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Research article

Genome-wide characterization of the *CBF/DREB1* gene family in *Brassica rapa*

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ABSTRACT

The C-repeat/dehydration-responsive element binding transcription factors (CBF/DREBs) are important proteins in involved in responses to abiotic stress in plants. We identified ten *BrDREB1* genes belonging to the *CBF/DREB1* gene family in the *Brassica rapa* whole genome sequence, whereas six genes are found in the *Arabidopsis thaliana* genome. The deduced amino acid sequences of the *B. rapa* genes showed conserved motifs shared with other known plant CBF/DREB1s. Comparative analysis revealed that nine of the *BrDREB1* genes were derived from the recent genome triplication in the tribe *Brassiceae* and the other one was translocated. The nine genes were located in seven of the 12 macrosyntenic blocks that are triplicated counterparts of four *Arabidopsis* macrosyntenic blocks harboring six *CBF/DREB1* genes: one gene on each of three blocks and three tandemly arrayed genes on another block. We inspected the expression patterns of eight *BrDREB1* genes by RT-PCR and microarray database searches. All eight genes were highly up-regulated during cold (4 °C) treatment, and some of them were also responsive to salt (250 mM NaCl), drought (air drying), and ABA (100 μM) treatment. Microarray data for plant developmental stages revealed that *BrDREB1C2* was highly expressed during a period of cold treatment for vernalization, similar to abiotic stress-inducible genes homologous to *Bn28a*, *Bn47*, *Bn115*, and *BoRS1*, but almost opposite of *BrFLC* genes. Taken together, the number of *BrDREB1* genes increased to 10 by genome triplication and reorganization, providing additional functions in *B. rapa* abiotic stress responses and development, as distinct from their *Arabidopsis* homologs.

1. Introduction

Plant growth is constantly affected by various abiotic stresses such as cold, drought, and high salinity. These stresses result in decreased growth and consequently significant yield losses of crops. The responses of plants to abiotic stresses are complex and controlled by various mechanisms including physiological, biochemical, and molecular regulation. Among them, molecular regulation can be related to stress perception, signal transduction, gene expression, and finally metabolic modifications for preventing cellular damage under abiotic stresses [1]. Many genes are regulated through these molecular regulations and they can be classified into two groups [2–5]. The first group consists of genes encoding proteins to protect cells from abiotic stresses; these include genes for antioxidant enzymes, osmolyte biosynthesis, and molecular chaperones. The second group is made up mainly of genes that regulate stress signal transduction and gene expression, such as transcription factors and protein kinases. Among those, C-repeat/dehydration-responsive element (CRT/DRE) binding factors (CBF/DREB) have been reported to be important stress-responsive transcription factors. CBF/DREB proteins bind specifically to the CRT/DRE sequence in promoters of stress-inducible genes, activating expression of the target genes [6–9].

The *AP2/EREBP* family is one of the largest gene families in plants and consists of transcription factors containing a conserved AP2 DNA-binding domain. Members of this family play important roles in various pathways including flowering control, hormone responses and environmental stress signaling. More than 100 *AP2/EREBP* genes have been found in plant species such as *Arabidopsis*, rice and grape and these can be divided into four subfamilies: DREB, AP2, ERF, and RAV. The DREB subfamily itself consists of six subgroups, of which the A1 subgroup includes CBF/DREB1 transcription factors [10]. To date, many CBF/DREB1 transcription factors have been identified in plant species including *Arabidopsis* and *Brassica* species. In addition, transgenic approaches with these genes have revealed that these factors play important roles in signaling and tolerance of abiotic stresses [11].

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In *Arabidopsis thaliana*, six *CBF/DREB1* genes have been characterized. *CBF1/DREB1C*, *CBF2/DREB1B*, and *CBF3/DREB1A* (hereafter, *AtDREB1C*, *AtDREB1B*, and *AtDREB1A*, respectively) are induced by cold stress but not by drought and salt stress, whereas *CBF4/DREB1D*, *DREB1E/DDF2*, and *DREB1F/DDF1* (hereafter, *AtDREB1D*, *AtDREB1E*, and *AtDREB1F*, respectively) are induced by osmotic stresses such as drought and salt [10,12–14]. In *Brassica napus*, more than 10 *DREB1* genes have been reported and their cold-responsive induction was determined [15–17]. *BjDREB1B* and *BoCBF/DREB1* are also highly expressed under cold stress in *Brassica juncea* [18] and *Brassica oleracea* [19], respectively. Among these genes, the roles of *BnCBF5*, *BnCBF17*, and *BjDRE1D* in stress tolerance were determined through transgenic approaches [18,20]. In *Brassica rapa*, *BcCBF1* and *BcCBF2* were identified as cold-inducible *CBF/DREB1*-homologs by RT-PCR analysis [21]. Recently, 20 genes encoding putative DREB proteins were identified by querying the *B. rapa* EST database and four of them belonged to the *DREB1* subgroup [22]. In addition, several putative *DREB1* genes have been isolated from *Brassica* species and deposited into the database without further study on their expression under abiotic stresses.

B. rapa species include various vegetable crops such as Chinese cabbage, PakChoi, turnip rape, and sarson [23]. Among them, Chinese cabbage (*B. rapa* ssp. *pekinensis*) is one of the most important vegetables and is widely cultivated in Asian countries such as Korea, Japan, and China. Various abiotic stresses cause critical limitations on the production of high quality *Brassica* crops. Recently, the whole genome sequence of *B. rapa* was made available to the public [24,25] and full-length cDNA clones became available [26], facilitating molecular genetic studies to improve agriculturally important traits, such as resistance to diseases and tolerance of abiotic stresses.

In this study, we conducted a genome-wide comparative analysis of the *DREB1* subgroup in *B. rapa* and *A. thaliana*, and named 10 *BrDREB1* genes based on syntenic relationships. We also characterized their expressions under various stress conditions and during development. To date, this is the first genome-wide study of the *DREB1* gene family in *B. rapa*. Our results enhance the understanding of the evolution of dynamic functions and fates for these abiotic stress-related genes, which were retained as a multi-gene family after several rounds of genome-level duplication.

2. Results

2.1. Identification of the CBF/DREB1 gene family in B. rapa

Genome-wide searches for conserved amino acids of DREB1 proteins revealed 10 *DREB1* gene candidates in 283.8 Mb of *B. rapa* whole genome pseudo-chromosome sequences [25]. A total of 147 and 276 genes encoding AP2/EREBP domain-containing proteins were also retrieved from *B. rapa* and *Arabidopsis* databases, respectively. Phylogenetic analysis revealed that all 10 *B. rapa DREB1* candidates belonged to the A1 subgroup, together with six *AtDREB1* genes (Fig. 1(A)). The *DREB* A1 subgroup showed four clades, which indicate their orthologous relationships (Fig. 1(B)). The first clade included three genes, *AtDREB1A*, *1B*, and *1C* that are present as a tandem array in *A. thaliana* and their five orthologs, *BrDREB1A*, *1B1*, *1C1*, *1B2*, and *1C2*, in *B. rapa*. The second and fourth clades included *AtDREB1D* and *1E* and their single copy orthologs from *B. rapa*, *BrDREB1D* and *BrDREB1E*, respectively. The third clade included *AtDREB1F* and three orthologs from *B. rapa*, *BrDREB1F1*, *F2*, and *F3*. Therefore, we designated the 10 genes as *B. rapa Dehydration-Responsive Element Binding factor 1* genes (*BrDREB1s*) and gave sub-names based on the names of their *Arabidopsis* counterparts. Detailed information about the *BrDREB1s* is shown in Table 1.

