B. nigra and B. rapa were higher, but lower for the B sub-genome in B. juncea, than that in the other species. For the gene retention ratios of different genes, more CRF10 genes and less CRF12 genes were maintained. Furthermore, no CRF9 gene existed in the Brassica species. In addition, the gene retention ratios of CRFs in the three sub-genomes of B. rapa, B. oleracea, and B. napus were analyzed (Additional file 8: Figure S5.). The result showed that the gene retention ratios of CRFs in the three sub-genomes were the same in B. oleracea. In B. rapa, B. napus A, and B. napus C, the gene retention ratios of CRFs were higher in LF and MF1 and lower in MF2. Furthermore, the gene retention ratios of CRFs in the three sub-genomes in B. rapa and B. oleracea were
higher (0.5 or 0.58) than that in *B. napus A* and *B. napus C* (0.33 or 0.42).

The *Ks* (synonymous substitution rates) and *Ka* (non-synonymous substitution rates) were determined to explore the gene divergence after duplication. The *Ka/Ks* value indicates the selection pressure on a gene pair. *Ka/Ks* < 1 means negative selection, *Ka/Ks* = 1 means neutral selection, and *Ka/Ks* > 1 means positive selection [64]. We obtained the *Ka/Ks* ratios from duplicated *CRF* orthologous gene pairs (Fig. 4a and b) between the *Brassica* species and *A. thaliana* and duplicated *CRF* paralogous gene pairs (Fig. 4c and d) in every *Brassica* genome. All the gene pairs underwent negative selection, and the *Ka/Ks* values ranged from 0 to 0.5. In different *Brassica* species, the *Ka/Ks* values of *CRF* gene pairs seemed to be similar. The results indicate that the *Brassica* species have encountered the similar selection pressure during the processes of domestication (Fig. 4a and c). For different *CRF* genes, in the evolution between *Brassica* species and *A. thaliana*, the *Ka/Ks* values of *CRF7s* were the lowest, whereas those of the *CRF11s* were the highest. However, in the *Brassica* species, the *Ka/Ks* values of the *CRF1s* and *CRF8s* were the lower, whereas those of *CRF6s*, *CRF7s*, *CRF10s*, and *CRF11s* were relatively higher (Fig. 4b and d).

Furthermore, we calculated the *Ka/Ks* values of the *CRF* orthologous gene pairs of *Br-At*, *Bol-At*, and *Bna-At* with a sliding window of 20 codons. Thus, we observed whether the *Ka/Ks* ratios changed in different parts of the protein (Additional file 9: Figure S6). We found that the *Ka/Ks* values of different protein parts were extremely distinct. Almost every protein contained at least one part with a *Ka/Ks* value more than 1. This result indicates that the mutations in these parts underwent positive selection. However, most of these parts avoided but were close to the conserved motifs. In addition, the C-termination parts of the proteins usually possessed relatively high *Ka/Ks* values of more than 1. Meanwhile, the orthologous genes manifested a similar variation trend.

**Analysis of the putative promoter regions of CRFs in Brassica species**

Gene expression variation among species is believed to be responsible for much of the phenotypic diversity [65], and many genes, with tissue-specific or stress-responsive expression patterns, are closely related to the cis-regulatory elements located upstream of these genes [66]. To thoroughly understand the function of the *CRF*
Expression pattern of CRFs in different tissues and organs of the Brassica species

The temporal and spatial expression of genes is the foundation of their function [67]. To obtain some clues about the functions of BolCRFs, BniCRFs, and BnaCRFs, qRT-PCR was used to analyze the transcription levels of these genes in the roots, stems, leaves, flowers, and siliques (Fig. 5). This result showed that the relative expressions of 4 BniCRFs (BniCRF3a, 3b, 5b, and 6a) and 11 BnaCRFs (BnaCRF1b, 2b, 2c, 3a, 3b, 3e, 4e, 4f, 5a, 5b, and 6a) among different tissues were more different than those of BolCRFs and other BniCRFs or BnaCRFs. Meanwhile, we found that almost all the CRFs exhibited higher relative expression levels in flowers and siliques. A total of 42, 59, and 48 out of 81 analyzed CRFs also showed more transcripts in the roots, stems, and leaves, respectively. For paralogous genes in one species, some genes showed similar expression patterns in different tissues (BolCRF3b with BolCRF3c, BniCRF8a with BniCRF8b, BnaCRF4a with BnaCRF4d, and so on), while some genes were expressed complementarily (BolCRF4a with BolCRF4b, BolCRF7a with BnaCRF7b, BnaCRF6a with BnaCRF6c, and so on). As regards the relationship between original genes and their corresponding genes, BolCRF1, BolCRF2, BolCRF3a, BolCRF3c, BolCRF4b, BolCRF5, BolCRF6a, BolCRF7a, and BolCRF8a exhibited similar expression patterns to their corresponding genes, but BolCRF4a, BolCRF6b, BolCRF7b, BolCRF8b, BolCRF10a, BolCRF10b, and BolCRF F11 displayed complementary expression profiles. Furthermore, by comparing the collinearity genes between B. oleracea and B. nigra, we noted the lacking orderliness that may have been derived from the distant relationship between such species.

