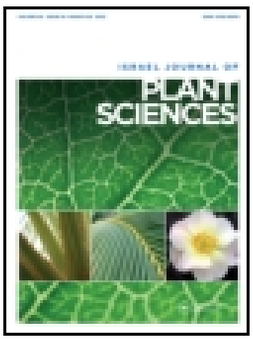

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Expression profiling and characterization of cold, freezing-related genes from *Brassica rapa* cultivars

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Brassica is a very important vegetable group worldwide. Cold and freezing stress are the major environmental factors that limit the productivity of *Brassica.* In this study, we retrieved 903 stress-chilled unigenes and the unique transcripts were classified functionally using gene ontology (GO) hierarchy, Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG orthology and structural domain data were obtained from the biological database to the unigene data set. Unigene data sets provide a wide outlook of functional characterization of *Brassica rapa. In silico* analysis revealed 94.2% of unigenes to be well annotated toward Reeds one-dimensional concept. On the basis of similarity searches and GO annotation of biological process (BP), 139, 113, and 57 sequences showed a response to stress (SR), abiotic stimulus (AS), and biotic stimulus (BS), respectively. To validate this observation, seven unigenes were randomly selected from AS that are known to be associated with cold stress from previous studies in other species and these genes were examined by real-time polymerase chain reaction to understand the change in expression pattern under cold and freezing stress. *In silico* study of occurrence in the AS subcategory revealed the presence of 138 single sequence repeats, some of which are being explored to assess genetic diversity among *B. rapa*. Thus, the unigene data set provides valid resources for discovering the potential genes related to cold and freezing resistance in *B. rapa* cultivars, and can be useful resources for genetic engineering of *Brassica* sp.

Keywords: *Brassica rapa*; unigenes; gene ontology; abiotic stimulus; cold; freezing; real-time PCR

Introduction

Plants vary greatly in their ability to survive cold and freezing temperatures. Chilling stress (<20°C) and/or freezing (<0°C) temperatures adversely affect the growth and development of plants (Chinnusamy et al. 2007). The genus *Brassica* includes many important vegetable crops such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale, and turnip (Abe et al. 2011). Chinese cabbage (*Brassica rapa*), which is an important vegetable crop worldwide, is widely cultivated in Asia, particularly in China, Korea, and Japan. Like many other crop plants, it is known to be sensitive to cold and freezing temperature, and individual genotypes of a single species of *B. rapa* may respond differently to cold and freezing conditions (Thamil Arasan et al. 2013). *B. rapa* ssp. *pekinensis* includes two distinct cultivars, chiifu and kenshin. Chiifu was previously established in the Shan Dong Province of northeast China and Kenshin was previously established in the Fu Jian Province of southeast China, both relatively cold and warm climates, respectively. It has been proposed that these two cultivars respond to temperature and vernalization differently (Lee et al. 2010).

Guy (1990) established an acclimation technique to identify cold-responsive genes (*CRG*) and determine whether they play roles in freezing tolerance. Thomashow (2001) suggested that many *CRG* mediated biochemical and physiological changes are required for growth and development at low temperatures. However, other genes might also play roles in freezing tolerance. Efforts to develop plants resistant to cold stress are currently under way, and functional genomics studies comprise one of the most important approaches to identifying potential genes related to stress resistance. Three of the most important techniques used to study gene expression are the sequencing and analysis of Expressed Sequence Tags (ESTs), DNA microarray and SAGE (Wu et al. 2005). ESTs are commonly used to identify genes involved in specific biological functions (Gueguen et al. 2003). One interesting application of ESTs is investigations of the gene expression pattern in response to various environmental stimuli (Zhang et al. 2001) and transcriptomics studies of plants in various stages of development under different experimental conditions using various plant tissues (leaf, stem, flower bud). Furthermore, combining ESTs with other molecular

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methods such as microarray analysis has made it possible to examine the expression of thousands of genes at the RNA level at once. Indeed, this technique was used to examine the transcript levels of~8000 genes by transferring *Arabidopsis* plants from warm to cold temperatures and identifying 306 *CRG*s (Fowler & Thomashow 2002).

Yu et al. (2011) have created 39 ESTs libraries and developed a *B. rapa* tissue-specific database (*Br*TED) with a large number of ESTs (147,217). Unigenes were successfully evaluated with real-time polymerase chain reaction (RT-PCR) using tissues-specific material. In this study, 535 and 368 stress-chilled tandem and consensus sequences, respectively, were retrieved from *Br*TED and have been applied to *in silico* approaches for functional classification on gene ontology (GO) vocabularies, transcript abundance estimation, and biochemical classification based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology from a range of biological databases for the respective unigene sequences. According to Reeds words, all one-dimensional data have been assigned to the putative sequences and those functional annotations helped to develop a further understanding of complex networks of cold- and freezing-related gene expression profiles with specific "Nunnara" (chiifu) and "Asia alphain" (kenshin) cultivars.