The *BrDREB1* genes showed 25–91% identity to each other and more than 49% similarity to other *DREB1* genes reported in the *Brassica* genus and *A. thaliana* at the amino acid level. Nine of the

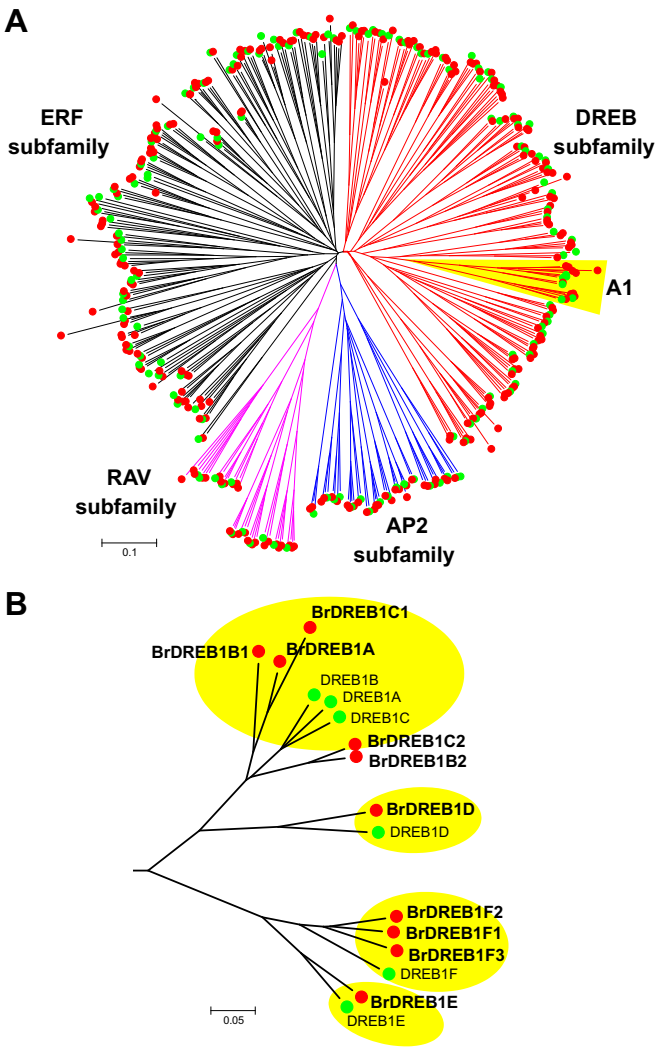

Fig. 1. Phylogenetic analysis of genes encoding proteins with an AP2/EREBP domain in *B. rapa* and *A. thaliana*. (A) Tree based on deduced amino acid sequences from the 276 and 147 genes encoding AP2/EREBP domain-containing proteins in *B. rapa* and *A. thaliana*, respectively. Four subfamilies, DREB, ERF, RAV, and AP2, are denoted based on the classification described by Dietz et al. [10]. The A1 subgroup of the DREB subfamily of CBF/DREB1 transcription factors is marked with a yellow triangle. (B) Enlarged phylogenetic tree of the DREB A1 subgroup in *B. rapa* and *A. thaliana*. The four clades and orthologous relationships based on collinear synteny between *B. rapa* and *A. thaliana* (see Fig. 2) are marked with yellow circles. BrDREB1B2 is located outside of the circle because it is found in a non-syntenic block. Red and green circles indicate genes derived from *B. rapa* and *A. thaliana*, respectively. The similarities among the proteins were analyzed using ClustalW and the phylogenetic tree was generated using MEGA 5 software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ten BrDREB1 proteins had a conserved nuclear localization signal (NLS), an AP2/EREBP domain, and CBF/DREB1 signatures similar to those found in AtDREB1s (Supplementary Fig. 1). The remaining protein, BrDREB1C1, had several deletions of amino acid residues in the NLS and the AP2/EREBP domain, but shared conserved amino acid residues in other regions.

2.2. Comparison of syntenic blocks harboring DREB1 genes in A. thaliana and B. rapa

In order to investigate syntenic relationships and genomic organization, we performed *in silico* mapping of the *DREB1* genes on chromosomes of *B. rapa* and *A. thaliana*. Six genes were located on four syntenic chromosome blocks in the *Arabidopsis* genome:

Table 1
List of *BrDREB1* genes identified in the *B. rapa* genome.

Gene name	BRAD gene ID	nt & Protein length[a]	Location on chromosome	EST sequence[b]	*Brassica* homolog deposited in Genbank (% ID)[c]	*Arabidopsis* homolog (% ID)[d]
BrDREB1A	Bra010461	777 bp, 258 aa	A08, 14636545 –14637321	Not found	ABQ18240 CBF-like [*B. rapa* ssp. *pekinensis*] (98)	AT4G25490, AtDREB1B (62)
BrDREB1B1	Bra010460	828 bp, 275 aa	A08, 14628261 –14629804	Not found	AAQ02701 CBF-like [*B. oleracea*] (91)	AT4G25490, AtDREB1B (60)
BrDREB1B2	Bra022770	645 bp, 214 aa	A03, 6951236 –6951880	EX089732	AAR20497 DREB2-2 [*B. napus*] (99)	AT4G25490, AtDREB1B (80))
BrDREB1C1	Bra010463	675 bp, 224 aa	A08, 14657134 –14657903	EX021200	AAM18961 CBF17 [*B. napus*] (86)	AT4G25490, AtDREB1B (59)
BrDREB1C2	Bra019162	645 bp, 214 aa	A03, 25970608 –25971252	EX110513, EX090298	AAD45623 DREB [*B. napus*] (100)	AT4G25470, AtDREB1C (78)
BrDREB1D	Bra028290	663 bp, 220 aa	A01, 18906624 –18907286	EX117521, EX120161	ABB17253 DREB1-2 [*B. juncea*] (99)	AT5G51990, AtDREB1D (79)
BrDREB1E	Bra027612	558 bp, 185 aa	A09, 7384782 –7385339	Not found	AAB17252 DREB1-1 [*B. juncea*] (53)	AT1G63030, AtDREB1E (85)
BrDREB1F1	Bra026963	618 bp, 205 aa	A09, 34509447 –34510064	not found	ABB17253 DREB1-2 [*B. juncea*] (51)	AT1G12610, AtDREB1F (80)
BrDREB1F2	Bra019777	621 bp, 206 aa	A06, 4457248 –4457868	Not found	ABB17253 DREB1-2 [*B. juncea*] (49)	AT1G12610, AtDREB1F (79)
BrDREB1F3	Bra016763	609 bp, 202 aa	A08, 19855900 –19856508	EX128373, EX125292, EX122624	ABB17253 DREB1-2 [*B. juncea*] (49)	AT1G12610, AtDREB1F (78)

[a] Lengths of the coding sequence and the deduced protein.
[b] *B. rapa* EST sequence showing more than 99% identity when Blastn was performed against the EST database (http://blast.ncbi.nlm.nih.gov/).
[c] *Brassica* homolog showing the best hit when Blastp was performed against the Genbank nr database (http://blast.ncbi.nlm.nih.gov/).
[d] *Arabidopsis* homolog showing the best hit when Blastp was performed against the TAIR10 protein database (http://www.arabidopsis.org/Blast/index.jsp).

one gene each on the A, D and W blocks, and three tandemly arrayed genes on block U. The four chromosome blocks appeared as triplicated, i.e. 12 collinear blocks in the *B. rapa* genome. Among the 12 macrosyntenic blocks, only seven blocks contained *BrDREB1* genes (Fig. 2). Overall, nine of the 10 *BrDREB1* genes resided on the blocks in the *B. rapa* genome. Unlike the other *BrDREB1* genes, *BrDREB1B2* was located in a non-syntenic genomic region between blocks W and J (Fig. 2(A)) although it was very similar to the *BrDREB1C2* gene. This implies that *BrDREB1B2* might have been translocated into that region.

