Identification and characterization of stress resistance related genes of *Brassica rapa*

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Abstract Two biotic stress resistance related genes from the full-length cDNA library of *Brassica rapa* cv. Osome were identified from EST analysis and determined to be pathogenesis-related (PR) 12 *Brassica* defensin-like family protein (*BrDLFP*) and PR-10 *Brassica Betv1* allergen family protein (*BrBetv1AFP*) after sequence analysis and homology study with other stress resistance related same family genes. In the expression analysis, both genes expressed in different organs and during all developmental growth stages in healthy plants. Expression of *BrDLFP* significantly increased and *BrBetv1AFP* gradually decreased after infection with *Pectobacterium carotovorum* subsp. *carotovorum* in Chinese cabbage. Expression of these two genes significantly changed after cold, salt, drought and ABA stress treatments. These two PR genes may therefore be involved in the plant resistance against biotic and abiotic stresses.

Keywords Biotic and abiotic stress · *Brassica* · Chinese cabbage · Pathogenesis-related genes · *Pectobacterium carotovorum* subsp. *carotovorum*

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Introduction

Brassicas are an important and diverse group of crops grown worldwide that belong mainly to *Brassica rapa*, as well as *Brassica oleracea* and *Brassica napus* (Cardoza and Stewart 2004). Chinese cabbage (*B. rapa*) is widely recognized for its economic importance and contribution to human nutrition (Salunkhe and Kadam 1998). Plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. Biotic stresses include fungi, bacteria, viruses, insects, and abiotic stresses include cold, salt, ABA, drought, high temperature, and heavy metal pollution. Each type of stress functions through different types of molecular mechanisms that affect plants and eventually cause damage. Plants respond to biotic and abiotic stress factors by producing various defense-related proteins, such as pathogenesis-related (PR) proteins, hydroxyproline-rich glycoproteins (HRGPs), and several key enzymes involved in the synthesis of phenylpropanoids,

phytoalexins, and lignins (Rajam et al. 2007; Pan et al. 2011; Korkina 2007).

One promising strategy to develop resistance against biotic and abiotic stresses is based on the exploitation of the PRs and other stress responsive genes in transgenic plants. Accumulation of PRs extends into non-inoculated plant parts that, upon challenge, exhibit acquired resistance (Van Loon and Van Kammen 1970; Ryals et al. 1996). PR-proteins are classified into 17 families based on sequence similarity and biochemical functions (Van Loon et al. 2006). It is well known that the birch allergen *Betv1* genes belong to the PR-10 family and the defensin genes belong to the PR-12 family (Van Loon and Van Strien 1999). An important common feature of most PRs is their antifungal effect; some PRs also exhibit antibacterial, insecticidal, nematicidal, and anti-viral activity (Van Loon and Van Strien 1999; Van Loon 2001; Selitrennikoff 2001). For instance, the expression of PR-10 protein was significantly higher in *Erwinia carotovora* resistant chicory inbred lines when compared with susceptible lines, suggesting its involvement in chicory resistance to pathogens attack (Molarda et al. 2004). The product from the defensin gene (*BSD1*), which belongs to the PR-12 family, shows antifungal activity against several phytopathogenic fungi (Park et al. 2002) and transgenic tobacco plants expressing a defensin gene (*Rs-AFP1*) showed enhanced resistance to fungal pathogens (Terras et al. 1995).

In this study, we analyzed the sequences of two stress resistance related genes that were identified from the full-length cDNA library of *B. rapa* cv. Osome and studied their homology with other stress resistance related genes. Expression of these genes was analyzed in different organs and developmental growth stages. In addition, expression was also analyzed after applying abiotic stresses in Chinese cabbage and by microarray data of biotic stress treatments, and their association with biotic and abiotic stress resistance was discussed.

Materials and methods

Plant materials

Chinese cabbage (*B. rapa* 'SUN-3061') plants were grown in the Department of Horticulture, Sunchon National University, Korea. Fresh roots, stems, leaves, and flower buds were harvested, frozen immediately in liquid nitrogen, and stored at −80°C for RNA or DNA isolation.