Materials and methods

Unigenes assembly and annotation

The 903 raw read sequences were composed of singlets and contigs and some short repetitive sequences were obtained from *Br*TED (http://s47.rna.kr/BrTED/index.php) using a cut off <0.05 of stress-chilled sequences (Yu et al. 2011). Obtaining high-quality sequences requires several steps, all of which are integrated in a freely accessible web interface, EGassembler (Masoudi-Nejad et al. 2006; Falgueras et al. 2010). For an EST assembly, EGassembler was used with the default analysis parameters. This method starts with a sequence cleaning stage in which low-quality stretches are removed and repetitive elements are then detected and removed. The resulting output is then searched and cleaned from organelle sequences.

The assembled unique transcripts were subsequently compared with those available in the public databases using Blast2go (Götz et al. 2008), a sequence-based tool to assign GO terms and annotation for each BLAST hit obtained by mapping the extant annotation associations. The GO terms for each of the three main categories (biological process, molecular function, and cellular component) were obtained from sequence similarity using the default parameters. From these annotations, the second group level and multilevel GO terms were based on the biological process, molecular function, and cellular components. This annotation was simplified and focused on plant-related functional categories using Plant GOslim.

Pathway assignment with KEGG

Pathway assignments were mapped according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html; Kanehisa & Goto 2000). Enzyme commission (EC) numbers were assigned to unique sequences with BLASTX scores with an E value cut-off of 10^{-10} by searching the KEGG protein databases. The unique sequences were mapped to specific biochemical pathways according to the corresponding EC distribution in the KEGG database.

In silico unigenes-derived single sequence repeats (SSRs)

The unigene data set was screened for the presence of microsatellite repeats using SSRIT (http://www.gramene.org/db/markers/ssrtool; Temnykh et al. 2001). It should be noted that monomeric repeating units were not taken into account owing to the presence of polyA/polyT sequences. SSR motifs with a repeat number of at least six for di-nucleotides, five for tri-nucleotides, five for tetra-nucleotides, four for penta-nucleotides and four for hexa-nucleotides were considered.

Plant sample and RNA extraction

For the expression study, Chinese cabbage cultivars of "Nunnara" (chiifu; www.nhseed.co.kr) and "Asia alphain" (kenshin) seeds (www.asiaseed.net) used in this study, which are winter and summer seasonal cultivars, respectively (Figure 1). Plants were grown under soil conditions that were maintained at a controlled temperature of 22°C to the fifth leaf stage. All samples were taken from the fifth emerged leaf of control and treated samples. To induce cold stress, the seedlings were maintained at 22°C, 3°C, −2°C and −6°C for 2 h. The samples were then frozen immediately in liquid nitrogen and stored at −80°C until RNA isolation. The total RNA was extracted from the frozen samples of the roots, stems, leaves, and flower buds of chiifu healthy plants and those exposed to cold stress using an RNeasy mini kit (Qiagen, USA). RNA was treated with RNase-free DNase (Promega, USA; Shoresh et al. 2011) to remove the genomic DNA contamination.

RT-PCR analysis

RT-PCR was performed using an Avian Myeloblastosis Virus (AMV) one-step RT-PCR kit (Takara, Japan) with primers specific for the stress-responsive genes listed in Table 1. RT-PCR was performed using 50 ng cDNA of

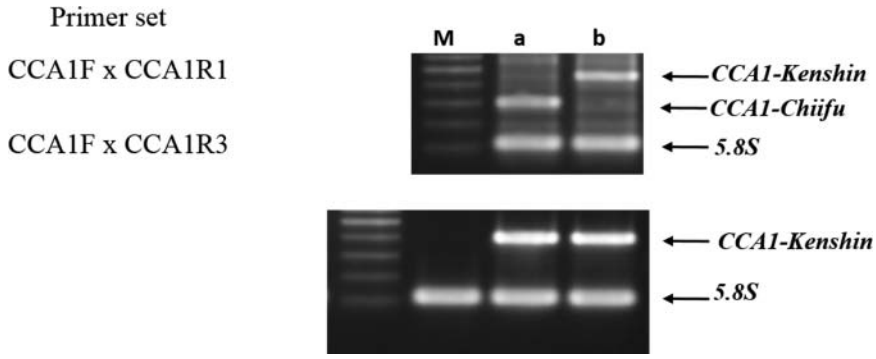

Figure 1. Genomic PCR amplification of gene-specific markers (CCA1F × CCA1R1; CCA1F × CCA1R3) using two cultivars of *B. rapa*: (a) "Nunnara"; (b) "Asia alphain".

plants exposed to different temperatures (22°C, 3°C, −2°C, −6°C). Reactions were conducted in 0.5 ml PCR tubes containing 20 pmol of each primer, 150 μM of each dNTP, 1.2 U of *Taq* polymerase, 1× *Taq* polymerase buffer, and double-distilled H_2O to a final volume of 20 μl. PCR consisted of pre-denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s, followed by final extension for 5 min at 72°C.