Sequence comparison revealed overall microsynteny with uneven InDel among the sequences flanking the *DREB1* genes on the U blocks (the one U block in *A. thaliana* and its three counterparts in *B. rapa*). The 42 kb sequence of block U on *Arabidopsis* chromosome IV harbors three tandem duplicates, *AtDREB1A*, *1B*, and *1C*. In the *B. rapa* genome, the U block on chromosome A08 harbors the same three tandem duplicates of *CBF/DREB1* genes, *BrDREB1A*, *1B1*, and *1C1*. Meanwhile, only one orthologous gene, *BrDREB1C2*, remained in the U block on chromosome A03 but none was found on the U block on chromosome A01, indicating that five orthologs might have deleted in the two syntenic blocks (Fig. 3(A)) and that one of the five, *BrDREB1B2*, might have been translocated into a non-syntenic region.

Block A, located on the upper region of *Arabidopsis* chromosome I, included *AtDREB1F*. The macrosyntenic block A is located on three chromosomes in *B. rapa* (A06, 08, and 09) and all three blocks harbor *AtDREB1F* homologs, named as *BrDREB1F2*, *BrDREB1F3*, and *BrDREB1F1*, respectively. At the nucleotide level, genomic regions harboring *BrDREB1F1*, *BrDREB1F2*, and *BrDREB1F3* showed microsynteny with various InDels among one another and also with their *Arabidopsis* counterpart (Fig. 3(B)).

2.3. Expression of BrDREB1 genes under abiotic stresses

To investigate the expression of *BrDREB1* genes in response to various abiotic stresses, we performed RT-PCR analysis for eight of the ten *BrDREB1* genes using *B. rapa* plants treated with cold (4 °C), salt (250 mM NaCl), and drought (air drying). Two genes, *BrDREB1F2* and *1E*, were not included in the experiment because they were discovered later. The eight tested candidate genes showed various expression patterns under abiotic stresses (Fig. 4). *BrDREB1A*, *1B1*, and *1C1* were up-regulated by cold and drought but not by salt treatment, whereas *BrDREB1B2*, *1D*, and *1F1* were up-regulated by cold, salt and drought treatment. *BrDREB1C2* was up-regulated by cold, and *BrDREB1F3* was up-regulated by cold and salt treatment. The expressions of *BrDREB1A*, *1B1*, and *1C1* were not changed and/or slightly decreased during abscisic acid (ABA) treatment, whereas *BrDREB1B2* and *1C2* were highly expressed under ABA treatment. *BrDREB1F1* and *1F3* were highly expressed at 30 min during treatment with ABA, and thereafter declined to an almost undetectable level. Similarly, *BrDREB1D* was slightly expressed at 30 min and down-regulated until 48 h of treatment.

In addition to RT-PCR analysis, we searched the *B. rapa* microarray database [27] and found expression values for four genes, *BrDREB1C1*, *1C2*, *1D*, and *1F3* (Fig. 5(A)). *BrDREB1C1* and *1C2* were up-regulated more than 2 fold during cold treatment but were not highly responsive to salt or drought treatment. *BrDREB1D* was up-regulated more than 2 fold compared to the control by cold, salt, and drought treatment. *BrDREB1F3* was up-regulated more than 2 fold by cold and salt treatment but down-regulated by drought. *BrDREB1D* and *1F3* were up-regulated early by ABA treatment and thereafter down-regulated, but *BrDREB1C1* and *1C2* were not affected by it. The microarray data for the four *BrDREB1* genes with regard to abiotic stresses were quite similar to the results of our RT-PCR analysis, although expression patterns of some genes, such as *BrDREB1C1* under drought and *BrDREB1C2* under ABA treatment, were different between the analyses, perhaps due to different conditions in sampling or stress treatment.

We also investigated the expressions of four *B. rapa* genes homologous to *Bn28a*, *Bn47*, *Bn115*, and *BoRS1* (Fig. 5(B)) that were reported as abiotic stress-inducible genes in *B. napus* and *B. oleracea* [28–30]. All four genes were highly expressed under cold, salt, and drought treatments. In particular, their strong expressions followed strong induction of *BrDREB1C1* and *BrDREB1C2* at 30 min of cold treatment, indicating that the expressions of the four genes are well correlated with those of *BrDREB1C1* and *1C2*.

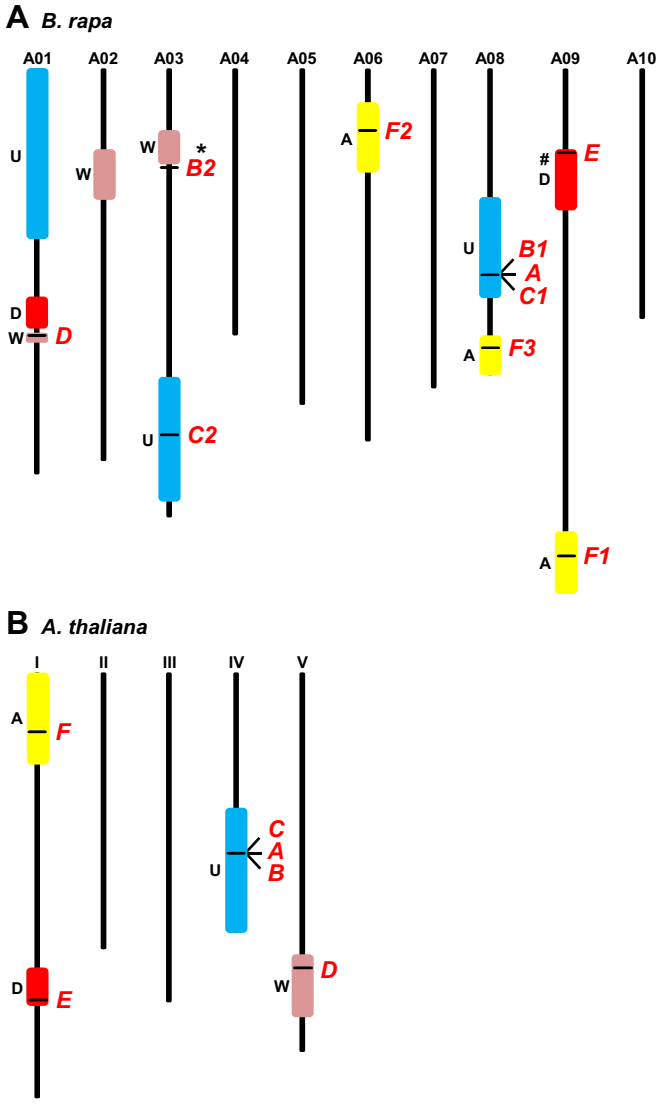

Fig. 2. Chromosomal locations of *CBF/DREB1* genes. The positions of *CBF/DREB1* genes in *B. rapa* (A) and *A. thaliana* (B) are marked with black lines on chromosomes depicted as vertical lines. Genome blocks showing macrosynteny between genomes of both species are drawn with vertical bars of the same color, based on the report of Wang et al. [25]. Asterisk indicates *BrDREB1B2*, which does not belong to any syntenic block. Block D, indicated by #, is considered to be duplicated internally.