Because CRFs belong to the AP2 super family [13] and AP2 is considered to be important for flower development [68], we further analyzed the transcriptional levels of the CRFs in sepals, petals, stamens, and pistils (Fig. 5). We found that most of the BolCRFs and BniCRFs showed smaller changes of relative expression levels in the four floral organs, whereas the relative expression levels of most BnaCRFs changed a lot among the four floral organs. Furthermore, some genes showed pistil-preferential expression among the four floral organs. These genes were BolCRF2, BolCRF3b, BolCRF4c, BniCRF11b, BnaCRF4e, and BnaCRF10b. Meanwhile, BolCRF1, BolCRF4a, BniCRF6c, BnaCRF1b, BnaCRF2b, BnaCRF3a, BnaCRF3b, BnaCRF3e, BnaCRF4f, BnaCRF5a, BnaCRF5b, and BnaCRF6a also revealed higher relative expression levels in pistil. On the contrary, genes such as BolCRF7b, BniCRF5a, BniCRF5b, BniCRF6a, BnaCRF7a, BnaCRF7b, and BnaCRF7d exhibited lower relative expression levels in the pistil but higher relative expression levels in other floral organs.

Effects of exogenous 6-BA, NAA, and ABA on the expression of CRFs in Brassica species

CRF genes were firstly discovered to respond to exogenous cytokinin, which explains their designation as CRFs [13]. To confirm whether the CRFs respond to cytokinin, qRT-PCR was applied to test the relative expression levels of the CRFs at 0.5 and 1 h after 6-BA treatment. Results showed most of the CRFs tested responded to exogenous cytokinin in various degrees (Fig. 6a). BolCRF3b, BolCRF6a, BolCRF6b, BolCRF10a, BniCRF1, BniCRF2b, BniCRF3a, BniCRF8a, BniCRF8b, BnaCRF10a, BniCRF10c, BnaCRF1b, BnaCRF2a, BnaCRF2b, BnaCRF2d, BnaCRF3c, BnaCRF3e, BnaCRF4b, BnaCRF4e, BnaCRF5a, BnaCRF5b, BnaCRF6b, BnaCRF6c, BnaCRF6d, BnaCRF7a, BnaCRF10c, BnaCRF10f, and BnaCRF11b were up-regulated significantly, whereas BolCRF3c, BolCRF4a, BolCRF4b, BolCRF5, BolCRF5b, BniCRF3c, BniCRF4a, BniCRF4b, BniCRF6c, and BnaCRF4c were obviously down-regulated. The genes initially up-regulated then down-regulated were BniCRF2a, BniCRF5a, BniCRF5b, BniCRF10b, and BniCRF11b, whereas those initially down-regulated then up-regulated were BniCRF6b, BnaCRF1a, BnaCRF2c, and BnaCRF6a. Three pairs of genes in B. oleracea and B. napus (BolCRF6a with BnaCRF6d, BolCRF6b with BnaCRF6b, and BolCRF10a with BnaCRF10f) showed the same response profiles. No significant regularity was recognized between B. oleracea and B. nigra.

In addition, more than 20 auxin-related genes can also be regulated by cytokinin, this observation suggests that auxin–cytokinin crosstalk may result from some genes co-regulated by the two hormones [69]. We analyzed the
relative expression levels of the CRFs at 0.5 and 1 h after NAA treatment; the results revealed that numbers of genes can be regulated by NAA (Fig. 6b). On one hand, some genes were significantly up-regulated and included BolCRF3a, BolCRF4a, BolCRF4b, BniCRF5a, BniCRF6a, BniCRF7a, BniCRF10c, BnaCRF1a, BnaCRF1b, BnaCRF2a, BnaCRF3b, BnaCRF3c, BnaCRF3e, and BnaCRF10a. On the other hand, several genes were down-regulated obviously, namely, BolCRF3b, BolCRF4a, BolCRF4c, BolCRF6a, BniCRF1, BniCRF2a, BniCRF4c, BniCRF6c, BnaCRF2c, BnaCRF3a, BnaCRF3d, BnaCRF4a, BnaCRF4d, BnaCRF4e, BnaCRF4f, BnaCRF5a, BnaCRF5b, BnaCRF6b, BnaCRF10b, BnaCRF10d, and BnaCRF10e. Meanwhile, BolCRF3c, BnaCRF2b, BnaCRF4b, BnaCRF4c, BnaCRF6a, BnaCRF7a, BnaCRF7b, BnaCRF7c, BnaCRF7d, BnaCRF8a, BnaCRF8b, BnaCRF8c, BnaCRF8d, BnaCRF10c, BnaCRF11a, BnaCRF11b, and BnaCRF12 were initially down-regulated and then up-regulated. Conversely, BniCRF4b and BniCRF8b were initially up-regulated then down-regulated. Significantly, the paralogous genes of BnaCRF1, BnaCRF5, BnaCRF7, BnaCRF8, and BnaCRF11 displayed similar response profiles. The response profiles to ABA of two genes, namely, BolCRF4a and BolCRF6a, were similar to those of their corresponding genes in B. napus (BnaCRF4f and BnaCRF6d, respectively).