RT-PCR (qPCR) was performed using 1 μl of cDNA in a 25 μl reaction volume with iTaq™ SYBR® Green Super-mix with ROX (California, USA). The primers used to perform RT-PCR are listed in Table 1. The reaction conditions were as follows: 10 min at 95°C, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s. The fluorescent product was detected at the last step of each cycle. Amplification, detection, and data analysis were carried out using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). The threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than the background in the initial exponential phase of PCR amplification.

Data analysis

The *Br-Actin* was used as the internal reference in all analyses and relative gene expression level was calculated on the basis of the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Means were separated using Tukey Pairwise Comparisons test ($P = 0.05$) in the Minitab 17 Statistical Software (State College, Pennsylvania, USA).

Results and discussion

Unigene assembly and functional annotation by searching against public databases

A review of the literature revealed that assembled unigenes identified a large number of stress-related genes and were well-expressed in the stressed condition (Ogundiwin et al. 2008; Ghangal et al. 2012). Based on these observations, to identify a *CRG*, stress-chilled tandem consensus and singleton sequences were retrieved from *Br*TED (Yu et al. 2011). The unique set of contig and singleton clusters were assembled into 903 unique sequences comprising 535 contigs and 368 singletons. The average read length of sequences in our data set was 863 bp. Ghangal et al. (2012) revealed polyA/T tails from unigenes, which signifies the presence of mature C termini in the translated proteins. Our results predicted that the polyA/T tails were present in 32 of the unigenes. A complete summary of sequences is presented in Table 2.

A homology-based functional annotation assignment for the stress-chilled sequences was accomplished using BLASTX queries against non-redundant databases. Nine

Table 1. *BrCRG* primer sequences used for RT-PCR.

Gene ID	Forward primer	Reverse primer	Product size (bp)
BrCRG1	GAATCTATCAGAGGTTCAG	AACAAGATCAATCGTCAA	199
BrCRG2	CAAGTCACCGAGCATCATCTG	CGTAACCGCCAGAGAACTTC	200
BrCRG3	ACCACTTCACCGAAGCAATC	GCTTCTTCGAGCTGGTTCAG	199
BrCRG4	TGGCTCATCCATAAAGTGACC	GTTTCCTCCCTGCCACTAAAC	203
BrCRG5	ATTGCGTTCAAGAGGGTAGTG	ATGGATCTCTCTGACGTTCAG	200
BrCRG6	CCTGCAAACCTCTTACTAACC	AGGACTGCGAGACCAAACG	180
BrCRG7	CCTAACCAAACAAGTCCTTGC	GCTCTAACAGCTTCACGATCC	200
BrActin	CAACCAATCGTCTGTGACAA	ATGTCTTGGCCTACCAACAA	

Table 2. Summary of unigenes, clustering and functional annotation.

Items	Records
Number of contigs	535
Number of singletons	368
Total number of sequences	903
Presence of polyA/T (AAUAAA)	32
Number of bases (singletons)	178,055
Number of bases (contigs)	601,102
Total number of bases (all sequences)	779,777
Minimum length (in singletons)	117
Maximum length (in singletons)	945
Average length of singletons	484
Minimum length (in contigs)	180
Maximum length (in contigs)	3288
Average length of contigs	1124
Average length of sequence	863
Number of sequences annotated	851
Total number of unannotated sequences	52

hundred and three tandem and consensus sequences were used to search for non-redundant protein databases in the Blast2go suite. Only matches with an E-value of 10^{-3} or less, an HSP cut-off of 33 and a maximum of 20 blast hits per sequence were considered (Conesa & Götz 2008; Götz et al. 2008). The average result for each BLASTX search was 17 sequences per unigene for 851 (94. 2%) sequences, while the remaining 52 unigenes (13 contigs and 39 singleton sequences; 5.8%) were left unannotated, because they did not show any significant similarity or contained genes that remained unassigned following the genome annotation effort. These sequences may be unique to *B. rapa*. We found that nearly 61.3% contigs (522) and 38.6% (329) singletons were annotated, indicating clearly that the clustering of contigs results in longer transcript and provides a better platform for annotation. The total blast hits was 15,026 (93.5%) from 16,055 sequences from the top 29 species and another 1029 (6.4%) sequences from other species of a non-redundant database (Table 3). All the plant species have been well studied and have more experimental data. The use of a homology-based functional annotation of those species with the BLAST score is acceptable (Sathiyamoorthy et al. 2010; Thamil Arasan et al. 2013).

Function analysis based on gene ontology

Assignment of gene ontology (GO) terms to unigenes was conducted using the Blast2go suite. GO is widely used to simplify the annotation process, gene functional annotation and classification of functional genomics (Sathiyamoorthy et al. 2010; The Gene Ontology Consortium 2010). GO describes the gene function using controlled vocabularies and hierarchies, including the three major categories molecular function, biological processes and cellular component. The unigene sequences were grouped in terms of the GO vocabularies belonging to only one combination of two, and all three vocabularies were organized in a Venn diagram. The total unigene sequences were classified into 732, 693, and 690 tandem and consensus sequences of molecular function (MF), biological process (BP) and cellular component (CC), respectively. In addition, 589, 597, and 641unigenes were mapped under CC and BP, CC and MF, and MF and BP, respectively. Additionally, 553 were annotated according to all GO subvocabularies (i.e. CC, BP, and MF; Figure 2). PlantGOslim was used to screen the plant-specific GO

Table 3. Classification based on species from BLAST hits sequence for 903 unigenes.