2.4. Expression of BrDREB1 genes during growth of B. rapa plants

We also investigated the expression patterns of four *BrDREB1* genes during development from seed through flowering using the microarray data. Among the four genes, only the expression of *BrDREB1C2* was dramatically changed by cold treatment (Fig. 6(A)). *BrDREB1C2* expression in seedlings without cold treatment was similar to that in seeds. However, its expression rapidly increased and was maintained at a high level during 49 days of cold treatment for vernalization, then gradually decreased, and finally was lower than that in seeds when the plant was returned to normal growth conditions for flowering. On the other hand, the expressions of the other three *BrDREB1* genes, *BrDREB1C1*, *1D*, and *1F3*, were not significantly changed during plant development.

The expressions of the genes homologous to *Bn28a*, *Bn47*, *Bn115*, and *BoRS1* were also investigated using microarray data (Fig. 6(B)). All of the genes were strongly expressed during the cold treatment for 49 days and then their expressions gradually decreased during the flowering period. This expression pattern was similar to that of *BrDREB1C2*, although the expression levels were different among the genes, indicating the possibility that *BrDREB1C2* is responsible for molecular regulation during vernalization in development of *B. rapa* plants.

We also investigated the expression of *BrFLC* genes during *B. rapa* development (Fig. 6(C)) because *BrFLC* genes are important for the vernalization response and flowering time. The expression of three genes, *BrFLC1*, *BrFLC2*, and *BrFLC3*, were maintained at high levels during the vegetative growth period, but drastically decreased by cold treatment. By contrast, the expression of *BrFLC5* remained low without significant change during the vegetative growth period and also during cold treatment. Thus, the expression of the three *BrFLC* genes other than *BrFLC5* was negatively correlated with the expression of *BrDREB1C2* and other abiotic stress-inducible genes.

3. Discussion

3.1. Ten genes in the B. rapa genome belong to the CBF/DREB1 gene family

The *CBF/DREB1* gene family encodes important transcription factors for abiotic stress responses and tolerance in plants. Furthermore, members of this family have become some of the most important targets for crop improvement [11]. However, the *CBF/DREB1* gene family has not been intensively studied in *B. rapa* species even though abiotic stresses are important challenges for *B. rapa* crops. We characterized ten *BrDREB1*s from the whole genome sequence of *B. rapa*. All ten genes contained a conserved NLS, an AP2/EREBP domain and CBF/DREB1 signatures and showed high similarity to other plant CBF/DREB1 proteins (Fig. 1, Supplementary Fig. 1), indicating that at least 10 *BrDREB1* genes are present in the *B. rapa* genome even though only six *AtDREB1* genes were found in the *Arabidopsis* genome [10]. Compared to the previous report identifying four *B. rapa CBF/DREB1* genes by EST database analysis [22], we identified additional six *CBF/DREB1* genes based on comparative genome analysis. The number of *DREB1* genes was increased 1.7 fold in *B. rapa* compared to that of *A. thaliana*, whose genome has six *AtDREB1* genes [10]. Considering that the tribe *Brassiceae* genome has been triplicated since divergence with *Arabidopsis* from the common ancestor [31], deletion might have reduced the number of *BrDREB1* genes from 18 to 10 during the diploidization process that followed the genome triplication, as shown by comparison of syntenic blocks between *B. rapa* and *A. thaliana* (Figs. 2 and 3) [32]. A total number of AP2/EREBP domain genes were 276 in *B. rapa*, which is about 1.8 fold more than that of *A. thaliana* that is also similar to the increase ratio for the homologous genes at the whole genome level [25,31,32].

3.2. Comparative genomics of BrDREB1s

Through comparison of the sequences flanking the 10 *BrDREB1*s with those of their *Arabidopsis* counterparts, we found that nine *BrDREB1*s had at least one syntenic homolog in *Arabidopsis* genome blocks. The microsynteny was well conserved, with various uneven InDels between and among the *A. thaliana* genomic regions and their three collinear counterpart regions in *B. rapa*. Three *Arabidopsis* genes, *AtDREB1A*, *AtDREB1B*, and *AtDREB1C*, were located tandemly on the U block of chromosome IV (Figs. 2 and 3) [33], and one of its triplicated counterparts showed same three gene tandem array: *BrDREB1A*, *BrDREB1B1*, and *BrDREB1C1* in the U block of *B. rapa* chromosome A08. Similarly, several plant species including tomato, potato, and rice also have *CBF/DREB1* genes located tandemly in their genomes [34–36]. This means that the tandemly