Analysis of the promoter regions of the CRFs showed the existence of several elements related to ABA. Therefore, ABA treatment was performed to test whether the CRFs can respond to exogenous ABA. qRT-PCR was performed to analyze the relative expression levels of the CRFs at 0.5 and 1 h after ABA treatment. In fact, many CRFs responded to ABA (Fig. 6c). BolCRF4b, BniCRF1, BniCRF2a, BniCRF3a, BniCRF3b, BniCRF3c, BniCRF4a, BniCRF4b, BniCRF4c, BniCRF5a, BniCRF5c, BniCRF6a, BniCRF6b, BniCRF7a, BniCRF7b, BniCRF9a, BniCRF9b, BniCRF10a, BniCRF10b, BniCRF10c, BniCRF11a, BniCRF11b, BnaCRF1b, BnaCRF2c, BnaCRF5a, BnaCRF5b, BnaCRF7a, BnaCRF10a, BnaCRF10c, and BnaCRF10d were up-regulated by ABA. On the contrary, ABA can significantly down-regulate BolCRF3a, BolCRF3b, BolCRF4a, BolCRF6a, BolCRF8a, BolCRF8b, BolCRF10a, BnaCRF2a, BnaCRF6a, BnaCRF6d, BnaCRF7b, BnaCRF8c, BnaCRF8d, BnaCRF10e, BnaCRF10f, BnaCRF11a, BnaCRF11b, and BnaCRF12. Meanwhile, BnaCRF2d, BnaCRF4c, and BnaCRF4e are down-regulated initially and subsequently up-regulated. Especially, paralogous genes of
BniCRF3, BniCRF4, BniCRF10, BniCRF11, BolCRF8, and BnaCRF5 show similar response profiles, and the response profiles of BolCRF6a, BolCRF8a, and BolCRF10a are consistent with those of their corresponding genes in B. napus (BnaCRF6d, BnaCRF8c, and BnaCRF10f).

Expression profiles of BolCRFs, BniCRFs, and BnaCRFs under salt stress

To date, many genes belonging to the AP2/ERF gene family from various plant species have been shown to be involved in abiotic stress responses [70, 71], especially in the drought and salt stress response [72]. The expression profiles of CRFs under salt stress for 4, 8, and 16 h were analyzed by qRT-PCR (Fig. 7). In B. oleracea, BolCRF3b and BolCRF11 were up-regulated, while BolCRF1, BolCRF2, BolCRF3a, BolCRF3c, and BolCRF4b were down-regulated continuously after salt treatment. The expressions of other BolCRFs were not changed. In B. nigra, 5 genes (BniCRF1, BniCRF2a, BniCRF10a, BniCRF11a, and BniCRF11b) were up-regulated, while 4 genes (BniCRF3a, BniCRF3b, BniCRF4c, and BniCRF5b) were down-regulated sustainedly, and the expression changes of the other BniCRFs were zigzag. In B. napus, 8 genes were up-regulated persistently, namely BnaCRF3e, BnaCRF4b, BnaCRF4e, BnaCRF5b, BnaCR10b, Bna CR10e, BnaCRF10f, and BnaCRF12. Five genes, consist
of BnaCRF2a, BnaCRF3b, BnaCRF10c, BnaCRF10d, and BnaCRF11a, were down-regulated continuously. However, the expression changes of the other BnaCRFs were fluctuant under salt stress.