Species	BLAST hits	Species	BLAST hits
Arabidopsis thaliana	3425	*Picea sitchensis*	101
Glycine max	1900	*Brassica rapa*	98
Vitis vinifera	1763	Unknown	65
Arabidopsis lyrata	1578	*Brassica napus*	64
Populus trichocarpa	1350	*Solanum lycopersicum*	61
Oryza sativa	1015	*Nicotiana tabacum*	61
Ricinus communis	780	*Gossypium hirsutum*	51
Medicago truncatula	684	*Solanum tuberosum*	47
Brachypodium distachyon	371	*Pisum sativum*	37
Zea mays	339	*Brassica oleracea*	33
Sorghum bicolor	337	*Silene latifolia*	31
Hordeum vulgare	271	*Arabidopsis halleri*	31
Selaginella moellendorffii	199	*Cucumis melo*	30
Physcomitrella patens	164	*Malus* ×	29
Thellungiella halophila	111	Others	1029

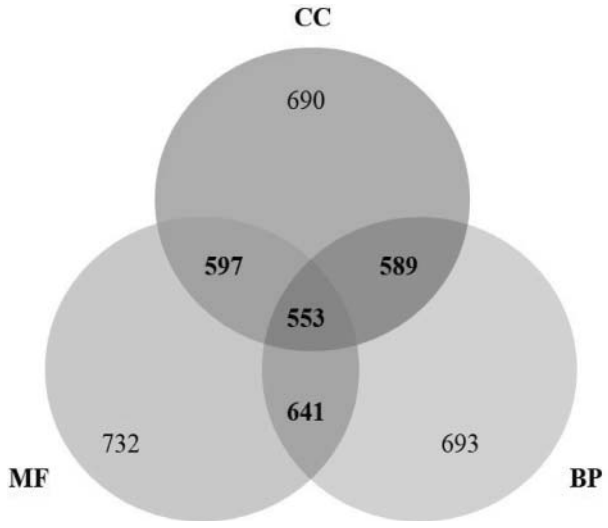

Figure 2. Venn diagram of the 903-unigene data set showing numbers annotated to a combination of two and all three GO vocabularies (MF, molecular function; CC, cellular component; BP: biological process).

vocabularies. A large number of unique sequences were grouped under the first category of MF, which included genes with nucleotide binding, structural molecule, RNA binding, and transcription factor activity. The second category, BP, included subcategories such as the response to stress, response to biotic stimulus, response to abiotic stimuli, protein modification process, catabolic process, and lipid metabolic process. CC included genes encoding plastids, plasma membranes, mitochondria, cytosol, and vacuoles. The GO results relied on well-annotated GO information pertaining to other plants (Figure 3). Within the BP category, 139 genes were related to response to stress (SR), indicating that they were likely involved in all kind of stresses. Nearly 113 and 57 genes in this category were also involved in response to abiotic (AS) and biotic stress (BS), respectively. Additionally, 81 genes were involved in SR and AB, 21 in AS and BS, and 42 genes in BS and SR. Finally, 16 genes were involved in all 3 subcategories (Figure 4). Many studies have implicated different genes regulated by cold stress, thus suggesting multistress interaction of genes in an organism (Seki et al. 2001; Kreps et al. 2002).

To identify the biological pathways which are active in *Brassica*, we used the Blast2go suite to map annotated sequences with reference to the canonical pathways in the KEGG on the basis of the enzyme commission (EC) number assigned to unigenes. Overall, we assigned 515 sequences to 97 KEGG pathways, the majority of which were unigenes related to metabolic pathways. Out of the six major classes of enzymes, EC: 1-oxidoreductase (115, unigenes) was the maximum represented, followed by EC: 2-transferase (99, unigenes).

To improve the theoretical evidence of the annotation, some data mining was performed from the biological databases. An alternative control vocabulary of biochemical for genes is KEGG orthology (KO). The use of the KOBAS (http://kobas.cbi.pku.edu.cn/program.inputForm.do?program=Annotate) bioinformatics pipeline assigned KOs of 51, 102, and 123 to 57 unigenes of BS, 113 unigenes of AS, and 139 unigenes of SR genes in that clusters, respectively. The use of these 51, 102, and 123 putative sequences was assigned to KOs, and 89.3% of the annotation was improved for the putative sequence. Interproscan was provided by EBI web service (Labarga et al. 2007). Based on the GO terms, the functional domains from a range of primary and secondary protein databases were obtained for ESTs using Blast2Go bioinformatics tool.