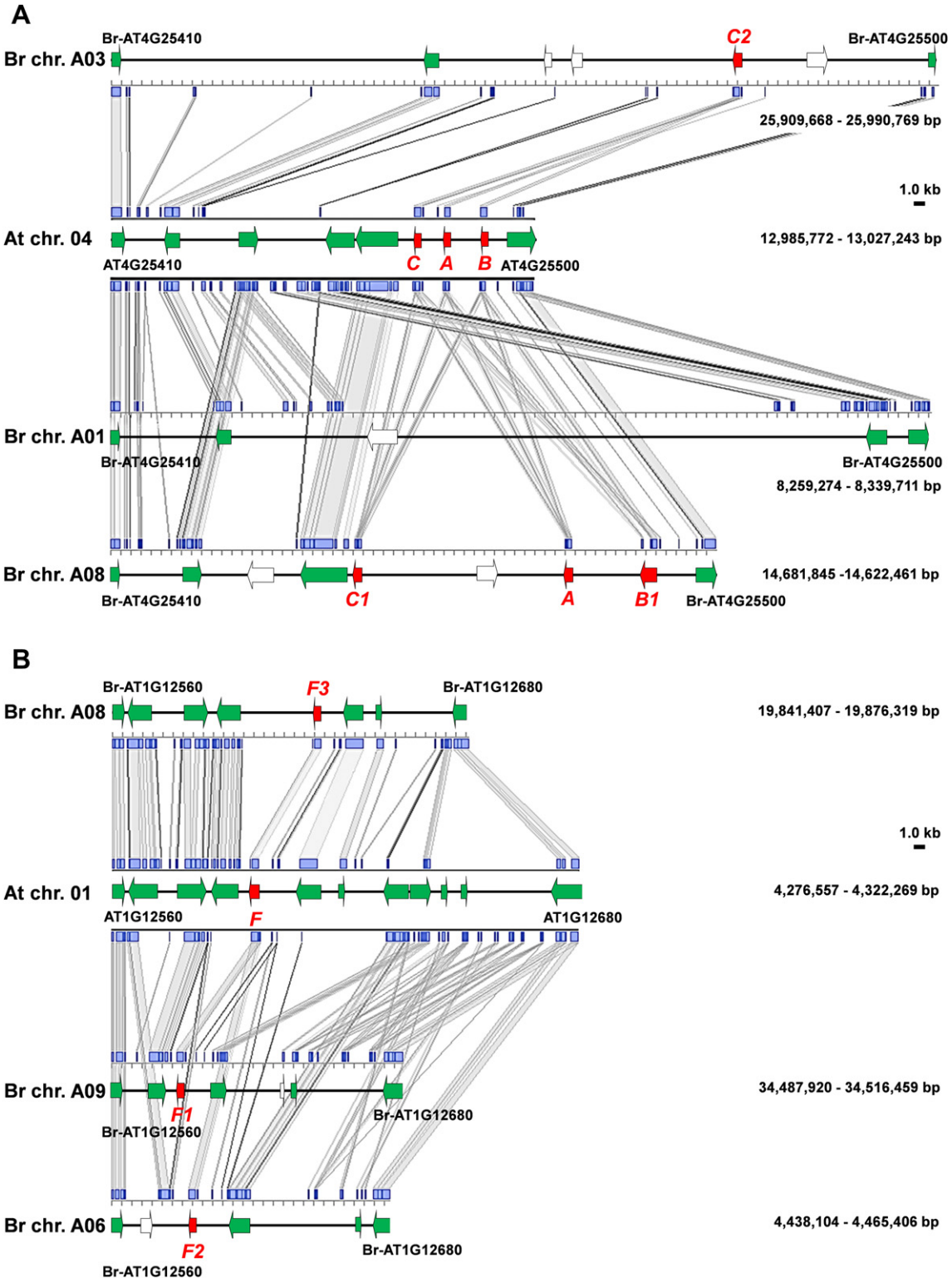

Fig. 3. Sequence level comparison of collinear genomic regions flanking *DREB1s* in *B. rapa* and *A. thaliana*. (A) Sequence comparison of the region around three tandem *DREB1* genes in synteny block U of *A. thaliana* and its three *B. rapa* counterparts. (B) Sequence comparison of the *DREB1F* gene regions in synteny block A of *A. thaliana* and its three *B. rapa* counterparts. Arrows indicate the position and orientation of annotated genes in the sequences. Green and red arrows indicate well-conserved flanking genes and *CBF*/*DREB1* genes, respectively, among collinear genomic regions. The gray bars connecting boxes between sequences indicate conserved sequences. The map was drawn using Blast viewer based on nucleotide sequence similarity from Blastn searches. Genomic regions showing similarity in less than 10 bp match length are not shown on the map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

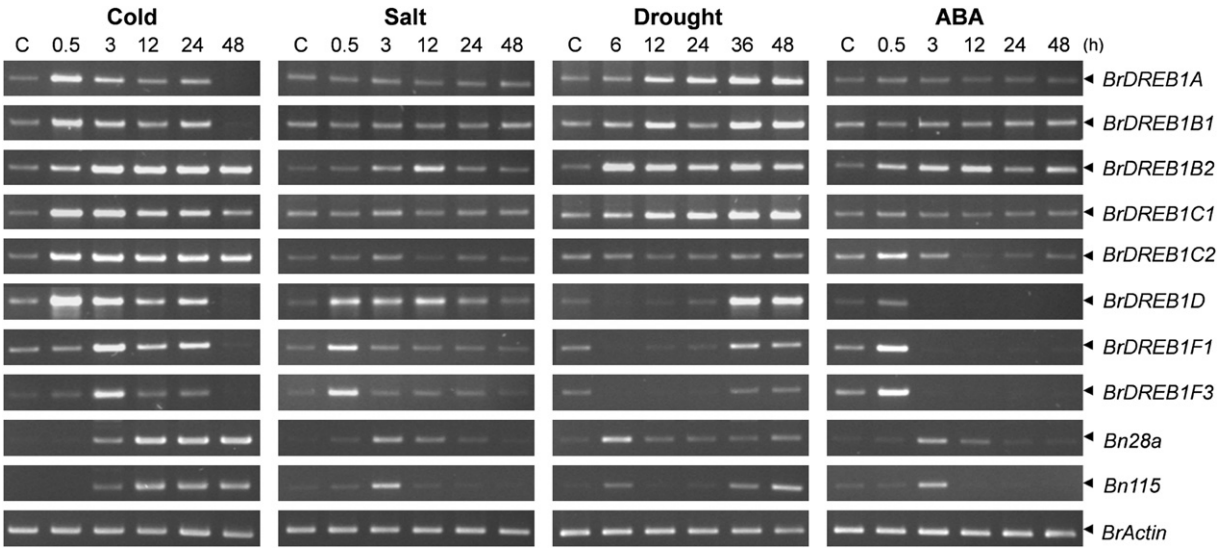

Fig. 4. RT-PCR analyses of the *BrDREB1* genes under various abiotic stresses. Total RNAs prepared from *B. rapa* plants subjected to stress treatments for the indicated times were used for RT-PCR analysis. *Bn28a* and *Bn115* were used as abiotic stress–response controls. *BrActin1* was used as a PCR control. C, control; cold, 4 °C; drought, air drying; salt, 250 mM NaCl; ABA, 100 μM abscisic acid. Numbers on the top indicate time (h) of treatment.

arrayed *DREB1* transcription factor gene sets evolved before the monocot and dicot divergence and remained well conserved in various plant species, suggesting that these transcription factors play an important role in abiotic stress responses and thus remain stable regardless of genome divergence. In *A. thaliana* and tomato, functional loss of at least one of the three *CBF/DREB1* genes significantly decreases the stress tolerance of these plants [34,37,38].

BrDREB1B2 was expected to be unique, because it was located in a non-syntenic region between blocks W and J (Figs. 1(B) and 2(A)). Considering the high similarity of *BrDREB1B2* to *Arabidopsis DREB1B*, it can be hypothesized that *BrDREB1B2* might have originated from the deleted regions in the U block on chromosome A03 or A01. Interestingly, the gene is flanked by *BrFLC5* (BRAD ID: Bra022771), which was also found in non-syntenic genomic regions despite other *B. rapa FLC* genes being present in well-conserved syntenic blocks [32,39].

In addition to differences at the genome level, the stress responsiveness of some *BrDREB1* genes was also different from that of their *Arabidopsis* counterpart genes. For example, the three tandem genes, *BrDREB1A*, *1B*, and *1C*, were highly expressed under cold and drought (Fig. 4), whereas the three *Arabidopsis* counterpart genes were induced only by cold [7]. This suggests that the different genomic organization of *CBF/DREB1* genes may allow different responses to stress between *B. rapa* and *A. thaliana*. Similarly, the orthologous *CBF/DREB1* gene set showed different capabilities in terms of tolerance to cold stress in *Thlaspi arvense* and *Solanum* species [36,40].