Functional analysis of AtCRF11 and BrCRF11a in the root growth of Arabidopsis

Many CRFs were found to be highly expressed in the root, and some can even modulate root growth in Arabidopsis [19, 22]. However, most studies focused on the CRFs in Clades I, II, III, and IV; studies on the CRFs of Clade V are limited. Interestingly, in our previous study, we found that some CRFs in Clade V, such as CRF11, exhibited preferential expression in the root of B. rapa. To examine whether AtCRF11 and its orthologous gene BrCRF11a play a role in the root growth of Arabidopsis, we grew three types of Arabidopsis (wild type Col-0, Atcrf11 mutant and BrCRF11a-overexpressing transgenic Arabidopsis p35S::BrCRF11a) on MS medium. Relative expressions of BrCRF11a and AtCRF11 in the three types of Arabidopsis seedlings were analyzed (Fig. 8b). BrCRF11a and AtCRF11 are orthologous genes, and the similarity of their nucleotide sequences is 81%. It is difficult to design primers for qRT-PCR to distinguish them perfectly. They might affect each other in the expression analysis, but we could find the up-expression and down-expression of the two genes to some extent. After 5 days, an obvious difference was noted in the root lengths of the three types of Arabidopsis (Fig. 8a). In the Atcrf11 mutant, the root length was shorter than that of the wild type, whereas that of p35S::BrCRF11a was much longer than that of the wild type. The statistical analysis of the changing root lengths among the three types of Arabidopsis showed that the differences were fairly significant (Fig. 8c).

Discussion

In this study, we identified the CRFs (Additional file 2: Table S2) in diploids (B. rapa, B. nigra, and B. oleracea) and amphidiploids (B. napus and B. juncea) of Brassica. Although no new member was found in B. rapa [16], we updated some of the species’ information, which is essential to subsequent investigations. In general, most of the new CRFs, except for BnaCRF1a, BnaCRF1b, BnaCRF2a, BnaCRF3a, and BjuCRF12a, do not contain introns (Fig. 2). Among these genes, BnaCRF1a and
BjuCRF12a, are consistent with their original genes BrCRF1 and BrCRF12, respectively, whereas the introns of BnaCRF1b, BnaCRF2a, and BnaCRF3a might emerge after the hybridization of the A and C genomes. In terms of CDS and protein lengths, as well as the molecular weights and pI of the CRFs in the same clade, similar characters were found among the Brassica species. In addition, the protein sequences of all the CRFs were aligned by ClustalX [48], and similar domains and motifs were identified (Additional file 3: Figure S1) [16, 45]. These results suggest that CRFs possess conserved functions among these species, which coincide with the previous result [14].

The Brassica diploids underwent an extra whole-genome triplication (WGT) event after diverging from A. thaliana [73, 74]. Thus, we calculated the gene retention of every gene and classified them by genome/subgenome and gene name (Additional file 7: Figure S4). This result showed that the gene retentions were similar among genomes but ranged from genes with no ortholog of AtCRF9 to those with almost no loss of AtCRF10 ortholog. This biased gene loss following whole-genome duplication is considered to indicate the gene functions [75].

To analyze the selective pressure suffered by the orthologous and paralogous gene pairs, the Ka/Ks ratios were calculated. Ka/Ks < 1 reveals a negative selection, whereas Ka/Ks > 1 indicates a positive selection. Generally speaking, the Ka/Ks ratios of all the CRFs ranged from 0 to 0.5, which suggests a stringent negative selection applied on these genes. Meanwhile, in different Brassica species, the Ka/Ks values of orthologs or paralogs were almost the same (Fig. 4a and c). However, the Ka/Ks values of the orthologs or paralogs showed an extraordinary difference when classified by gene name. For the orthologous gene pairs between Brassica species and A. thaliana, the selection pressures on the CRF7s were the severest, and the selection pressures on the CRF11s were the mildest along the evolution from A. thaliana to Brassica species (Fig. 4b). For the paralogs within each of the Brassica species, the selective pressures on the paralogous genes of CRF1s and CRF8s were likely stronger, and the paralogs of CRF6s, CRF7s, CRF10s, and CRF11s have encountered a milder natural selection within a Brassica genome. Meanwhile, the Ka/Ks values of orthologous gene pairs were around 0.3, which was higher than those of paralogous gene pairs at about 0.2. This result suggests a weaker natural selection between the Brassica species and A. thaliana than that in the Brassica species alone. Furthermore, meticulous works were carried out to analyze the Ka/Ks values of the different regions in one gene compared with its ortholog in A. thaliana with a sliding window (Additional file 9: Figure S6). We found that most of the CRFs in B. rapa, B. oleracea, and B. napus achieved a peak of Ka/Ks value of
more than 1 in the last 100 codons of the C-terminal region. This result implies that the mutations in this region are positively selected, which highly likely embodies their functional differentiation. This opinion is supported by previous research; for instance, the transcriptional activity of AtCRF5 was found to be governed by the C-terminal domain [44], and the C-terminal sequence divergence was correlated with vascular expression [45]. Besides, some Ka/Ks peaks exceeding 1 were located in the N-termination and in regions close to the AP2 domain; the functions of these regions remain to be elucidated.