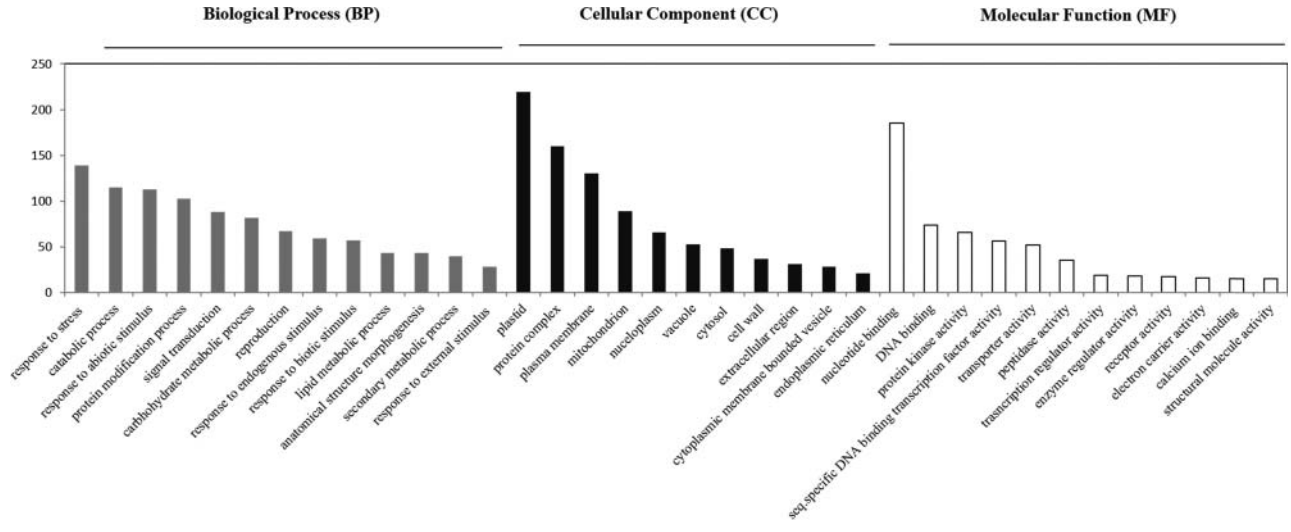

Figure 3. Assignment of GO terms to *B. rapa* stress-chilled unigenes in different categories of cellular component (CC), molecular function (MF) and biological process (BP).

Databases, including PFAM (Finn et al. 2006), SMART (Letunic et al. 2009), GENE3d, PROSITE, PROFILE (Hulo et al. 2006), SUPERFAMILY (Gough et al. 2001), PANTHER, PIR (Wu et al. 2003), PRINTS (Attwood et al. 2000) and TIGRFAMs (Selengut et al. 2007), TMHMM (Krogh et al. 2001) and SINGNALP (Bendtsen et al. 2004) were used to identify the transmembrane regions and signaling part of the EST sequence. The total unique ids were 639 PFAM, 190 SMART, 490 GENE3d, 125 PROSITE, 225 PROFILE, 571 SUPERFAMILY, 918 PANTHER, 28 PIR, 79 PRINTS, and 57 TIGRFAMs. For the transmembrane regions, 316 TMHMM and 290 SINGNALP were found. Functional domains were identified for the genes involved in the response to abiotic stress, cold-shock protein, DNA-binding PF00313 (PFAM), zinc finger, CCHC-type PF00098 (Wistow 1990; Klug 1999). Using these merging results with the GO annotation, a mean of 24.4% totally augmented the functional annotations for the putative sequences.

In silico analysis of unigene-derived SSRs

Among the different classes of molecular markers available, microsatellites or SSRs provide the most useful molecular marker system for diverse applications including genetic diversity assessment, molecular mapping and marker-assisted breeding. Ramchiary et al. (2011) explored some of these SSRs from a unigenes data set and developed polymorphic microsatellite markers for *B. rapa.* A total of 138 SSRs were identified in 113 unigenes. Additionally, more than 52% of the SSRs were tri-nucleotide repeats, while 45%were di-nucleotide repeats (Table 4).Within the di-nucleotide repeats, the TC motif was found to be most abundant followed by the GA motif,

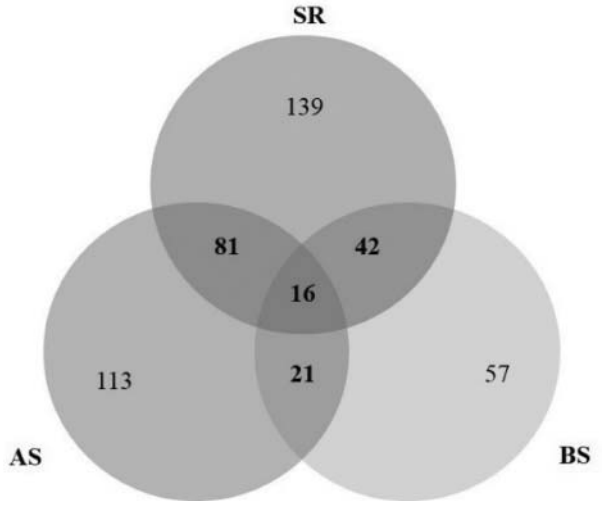

Figure 4. Venn diagram of a 309 stress-related unigene data set showing numbers of annotated, response to stress (SR), response to abiotic stimulus (AS), and response to biotic stimulus (BS).