3.3. Expression of BrDREB1s under abiotic stresses

Expression analysis revealed that all eight *BrDREB1* genes tested here were strongly expressed in response to cold stress and some were also responsive to other abiotic stresses, such as salt and drought (Figs. 4 and 5). During ABA treatment, some *BrDREB1* genes, such as *BrDREB1B2* and *BrDREB1C2*, were strongly up-regulated, whereas *BrDREB1A*, *BrDREB1B1*, and *BrDREB1C1* were not responsive or were even slightly down-regulated. This indicates that some *BrDREB1* genes may be involved in ABA-dependent signaling but some may not be. Similarly, various responses to the ABA hormone were also identified in other plant *CBF/DREB1* gene families [7,8,13,35].

A CBF/DREB1-involved signaling pathway is reported to be well conserved in many plant species [11]. In *A. thaliana*, HOS1 and ICE1 are involved in the signaling pathway as upstream regulator of CBF/DREB1. HOS1 plays a role as a negative regulator of ICE1 which is an MYC transcription factor activating *AtDREB1A* expression [37,41]. We tried to identify genes related to these two components and inspect their expressions and relationships in a potential CBF/DREB1-mediated signaling pathway in *B. rapa*. A *HOS1* homolog was found in the *B. rapa* microarray database and its expression (*B. rapa* 24K oligo microarray chip ID BRAS0001S00019635) was drastically decreased under cold stress, opposite from *BrDREB1* gene expression. Five *ICE1* candidate genes (BRAD ID: Bra016749, Bra019794, Bra025226, Bra032947, and Bra034246) were also identified in the genome database but we could not determine their expression levels because they were not included in the 24K microarray chip. Furthermore, we inspected genes downstream of CBF/DREB1. *Bn28a*, *Bn47*, and *Bn115* were found as orthologs of *Arabidopsis COR6.6*, *COR47*, and *COR15*, respectively, that are known as CBF/DREB1-target genes [16,20]. Three homologous genes from *B. rapa* were strongly expressed immediately after *BrDREB1* induction by cold treatment (Figs. 4 and 5), a similar expression pattern to that of other plant CBF/DREB1-target genes. Collectively, these results suggest that a CBF/DREB1-involved signaling pathway for abiotic stress responses also functions in *B. rapa*.

3.4. Expression of BrDREB1s during vernalization

B. rapa plants need low-temperature treatment for flowering, a process known as vernalization [42]. During the vernalization period, plant growth is adversely affected by cold stress. Thus, *B. rapa* plants highly express genes that are important not only for flowering, but also for survival during cold treatment [27,43]. Among the *BrDREB1* genes, *BrDREB1C2* was highly expressed during the long period of cold treatment for vernalization (Fig. 6(A)). This implies that *BrDREB1C2* plays a role in regulation of downstream genes to protect cells from damage induced by cold stress. This assumption is also supported by *BrDREB1C2*'s correlated expression with homologs of known abiotic stress-inducible genes

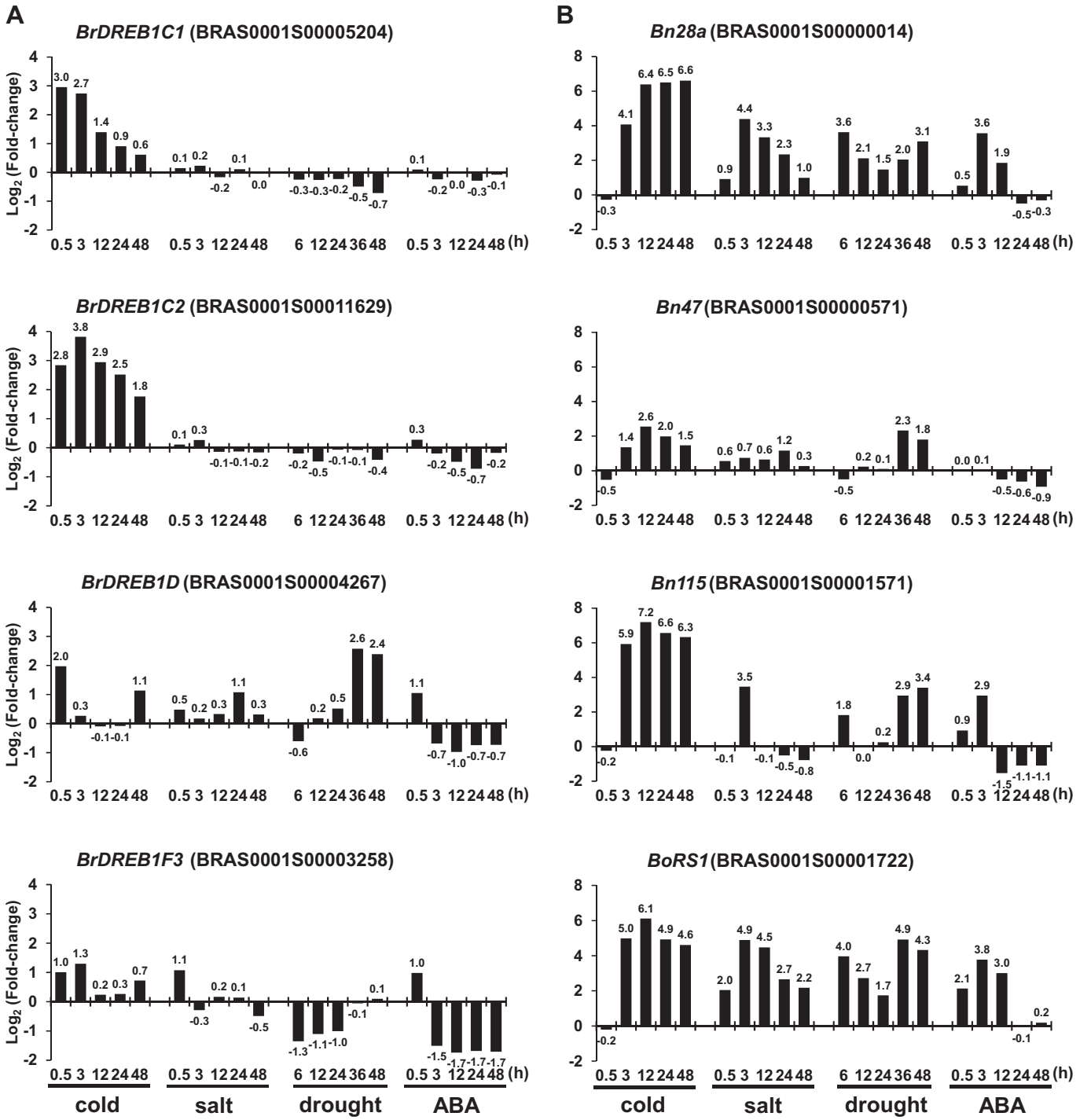

Fig. 5. Expression levels of *BrDREB1* and *COR* genes in response to cold (4 °C), salt (250 mM NaCl), drought (air drying), and ABA (100 μM) treatment according to 24K microarray analysis. (A) The expression of four *BrDREB1* genes: *BrDREB1C1*, *BrDREB1C2*, *BrDREB1D*, and *BrDREB1F3*. (B) The expression of *B. rapa* homologs of *Bn28a*, *Bn47*, *Bn115*, and *BoRS1*, which are reported abiotic stress-inducible genes in *Brassica* species. The expressions of the genes are represented using fold change compared to control (0 h). Numbers at the bottom indicate time (h) of treatment. Log_2 (fold-change value) is shown at the top of bar. Microarray probe IDs for the genes are in parentheses. The *B. rapa* 24K oligo microarray database provides only averaged PM values of repeated microarray experiments after statistical analysis [27], and therefore standard error or deviation was not calculated in this study.