Given the wide application of synteny analysis in genome comparison and evolutionary analyses [76, 77], we performed a meticulous synteny analysis for the CRFs between the amphidiploids and their parental diploids to ascertain where the CRFs in the amphidiploids originate (Additional file 4: Figure S2). As a result, we obtained the original genes in the diploids and their corresponding genes in the amphidiploids (Table 2 and Additional file 5: Table S3). On this basis, the gene rearrangements that occurred after hybridization were discovered, such as BolCRF3c with BnaCRF3d, BnaCRF4a with BrCRF4a, BjuCRF8a with BrCRF8a and so on. Moreover, many genes, such as BrCRF11b, BnaCRF3e, BnaCRF4c, and BjuCRF5b, do not possess an original gene or corresponding amphidiploid gene probably because of incomplete genome information or the degeneration of corresponding genes during evolution after hybridization. The CRF distribution in the B. nigra genome differed from those in B. rapa and B. oleracea; this result is consistent with a previous conclusion on the close relativity of the Brassica A and C genomes and the distinctiveness of the B genome [78, 79]. However, the CRF distribution significantly differed between the genome of B. nigra and the B subgenome of B. juncea. This result implies that the CRFs in the B subgenome were considerably influenced by the A subgenome of B. juncea. Otherwise, this result was derived from the low sequencing proportion (68%) of the B. nigra genome [43]. Notably, four gene pairs (BnaCRF2b with BrnCRF2d, BnaCRF6b with BnaCRF6c, BjuCRF6b with BjuCRF6c, and BjuCRF12a with BjuCRF12b) were extraordinarily similar to each other and located in different subgenomes of an amphidiploid. On one hand, one gene in the pair may be a copy of the other, which was rearranged [80, 81]. On the other hand, a similar gene may exist in their parental genomes but degenerated after hybridization.

To find clues to the functions of the CRFs in B. nigra, B. oleracea, and B. rapa, we analyzed the expression patterns of these genes in different organs. All the CRFs were constitutively expressed genes, but different relative expression levels were noted in different organs (Fig. 5). The relative expression levels of almost all of the CRFs were higher in the flowers and siliques; this observation suggests the genes’ important roles in reproductively development, which was coincident with previous research [20]. Among the four floral organs, many CRFs presented higher relative expression levels in the pistils; this finding indicates the link between CRFs and pistil development. The crosstalk of cytokinin with other phytohormones is well known to be involved in many plant growth processes [3, 82]. In this present research, numerous cis-elements related to hormones were found in the putative promoter region of the CRFs (Additional file 10: Table S4 and Additional file 11: Table S5). Additionally, most CRFs were found to respond to cytokinin (6-BA), auxin (NAA), and ABA treatments, although the response patterns are dissimilar (Fig. 6). Such result agrees with those of the CRFs that highly participate in the crosstalk between cytokinin and other hormones, such as auxin, ABA, and salicylic acid [20, 25, 83]. To date, numbers of AP2/ERF TFs have been identified to be related to abiotic stress responses in various plant species [70, 71]. Since a subfamily of the AP2/ERF family consist of ERF TFs, some members of the ERF subfamily have been proven to respond to many abiotic stresses, such as high salinity and drought [84]. Thus, CRFs, as Group VI and VI-L members of the ERF subfamily, also share the capability to respond to abiotic stresses [16, 23]. A similar result was obtained in this study (Fig. 7), and cis-elements related to stresses in the putative promoter region of the CRFs (Additional file 10: Table S4 and Additional file 11: Table S5) also supported our results. B. napus and b. juncea are known to be formed ~7500 years or 0.038–0.055 MYA by the hybridization between B. rapa and B. oleracea/B. nigra [40, 43]; such event is far behind the divergence time of B. rapa (13–17 MYA) [42]. Although the evolution time is relatively shorter for the amphidiploid B. napus, the CRFs in this genome considerably differ from those of its parental genomes. For example, only 3 of 16 CRF gene pairs between B. napus and B. oleracea exhibited metastable expression patterns in at least two situations in this research. The orthologous gene pairs among the three diploids also shared low similarities in expression profiles [16]. All these results indicate that CRFs likely serve species-specific functions despite their numerous common functions.

In our previous study [16], we found that the relative expression level of BrCRF11a in the root of B. rapa was higher. In this present study, we found that the relative expression levels of BolCRF11, BniCRF11a, and BniCRF11b in the root were also higher (Fig. 5). These results indicate that the CRF11s may be related to the root growth. We analyzed the functions of AtCRF11 and BrCRF11a in the Arabidopsis root. Knocking out AtCRF11 inhibited primary root growth, whereas overexpressing BrCRF11a in Arabidopsis resulted in primary root growth promotion. These findings confirmed our
hypothesis and agreed with those of previous works, which indicated the expression or function of the CRF genes in the root in many species, such as A. thaliana [19], S. lycopersicum [24], and B. rapa [16]. However, the relative expression levels of BnaCRF11a and BnaCRF11b in the root were lower. It needs further study to analyze the functions of CRF11s in B. rapa, B. oleracea, and B. napus, to find whether the functional differentiation exists and to explore their precise biological roles.