Stress treatments

Chinese cabbages (*B. rapa* 'SUN-3061') seeds were aseptically grown on MS agar medium in a culture room under a 16 h light photoperiod at 25°C. After 3 weeks of growth, the seedlings were transferred to fresh liquid MSH (half-strength MS medium without sucrose) medium containing 250 mM NaCl and 100 mM abscisic acid (ABA) for 24 h. To induce cold stress, the seedlings were maintained at 4°C for 24 h. Drought treatment was applied by keeping the seedling on the filter paper at 28°C for 24 h. The samples were treated with all the stresses for 0 h (wild type), 30 min, 1, 2, 4, 8, and 24 h and then frozen immediately in liquid nitrogen, and stored at −80°C for RNA or DNA isolation.

RNA extraction

Total RNA was extracted from roots, stems, leaves, flower buds of healthy plants and abiotic stress applied frozen samples using the Rneasy mini kit (Qiagen, USA). RNA was treated with RNase-free DNase (Promega, USA) to remove genomic DNA contaminants.

Sequence analysis of genes

DNA and protein sequences were processed or deduced using Software Editseq (DNAstar Lasergene, USA). ORF was obtained by ORF finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The primary structure of genes was analyzed using protParam (http://expasy.org/tools/protparam.html) and Augustus (http://augustus.gobics.de/submission). The alignment search was carried out using BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The program used was BLASTp and the database "nr" was selected. Typical domains were analyzed using the web tool from EMBL (http://smart.embl.de/smart/set_mode.cgi?GENOMIC=1). Multiple protein sequences were aligned using PIR (http://pir.georgetown.edu/pirwww/search/multialn.shtml).

Expression analysis

RT-PCR was performed using AMV one step RT-PCR kit (Takara, Japan). The specific primers for *BrDLFP*, FW-GAGTATCACCCAAGTTTCTGA and RV-TC ATTATAGCTTCCTCCTACG, and for *BrBetv1AFP*, FW-TAGAAAATGGGATTGAGTGG, and RV-AAC TGATAGAGATGATCGAACC were used for RT-PCR. Actin primers of *Brassica*, FW-ATGGTTGG GATGGGTCAAAAA, and RV-TCTTTAATGTCAC GGACGATT, were used as control. PCR was performed in 20 μl containing 50 ng cDNA as template in 0.5 ml PCR tubes, 20 pmol of each primer, 150 μM of each dNTP, 1.2 U *Taq* polymerase, *Taq* polymerase buffer, and double-distilled H_2O. The procedure was pre-denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and was terminated with an additional extension for 5 min at 72°C. The microarray data on *B. rapa* subsp. *pekinensis* were obtained from the *B. rapa* Genomic Project of National Academy of Agricultural Science (Suwon, Korea) (http://brassica-rapa.org/BRGP/index. jsp). Real-time quantitative PCR was performed using 1 μl cDNA in 20 μl using iTaq SYBR Green Super-mix with ROX (California, USA). Specific primers for *BrDLFP*, FW-GAGTATCACCCAAGTTTCTGA, and RV-CAGCTGAAATCAACAAGACA and for *BrBet-v1AFP*, FW-CTGCTGAGTTTGAGAAGACC and RV-AACTGATAGAGATGATCGAACC were used to perform real-time PCR. The thermal cycler conditions recommended by the manufacturer were used as follows: 10 min at 95°C, followed by 40 cycles at 94°C for 30 s, 55°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 with a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). Threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than the background at the initial exponential phase of PCR amplification. Means were separated using Duncan's multiple range test ($P = 0.05$) in the SAS (2010) program.

Results and discussion

Identification and sequence analysis

We constructed a full-length cDNA library of *B. rapa* cv. Osome (Park et al. 2010) and obtained 3,429 ESTs from this library. These ESTs were then annotated and 140 biotic stress related genes were selected (unpublished). From these genes, three chitinase genes have been characterized and their expression after pathogen infection was previously evaluated (Ahmed et al. 2011). In this study, we selected two cDNA clones which are small, cysteine-rich polypeptides and play vital role for stress resistance from these genes and sequenced as full-length cDNA. Several families of small, cysteine-rich basic antimicrobial polypeptides play important roles in protecting plants against invading phytopathogens (Garcia-Olmedo et al. 1998). Two full-length cDNAs encoding biotic stress resistance related genes, designated as *Brassica* defensin-like family protein (*BrDLFP*) and *Brassica Betv1* allergen family protein (*BrBetv1AFP*) were characterized. The sequence data for *BrDLFP* and *BrBetv1AFP* are deposited in GenBank under accession number of EU186319 and EU186326, respectively.