Bn28a, *Bn47*, *Bn115*, and *BoRS1* (Fig. 6(B)). Meanwhile, *BrDREB1C2* expression showed a negative correlation with those of *BrFLC1*, *2*, and *3* (Fig. 6(C)). Among these three *BrFLC* genes, *BrFLC1* and *2* were also reported to be associated with flowering time [39,44,45]. These results also support the view that *BrDREB1C2* plays a role in the vernalization process. In addition, *BrDREB1C2* might be involved in regulation of endogenous GA levels to repress growth for stress adaptation under adverse growth conditions, such as a long period of cold stress, as mentioned in Magome et al. [46]. It is interesting to note that *BrDREB1C2* (BRAD ID: Bra019162) is flanked in the genome by genes encoding GA20 oxidase (BRAD ID: Bra019163) and AGAMOUS-LIKE protein (BRAD ID: Bra019165).

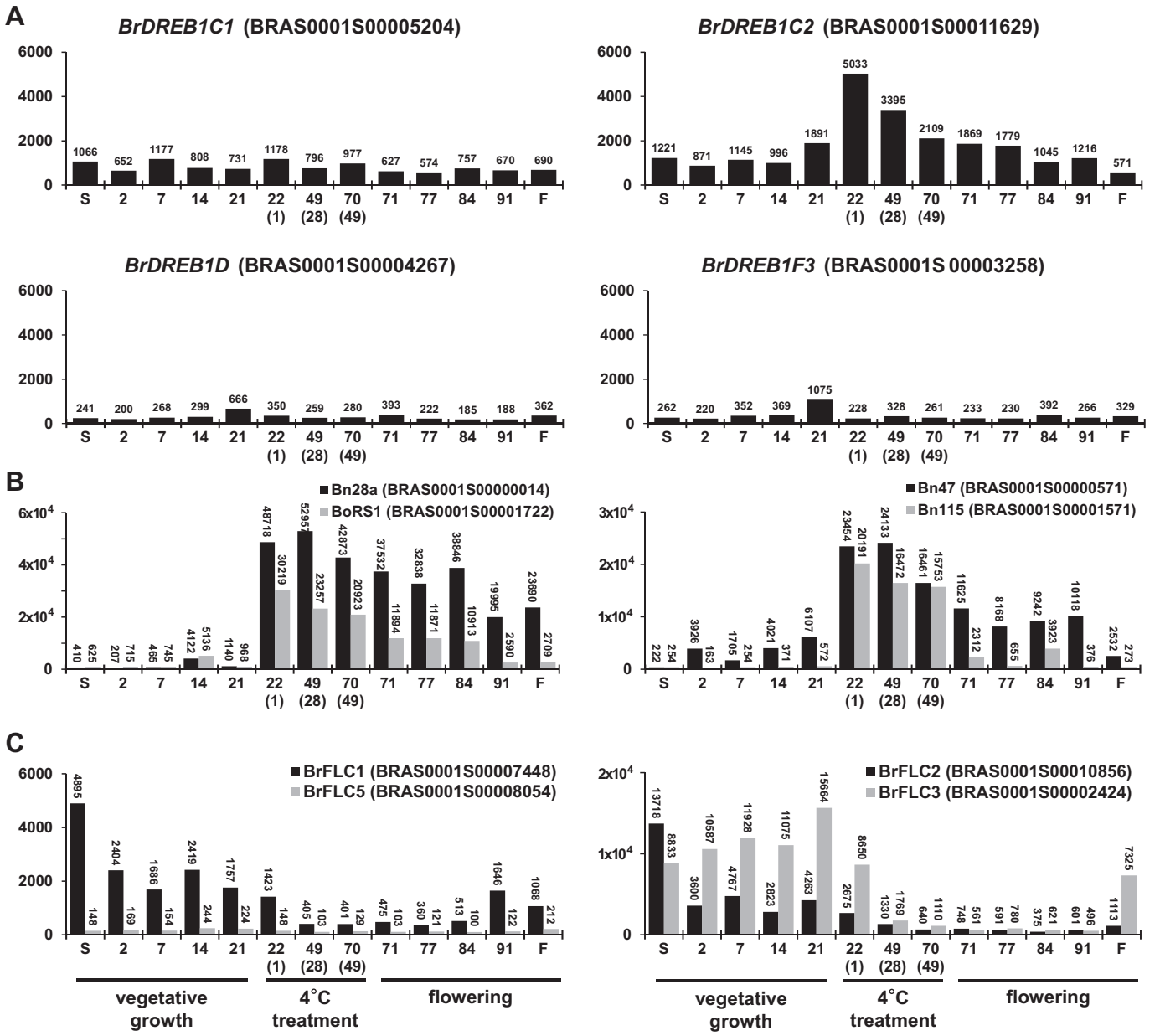

Fig. 6. The expressions of *BrDREB1*, abiotic stress-inducible genes, and *BrFLC* genes during development from seed through flowering according to 24K microarray analysis. The perfect match (PM) values for *BrDREB1* (A), abiotic stress-inducible genes (B), and *BrFLC* genes (C) were retrieved from the *B. rapa* microarray database and used to determine expression patterns. Numbers at the bottom indicate days after germination. S and F indicate seeds and flowers, respectively. Numbers in parentheses indicate days after cold (4 °C) treatment for vernalization. PM values are shown at the tops of the bars. Microarray probe IDs for the genes are in parentheses. The *B. rapa* 24K oligo microarray database provides only averaged PM values of repeated microarray experiments after statistical analysis [27], and therefore standard error or deviation was not calculated in this study.

BrDREB1C2 shows a high degree of similarity in predicted amino acid sequence and expression pattern to *AtDREB1C*. Both are highly induced by cold but not by salt and drought (Figs. 4 and 5) [7]. Taking into account expression pattern during *B. rapa* development, it is suggested that *BrDREB1C2* gained an extra function in vernalization during genome evolution, and thus plays an additional role different from its orthologs in *A. thaliana*. Gene duplication is believed to be an important driving factor for acquisition of new gene functions, because an additional copy of a gene can relieve functional constraint on the other gene [47]. Further characterization of these genes will give insight to their distinctive functions under abiotic stress as well as during development in *B. rapa*.

In conclusion, we identified 10 *BrDREB1* genes belonging to the *CBF/DREB1* gene family through genome-scale analysis and then characterized their genomic organization and stress responsiveness in *B. rapa*. These *BrDREB1* genes will be valuable for understanding stress–response mechanisms in *Brassica* species and for molecular breeding approaches to develop new cultivars with high stress tolerance.