Conclusion
In this study, we characterized 141 CRF genes in three diploids and two amphidiploids of Brassica U-triangle species. On the basis of the comparisons among their sequences and expression patterns, we analyzed the functional inheritance and differentiation of CRFs among the species during the evolution. Our results showed the close relativity of the Brassica A and C genomes and the distinctiveness of the B genome, and the B subgenome was considerably influenced by the A subgenome of B. juncea. Furthermore, we firstly discovered the function of a Clade V CRF, CRF11, related to root growth. This study provided insights into the functional genomics and evolutionary biology of plants and obtained useful information for fine farming and improved breeding.

Additional files

Additional file 1: Table S1. Primers used for qRT-PCR. (XLS 47 kb)

Additional file 2: Table S2. Characterization of CRFs in Brassica species. (DOC 255 kb)

Additional file 3: Figure S1. Conserve motif alignment of three types CRFs. The figure was obtained by ClustalX and WedLogo 3 (https://weblogo.threebpusone.com/). (DOC 3169 kb)

Additional file 4: Figure S2. Synteny analysis of CRFs among Brassica species. (A) Alignment between genes in diploid and in allotetraploid. (B) Alignment between similar genes in allotetraploid. Gene models are colored in grey (gene) and green (CDS). Red blocks indicate the high-scoring segment pair (HSP), and pink links show the connectors between HSP. The figure was formed on Gevo (https://genomewolution.org/CoGe/Gevo/). (TIF 8633 kb)

Additional file 5: Table S3. List of origin genes in B. rapa, B. nigra and amphidiploid genes in B. napus, B. juncea. High-similarity genes are marked in bold. (DOC 58 kb)

Additional file 6: Figure S3. Chromosomal mapping of CRFs in B. nigra and B. juncea. The CRFs in B. nigra (A) and B. juncea (B) are shown except those located on the scaffolds. The locations were shown on the left of the chromosomes, whereas the gene names were on the right. The arrows next to gene names show the direction of transcription. The bar indicates the size of 5 Mb. (TIF 9375 kb)

Additional file 7: Figure S4. Box-plots of gene retention ratio. Statistics by genomes (A) and by gene names (B) are shown. The Violin plots indicate the number of the same value. (TIF 3464 kb)

Additional file 8: Figure S5. Gene retention ratios of CRFs in the three sub-genomes of B. rapa, B. oleracea, and B. napus. (TIF 1003 kb)

Additional file 9: Figure S6. Ka/Ks values of CRF orthologous gene pairs of Br-At, Bo-At and Bna-At over a sliding window of 20 codons. The x-axis indicates the starting codon of sliding window. The y-axis shows the Ka/Ks values. (DOC 1218 kb)

Additional file 10: Table S4. Elements relate to hormone and stress in the promotor of CRFs predicted by PLACE. (XLS 39 kb)

Additional file 11: Table S5. Elements relate to hormone and stress in the promotor of CRFs predicted by PlantCARE. (XLS 37 kb)

Abbreviations
6-BA: 6-Benzylaminopurine; A: Arabidopsis; ABA: Abscisic acid; ARRs: Arabidopsis response regulators; B: Brassica; CRF: Cytokinin Response Factor; HPS: His-containing phosphotransfer proteins; Ka: Non-synonymous substitution rates; Ks: Synonymous substitution rates; MS: Murashige and Skoog; MYA: Million years ago; NAA: 1-naphthylacetic acid; qRT-PCR: Quantitative reverse-transcription PCR; S: Solanum; TFs: Transcriptional factors; WGT: Whole-genome triplication

Acknowledgements
The authors gratefully acknowledge Dr. Longjiang Fan and Dr. Enhui Shen for their technological guidance, and thank Dr. Gang Lu, in particular, for the stimulating discussions and critical reading of the manuscript.

Funding
This work was partially supported by the National Key Research and Development Program of China (2016YFD0100204–31), the 948 Project of Agricultural Ministry of China (2014-228), National Natural Science Foundation of China (3146521, 31700272 and 3187210), the Breeding Project of the Sci-tech Foundation of Zhejiang Province (2016C02085–6–1), the Project of the Sci-tech Foundation of Ningbo City (2015C10008), the Development Project of Wuxi City (CJEGQ1603), and the Project of Application on Public Welfare Technology in Zhejiang Province (LGN18C150003). The funding body had not involved in the design of the study and collection, analysis, and interpretation of the data or in writing the manuscript.