Table 4. Distribution of SSRs in unigenes sequences.

Items	Records
Total number of unigenes examined	903
Number of unigenes containing SSRs	114
Total number of SSRs identified (excluding mononucleotide repeats)	138
Unigenes having more than 1 SSR	32
Number of di-nucleotide repeats [$\geq(NN)_6$]	63
Number of tri-nucleotide repeats [$\geq(NNN)_5$]	72
Number of tetra-nucleotide repeats [$\geq(NNNN)_5$]	1
Number of penta-nucleotide repeats [$\geq(NNNNN)_4$]	1
Number of hexa-nucleotide repeats [$\geq(NNNNNN)_4$]	1

whereas among tri-nucleotide repeats, the GAA motif was most abundant followed by the AAG motif. Rudolph et al. (1999) have also explored the motif repeats from the rape seed genome. Thus, we have shown that the mining of AS unigenes is an effective strategy to identify functional microsatellites, with perfect repeats, in *B. rapa*.

Characterization of BrCRG

We functionally characterized the stress-chilled unigene sequences from the *Br*TED database based on GO and obtained 12.5, 21.4, and 14.8% of AS, AS and SR, and AS and BS, respectively (Figure 4). This study was extended with analysis of the responses of cold- and freezing-related genes against cold and freezing stresses as experimental evidence of such unigene clustering, and 7 unigenes were randomly selected from 113 AS unigenes that are known to be associated with cold stress from previous studies in model plants. Based on these unigenes, gene sequences were collected from the *Brassica* database (BRAD) using a BLASTN search. These seven genes were designated as *B. rapa* cold-responsive genes (*BrCRG*) and are summarized in Table 5. Gong et al. (2005) suggested that RNA helicase may be directly involved in temperature-sensing activity and is of importance for mRNA export during stress responses in *Arabidopsis*. *BrCRG1* was hypothetical proteins and *BrCRG5* was a dead box RNA helix, but two genes had the same domain as DEAD-like helicases, which might be involved in mRNA export in plants sensitive to cold and freezing temperatures. Nishizawa-Yokoi et al. (2011) suggested that heat stress transcriptional factor A-1e (H*sf*A-1e) controls the expression of Hsfs genes in response to various environmental stresses. *BrCRG2* is a heat stress transcription factor A-1e gene has heat shock factor domain. *BrCRG3* has a heat shock chaperonin binding motif and *BrCRG4* has calmodulin-binding motifs; both functions are associated with the cold stress gene (Tabata et al. 2000; Bouché et al. 2002). *BrCRG6* propyzamide-hypersensitive 1 (Psh1) gene was identified in a previous study

Table 5. List of seven *BrCRG* identified in *Brassica rapa* and their sequence identity with functional homology.

Designated Id.	BRAD Id.	Nucl. (bp)	Chr. no.	Protein		Top matched clones	Identity (%)	Top homologous	Description	Function	References
				Length (aa)	Domain						
BrCRG1	Bra031349	1809	A05	602	DEAD-like, helicase superfamily c-terminal	XM002883308	86	*Arabidopsis lyrata* subsp. *lyrata*	Hypothetical protein	Unknown	Unpublished
BrCRG2	Bra032023	1371	A02	456	Heat shock factor	NM111169	84	*A. thaliana*	Heat stress transcription factor A-1e	Environment stress	Salanoubat *et al.* 2000, Nishizawa-Yokoi *et al.* 2011
BrCRG3	Bra036648	1662	A09	553	Tetratricopeptide repeats, heat shock chaperonin-binding motif	NM104951	91	*A. thaliana*	Putative stress-inducible protein	Cold stress	Theologis *et al.*2000; Seki *et al.* 2001
BrCRG4	Bra009382	3024	A10	1007	CG-1, ANK c almodulin-binding motif	AF491304	96	*Brassica napus*	Calmodulin-binding transcription activator	Cold stress	Bouche *et al.* 2002, Doherty *et al.* 2009
BrCRG5	Bra009356	1686	A10	561	DEAD-like helicases, helicase superfamily c-terminal	XM002871306	88	*A. lyrata* subsp. *lyrata*	Dead box RNA helicase	Cold stress	Unpublished, Tripurani *et al.* 2011
BrCRG6	Bra009705	2802	A06	933	Act-Frag_cataly, dual-specificity phosphatase, catalytic, zinc finger	XM002872023	87	*A. lyrata* subsp. *lyrata*	Propyzamide-hypersensitive 1	Response to ABA	Unpublished, Gosti *et al.* 1999
BrCRG7	Bra020611	1887	A02	628	FAR1, MULE, ZnF_PMZ	NM122736	81	*A. thaliana*	Putative protein FAR1-related sequence 10	Response to light control	Unpublished, Lin &Wang 2004