4. Materials and methods

4.1. Plant material and stress treatments

Plants of *B. rapa* ssp. *pekinensis* (inbred line 'Chiifu') were grown in soil for 3 weeks, as described in Lee et al. [27]. Three-week-old plants in 5 cm diameter pot were used for abiotic stress treatments. Plants were kept in 4 °C growth chambers for cold stress

treatment and irrigated with 250 mM NaCl solution for salt stress treatment under continuous light. We carefully picked up the whole plants from the pots with the intact pot-shape root and soil architecture and remained the plants in same condition without watering for drought stress treatment. The plants were sprayed with 100 μM abscisic acid (ABA) in 0.02% (v/v) Tween 20 solution for ABA treatment. For controls, whole plants were sampled immediately before cold, salt, drought, or ABA treatment. Among the plants subjected to treatments, six plants, two replications with three combined plants, were sampled at 0.5, 3, 12, 24, and 48 h after cold, salt, and ABA treatment and at 6, 12, 24, 36, and 48 h after drought treatment. The sampled plants were immediately frozen in liquid nitrogen for isolation of total RNA.

4.2. Identification of DREB1-homologous genes

To identify members of the *CBF/DREB1* gene family in *B. rapa*, we searched the genome database (http://brassicadb.org/brad/; BRAD) [24] using the tBlastn algorithm (with default parameters except for a cutoff *E*-value of $1E^{-13}$) [48] with the conserved amino acid residues of DREB1s as queries. The resulting sequences were retrieved and annotated using BRAD annotation information, FGENESH (http://www.softberry.com/berry.phtml), and the Blast algorithm (http://blast.ncbi.nlm.nih.gov/). To find *B. rapa* expressed sequence tags (ESTs) encoding DREB1, we searched EST databases at NCBI (http://blast.ncbi.nlm.nih.gov/), BRGP (http://www.brassica-rapa.org/BRGP), and RIKEN (http://www.brc.riken.go.jp/lab/epd/Eng/species/brassica.shtml). In addition, AP2/EREBP domain-containing proteins of *B. rapa* and *A. thaliana* were obtained from BRAD and TAIR (http://www.arabidopsis.org/index.jsp), and used for phylogenetic comparison. The phylogenetic tree was generated based on the similarities among the proteins by using ClustalW (http://www.genome.jp/tools/clustalw/) and MEGA 5 software (http://www.megasoftware.net/) [49] with default parameters.

4.3. Determination of syntenic relationships among DREB1 genes in B. rapa and A. thaliana

The locations of *BrDREB1* and *AtDREB1* genes on chromosomes were determined based on the information in BRAD and TAIR database. We have used collinear flanking sequences harboring *DREB1* genes on exhibiting 24 macrosyntenic genome blocks between the genomes of *B. rapa* and *A. thaliana* [24]. To examine genomic organization and microsynteny, genomic sequences containing *BrDREB1*s and their flanking genes were retrieved from BRAD and then used to search for collinear genomic sequences in the *B. rapa* and *A. thaliana* genomes by Blastn algorithm. Genomic organization such as gene position and order among collinear genomic regions was determined based on annotation information provided by BRAD and TAIR. A synteny paralog search tool provided in BRAD was also used to investigate microsynteny existing in the *Arabidopsis* genome and its three counterpart subgenomes of *B. rapa*. In addition, microsynteny among the collinear genomic sequences was also analyzed at the nucleotide level by local Blastn searches (expect value $1E^{-06}$) and a comparative view for a synteny map was generated using Blast viewer (http://nature.snu.ac.kr/tool/blastz.php).

4.4. Expression analyses of BrDREB1 genes

The expression of *BrDREB1* genes was investigated through RT-PCR analysis and microarray database searches. For RT-PCR analysis, total RNAs from stress-treated *B. rapa* plants were isolated using the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, and the quality and concentration were measured using a spectrophotometer and formaldehyde-agarose electrophoresis. 5 μg of total RNA was reverse transcribed into first strand cDNAs using oligo $(dT)_{18}$ primer and PowerScript reverse transcriptase (Clontech, USA) and then the synthesized cDNAs were used as templates for PCR amplification. Gene-specific primers of *BrDREB1* genes were used for PCR analysis. Specific primers were designed based on the coding sequence of each gene annotation (Table 2). As a control, we used primers specific to the *B. rapa* actin gene, *BrActin1* (Genbank ID: EX087730) [27]. As controls for stress-responsive expression, specific primers for *Bn28a* [28] and *Bn115* [29] were used. PCR conditions were as follows: 94 °C for 5 min; followed by cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and finally extension at 72 °C for 7 min. The number of PCR cycles used ranged between 22 and 35 for specific amplification of the genes. The RT-PCR products of *BrDREB1* genes were confirmed by nucleotide sequencing. RT-PCR analysis was performed at least twice with biologically independent samples and repeated technically several times. The repeated analyses showed almost identical results and therefore a representative RT-PCR result was shown in Fig. 4. For microarray database analysis, probe sequences and IDs in the *B. rapa* 24K oligo microarray database (http://www.brassica-rapa.org/BrEMD/microarray_overview.jsp) [27] were searched using nucleotide sequences of *BrDREB1* genes as queries. The perfect match (PM, a measure of signal intensity) values of probes for *BrDREB1*s were retrieved and processed to determine expression patterns, as described in Lee et al. [27].

Table 2
List of primers used for RT-PCR analysis.

Primer name	Nucleotides
BrDREB1A-forward	5′-TCAGCCTTATCCAGTTTTCAA-3′
BrDREB1A-reverse	5′-AAAATATTATCGTCCAAAAGTAAA-3′
BrDREB1B1-forward	5′-CTTACTGTACTCAGCCTTATCCAGTTT-3′
BrDREB1B1-reverse	5′-TCAGTCGTGAATCAGTCGGTA-3′
BrDREB1B2-forward	5′-ACTCAACCTTATCCGGTTAACAA-3′
BrDREB1B2-reverse	5′-ACAAAAACCGGAAATAAAAA-3′
BrDREB1C1-forward	5′-TTTTTCAAAAGAAGTTTTCAACG-3′
BrDREB1C1-reverse	5′-AGGTAGAATCCGAATTCAATAAAA-3′
BrDREB1C2-forward	5′-GTTACTTATCCAGTTATTTT-3′
BrDREB1C2-reverse	5′-AAAAACTTTGCACTAATA-3′
BrDREB1D-forward	5′-CAGATCTCCAGTTTCCGACAG-3′
BrDREB1D-reverse	5′-GGTAGTTGAACTTTTGACCAATCC-3′
BrDREB1F1-forward	5′-TTACCGACGAACTAGCGTTCT-3′
BrDREB1F1-reverse	5′-AACAACTCGCACACTATTGCT-3′
BrDREB1F3-forward	5′-CGACAATCATTACAAGAAATAGCA-3′
BrDREB1F3-reverse	5′-GCACGCATTTTACCATATTACA-3′
Bn28a-forward	5′-GGACCAATCAAAACACAAAAA-3′
Bn28a-reverse	5′-AAGATACATTGTTGGACTTGAAGC-3′
Bn115-forward	5′-CATGGCTATGTCACTCTCAGGA-3′
Bn115-reverse	5′-AAATAATAAACATGAGACAAGAAGGAT-3′
BrActin1-forward	5′-CCCTAAGGCTAACAGGGAGAA-3′
BrActin1-reverse	5′-AGCTCCGATGGTGATGACTT-3′

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plaphy.2012.09.016.

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