Availability of data and materials
Plant materials are available under request to the respective owner institutions. The datasets supporting the results of this article are included within the article and its additional files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
XY and LK proposed and designed the research. LK and KZ performed the PCR analysis. LM, HD, CC, and LK performed the statistical analyses, interpretation of experimental results. LK, XY and ZL wrote and revised the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable. This study has not directly involved humans and animals. The plant materials have not been directly used. The seeds of Brassica oleracea were obtained from Zhejiang Academy of Agricultural Sciences. The seeds of B. nigra, B. juncea and Arabidopsis thaliana were obtained from our laboratory. All transgenic plants were die out. We comply with the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Laboratory of Cell and Molecular Biology, Institute of Vegetable Science, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, Zhejiang, China. 2Key Laboratory of Horticultural Plant Growth, Development, and Quality Improvement, Ministry of Agriculture, Zhejiang Provincial Key
Laboratory of Horticultural Plant Integrative Biology, Hangzhou 310058, Zhejiang, China. 4College of Agriculture and Forestry Sciences, Linyi University, Linyi 276000, Shandong, China.

Received: 23 April 2018 Accepted: 25 September 2018

Published online: 03 October 2018

References

1. Riefler M, Novak O, Strnad M, Schmulling T. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell. 2006;18(1):40–54.

2. Ma XQ, Zhang J, Burgess P, Rossi S, Huang BR. Interactive effects of melatonin and cytokinin on alleviating drought-induced leaf senescence in creeping bentgrass (Agrostis stolonifera). Environ Exp Bot. 2018;145:1–11.

3. Liu J, Moore S, Chen CL, Lindsey K. Crosstalk complexities between auxin, cytokinin, and ethylene in Arabidopsis root development: from experiments to systems modeling, and back again. Mol Plant. 2017;10(12):1480–96.

4. Cortellessa I, Taniguchi M, Schlick H, hill K, Grimm B, Schaller GE, Schmulling T. Cytokinin regulates the etoposide-chloroplast transition through the two-component signaling system and activation of chloroplast-related genes. Plant Physiol. 2016;172(1):464–78.

5. O'Brien JA, Benkova E. Cytokinin cross-talking during biotic and abiotic stress responses. Front Plant Sci. 2013;4:451.

6. Wastef J, Cheng ST, Wu Y, Ren BZ, Qian W. A bacterial receptor PchK senses the plant hormone cytokinin to promote adaptation to oxidative stress. Cell Rep. 2017;21(10):2940–51.

7. Zwick PI, Rashotte AM. Interactions between cytokinin signalling and abiotic stress responses. J Exp Bot. 2015;66(16):4863–71.

8. Kakimoto T. Perception and signal transduction of cytokinins. Annu Rev Plant Physiol Plant Mol Biol. 2003;54:605–27.

9. To JPC, Kieber JJ. Cytokinin signaling: two-components and more. Trends Plant Sci. 2008;13(2):85–91.

10. Shi X, Gupta S, Rashotte AM, Schaller GE, Kieber JJ. A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Plant Physiol. 2006;142(3):910–21.

11. Mason KG, Mathews DE, Argyros DA, Maxwell BB, Kieber JJ, Alonso JM, Ecker JR, Schaller GE. Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell. 2005;17(11):3007–19.

12. Ferreira FJ, Kieber JJ. Cytokinin signaling. Curr Opin Plant Biol. 2005;8(5):518–27.

13. Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ. A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Plant Physiol. 2006;142(3):910–21.

14. Rashotte AM, Goertzen LR. The CRF domain defines Cytokinin response factor proteins in plants. BMC Plant Biol. 2010;10:74.

15. Shi X, Gupta S, Rashotte AM. Solanum lycopersicum cytokinin response factor (SCRF) genes: characterization of CRF domain-containing ERF genes in tomato. J Exp Bot. 2012;63(2):973–82.

16. Liu Z, Kong L, Zhang M, Lv Y, Liu Y, Zou M, Lu G, Cao J, Yu X. Genome-wide identification, phylogeny, evolution and expression patterns of AP2/ERF genes and cytokinin response factors in Brassica rapa ssp. pekinensis. PLoS One. 2013;8(12):e83444.

17. Kim J. CYTOKININ RESPONSE FACTORS gating environmental signals and hormones. Trends Plant Sci. 2016;21(2):99–6.

18. Zwick PI, Robinson BR, Riskey MG, Rashotte AM. Cytokinin response factor 6 represses cytokinin-associated genes during oxidative stress. Plant Physiol. 2016;172(2):1249–58.

19. Gupta S, Rashotte AM. Expression patterns and regulation of SCRF3 and SCRF5 in response to cytokinin and abiotic stresses in tomato (Solanum lycopersicum). J Plant Physiol. 2014;171(4–5):549–58.

20. Shi X, Gupta S, Rashotte AM. Characterization of two tomato AP2/ERF genes, SCRF1 and SCRF2 in hormone and stress responses. Plant Cell Rep. 2014;33(1):35–45.