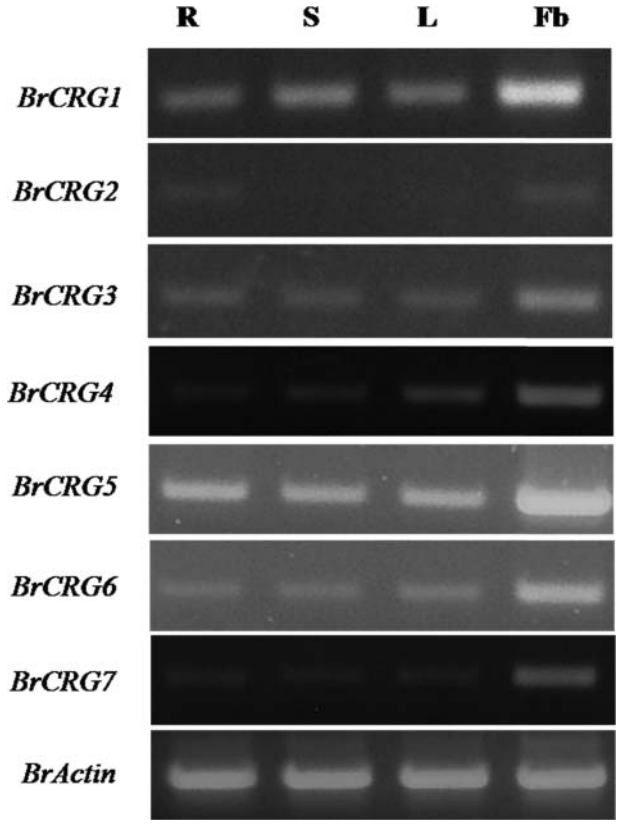

Figure 5. Analysis of the organ-specific expression pattern of seven *BrCRG* by RT-PCR, cDNA isolated from chiifu. R, root; S, stem; L, leaves; FB, flower buds.

and was suggested to be hypersensitive to ABA signaling in response to ABA stress (Bouché et al. 2002). *BrCRG7* is a putative protein FAR1-related gene identified in a previous study. It was found that this group of family proteins could be involved in distinct signaling processes in response to various biotic and abiotic stresses (Lin & Wang 2004). Further characterization of these genes will ascertain their role in cold stress management.

Organ-specific expression analysis

In a previous study, the expression of cold-inducible genes was observed in flowers and leaves of rice (Kim et al. 2007). Conversely, Fernandez et al. (2008) also identified chilling-responsive genes in sunflower leaves, stems and flowers. We observed the expression of *BrCRG* in an organ-specific pattern. Among these genes, *BrCRG1*, *3*, *4*, *5*, *6* and *7* showed expression in all organs. *BrCRG2* was only expressed in roots and flower buds. It should be noted that the expression of most genes was comparatively higher in flower buds than other organs. *BrCRG* was expressed in an organ-specific manner, suggesting possible roles of these genes in specific organs through cold and freezing stress conditions (Figure 5).

Expression analysis of BrCRG *in cold and freezing stress conditions*

To validate our analysis of seven *BrCRG* associated with increased freezing survival, we compared the gene expression profiles of a "Nunnara" (chiifu) and "Asia alphain" (kenshin) cultivar. A review of available literature revealed cold and freezing tolerance is a multigenic trait (Thomashow 2001). Monroy et al. (2007) have detected new genes from winter and spring cultivars of wheat in cold acclimation conditions. Zalunskaite et al. (2008) also investigated *CRG* in different cultivars of orchard plants that were sensitive and tolerant to cold stress. To explore such responses, we attempted to investigate the expression in cold (3°C) and freezing (−2°C and −6°C) stress condition over the same time course of 2 h (Figure 6). *BrCRG1*, *2*, *3* and *5* genes are substantially upregulated during freezing stress conditions in the "Nunnara" cultivar relative to the "Asia alphain" cultivar. (a) *BrCRG1* was differentially expressed in both cultivars. However, this gene gradually increased the level of tolerance in "Nunnara" was twice as high at −2°C relative to the "Asia alphain" cultivar. (b) *BrCRG2* had substantially upregulated gene expression in "Nunnara" compared with "Asia alphain". There is no notable expression pattern in "Asia alphain". This gene has tolerance to cold and freezing stress conditions. (c) *BrCRG3* expressed two differential expression patterns in the respective cultivars. Specifically, this gene has revealed the tolerance level in "Nunnara" and susceptible level of "Asia alphain" cultivars at cold and freezing stress conditions. (d) *BrCRG4* and (e) *BrCRG5* had similar expression patterns in both the cultivars. *BrCRG4* was well-expressed at 3°C, showing a cold tolerance level in both cultivars. It is noted here that the *BrCRG4* has gradually increased tolerance levels in freezing stress conditions for both cultivars. *BrCRG5* was gradually expressed in 3°C to −2°C followed by a decrease at −6°C. This gene was substantially expressed in "Nunnara", which shows that the gene has participated in the tolerance level of this cultivar. (f) *BrCRG6* was highly induced at 3°C in "Asia alphain", showing the tolerance level in a susceptible cultivar. However, this gene gradually increased the tolerance level in both cultivars at freezing stress conditions. (g) *BrCRG7* was downregulation in the "Asia alphain" cultivar, showing the susceptible levels of this gene. However, no differential expression pattern after stress treatment was seen in the "Nunnara" cultivar. These expression patterns suggest that *BrCRG1*, *2*, *3* and *5* have participated in freezing tolerance genes and *BrCRG4* and *6* have participated in cold tolerance genes that were identified as differentially regulated between the *B. rapa* cultivars. These results show that