21. Kwon T. Cytokinin response factor 2 positively regulates salicylic acid-mediated plant immunity in Arabidopsis thaliana. Plant Biotechnol. 2016;33(3):207–10.

22. Liang Y, Ernawati N, Chu YJ, Jung MH, Suud M, Kim MG, He SH, Park CG, Son D. Overexpression of an AP2/ERF-type transcription factor CRF5 confers pathogen resistance to Arabidopsis plants. J Korean Soc Appl Biol. 2010;53(2):142–8.

23. Kanwal M, Rabbeni MA, Iqbal S, Fayaz L, Afzal M. Genetic diversity in Brassica species and Erca sativa for yield associated parameters. Genetika-Belg. 2014;46(2):537–43.

24. N U. Genomic analysis of Brassica with special reference to the experimental formation of B. napus and peculiar mode of fertilization. Jpn J Bot. 1935;7:389–452.

25. Chen S, Nelson MN, Chevre AM, Jenczewski E, Li ZY, Mason AS, Meng JL, Plummer JA, Pradhan A, Siddique KMH, et al. Trigenic bridges for Brassica improvement. Crit Rev Plant Sci. 2011;30(6):524–47.

26. Snowden RJ, Kohler W, Friedt W, Kohler A. Genomic in situ hybridization in Brassica amphiploids and interspecific hybrids. Theor Appl Genet. 1997;95(3):1320–4.

27. Maluszynska J, Hasterok R. Identification of individual chromosomes and parental genomes in Brassica juncea using GISH and FISH. Cytogenet Genome Res. 2005;109(1–3):310–4.

28. Snowden RJ, Friedrich T, Friedt W, Kohler W. Identifying the chromosomes of the A- and C-genome diploid Brassica species B. rapa (syn. campestris) and B. oleracea in their amphiploid B. napus. Theor Appl Genet. 2002;104(4):533–8.

29. Tonoaki K, Nishio T. Identification of species in tribe Brassiceae by dot-blot hybridization using species-specific ITS probes. Plant Cell Rep. 2010;29(10):1179–86.

30. Pankin AA, Khakvin EE. Genome-specific marker sets help solve taxonomy issues: a case study with Sinapsis arvensis (Brassicaceae, Brassicaceae). Am J Bot. 2011;98(3):554–7.

31. Alix K, Heslop-Harrison J. Diversity of retroelements in diploid and allotriploid Brassica species. Plant Mol Biol. 2004;54(6):895–909.

32. Alix K, Ryder CD, Moore J, King GJ, Heslop-Harrison JS. The genomic organization of retrotransposons in Brassica oleracea. Plant Mol Biol. 2005;59(6):839–51.

33. Mason AS, Nelson MN, Castello MC, Van GJ, Cowling WA. Genotypic effects on the frequency of homoeologous and homologous recombination in Brassica napus × B. carinata hybrids. Theor Appl Genet. 2011;123(3):543–54.

34. Thakur AK, Singh HK, Singh L, Nanjundan J, Khan YJ, Singh D. SSR marker variations in Brassica species provide insight into the origin and evolution of Brassica amphiploids. Hereditas. 2017;155(6).

35. Yu JY, Tehrim S, Zhang FQ, Tong CB, Huang JY, Cheng XH, Dong CH, Zhou YQ, Qin R, Hua W, Liu SY. Genome-wide comparative analysis of NBS-encoding genes between Brassica species and Arabidopsis thaliana. BMC Genomics. 2014;15:13.

36. Chalhoub B, Denoeud F, Liu S, Parinck IA, Tang H, Wang X, Chiquet J, Belcram H, Tong C, Samani B, et al. Plant genetics. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. Science. 2014;345(6199):950–3.

37. Liu S, Liu Y, Yang X, Tong C, Edwards D, Parinck IA, Zhao M, Ma J, Yu J, Huang S, et al. The Brassica oleracea genome reveals the asymmetrical evolution of polyploid genomes. Nat Commun. 2014;5:3930.

38. Wang X, Wang X, Wang J, Sun R, Wu J, Liu S, Bai Y, Mun JH, Bancroft I, Cheng F, et al. The genome of the mesopolyploid crop species Brassica rapa. Nat Genet. 2011;43(10):1035–9.

39. Yang J, Liu D, Wang X, Ji C, Cheng F, Liu B, Hu Z, Chen S, Pental D, Ju Y, et al. The genome sequence of allopolyploid Brassica juncea and analysis of differential homoeolog gene expression influencing selection. Nat Genet. 2016;48(10):1225–32.

40. Stribberny B, Melton AE, Schwark R, Krause K, Fischer K, Goertzen LR, Rashotte AM. Cytokinin response factor 5 has transcriptional activity governed by its C-terminal domain. Plant Signal Behav. 2017;12(2):e127684.