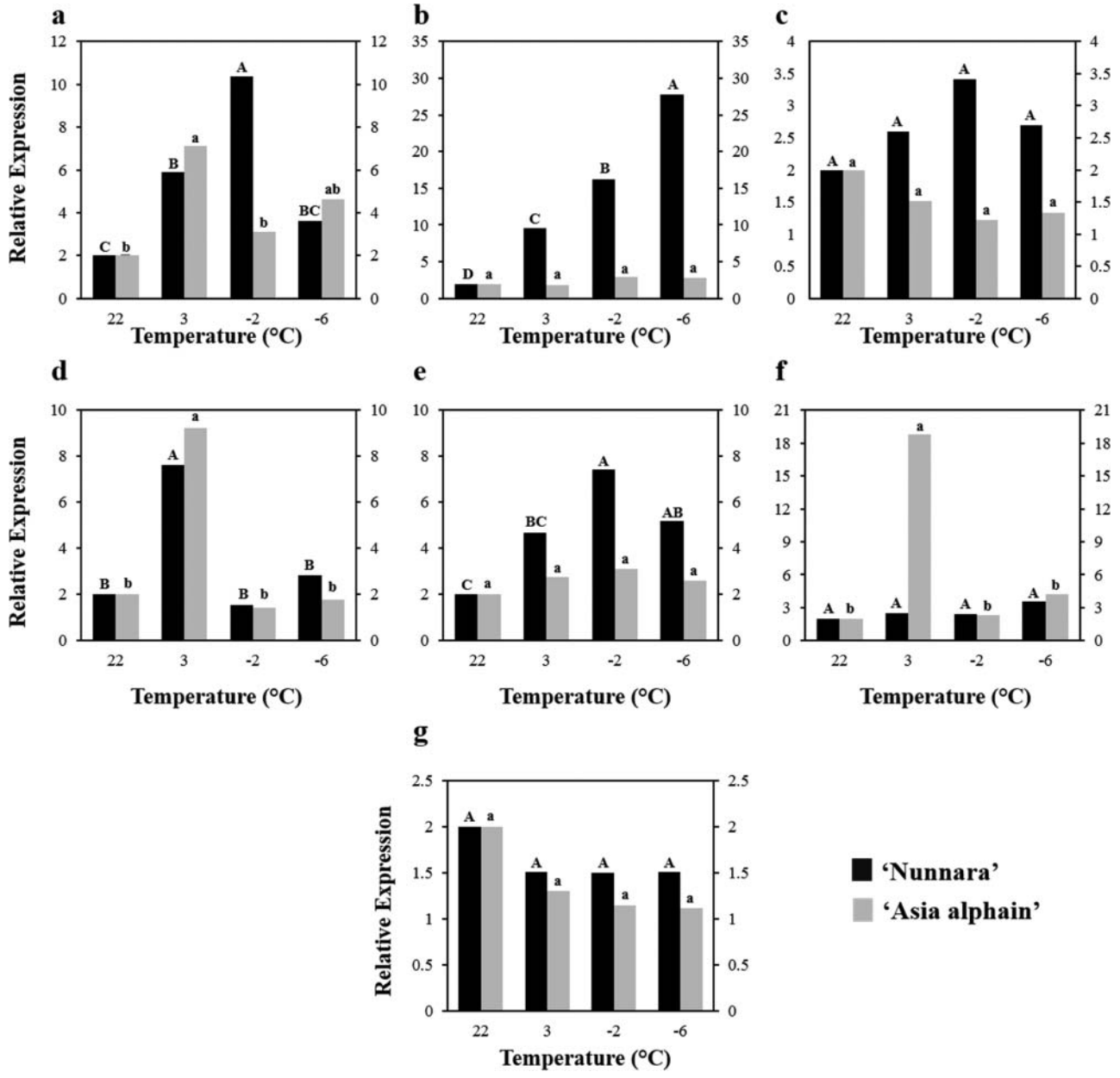

Figure 6. RT-PCR expression analysis of *BrCRG 1−7* (a−g) after cold and freezing stress treatment (22°C, 3°C, −2°C, −6°C) in two contrasting cultivars ("Nunnara" and "Asia alphain") of *B. rapa*. Values within the cultivar denoted by the same letters (caps for Nunnara and small letters for Asia alphain) do not differ significantly at $P < 0.05$ according to the Tukey HSD test.

the unigenes were well clustered by such unigene analysis.

Overall, the unigene data set provides valuable resources for discovering potential tolerance genes associated with cold and freezing stress and suggests a possible way to characterize them.

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