In the sequence analysis, these genes provided complete coverage of the transcribed region and no introns were identified in the coding region of both genes. The primary structure of these genes was analyzed using bioinformatic tools and the obtained data are presented in the Table 1.

The full-length sequence of the *BrDLFP* cDNA contained 579 nucleotides and had a putative ORF of 273 bp encoding a defensin protein that contains 90

Table 1 Primary structure analysis of *BrDLFP* and *BrBetv1AFP* genes of *Brassica rapa* cv. Osome

Name of genes	Molecular mass (kDa)	Isoelectric point (pI)	Instability index (II)	Positively charged residue (Arg + Lys)	Negatively charged residue (Asp + Glu)
BrDLFP	10.08	5.77	35.41	7	13
BrBetvIAFP	17.16	5.27	29.75	18	23

Analyzed using protParam, http://expasy.org/tools/protparam.html

amino acids, beginning at the initiation codon ATG (position 78) and ending at the stop codon TAA (position 350) of the cDNA. Defensins are small cysteine-rich peptides with a molecular mass between 5 and 10 kDa and some of these peptides have been shown to exhibit potent antifungal activity (Olli and Kirti 2006). *BrDLF*P has a low molecular weight and contains cysteine residues in identical positions with other published defensin proteins (Fig. 1a). The cysteine-rich motif is believed to be typical for proteins belonging to the plant defensin family.

The full-length sequence of the *BrBetv1AFP* cDNA contained 636 nucleotides and had a ORF of 468 bp encoding an allergen family protein that contains 155 amino acids, beginning at the initiation codon ATG (position 50) and ending at the stop codon TAA (position 517) of the cDNA. The *Betv1* family was named after the major birch pollen allergen and belongs to the PR-10 family (Radauer et al. 2008). In the *BrBetv1AFP* gene, one *Betv1* domain was observed between amino acid residues 2 and 152,

which suggest that this protein is a *Betv1* family protein and belongs to PR-10 family.

Amino acid sequence comparison

The alignment search was carried out using BLAST from NCBI. The deduced protein sequence of *BrDLFP* (ABV89608) shared a high homology with the defensin-like protein of *Arabidopsis thaliana* (NP001030645 and NP187224) (59 and 62% identity, respectively) (Salanoubat et al. 2000). *BrBetv1AFP* (ABV89615) showed high homology with the pollen allergen-like protein of *A. thaliana* (AAM65899) (90% identity) (Haas et al. 2002) and *Betv1* allergen family protein of *Arabidopsis lyrata* subsp. *lyrata* (XP002893317) (91% identity) (unpublished work) (Table 2). Multiple alignment also revealed high similarity among the deduced amino acids of *BrDLFP* and three other defensin genes (Fig. 1a) and *BrBet-v1AFP* and three other *Betv1* allergen family genes (Fig. 1b) indicating their relatively conserved

Fig. 1 Alignment of deduced amino acid sequences of **a** *BrDLFP* and three other stress responsive defensin genes of *A. thaliana* (NP_001030645.1 and NP_187224.1) and *R. sativus* (P30230.4). Cysteine residues in identical positions were indicated by *asterisk*, **b** *BrBetv1AFP* with three other stress responsive genes of *A. thaliana* (AAM65899.1), *A. lyrata* subsp. *lyrata* (XP_002893317.1), and *Zea mays* (NP_001105279.1) using PIR. Numbers on the *right margin* indicate the positions of amino acid residues. The identical amino acids are marked in *dark background*, and more than 50% similarities in *light*

Table 2 Overall analysis of *BrDLFP* and *BrBetv1AFP* genes of *Brassica rapa cv*. Osome

Gene	Length	Top matched clone	Top matched protein	Score	*E* value	Top homologous species
BrDLFP	90	NP_001030645.1	Defensin-like family protein	95.1	3e−18	*Arabidopsis thaliana*
BrBetvIAFP	155	AAM65899.1	Pollen allergen-like protein	288	1e−76	*Arabidopsis thaliana*

Analyzed using BLAST from NCBI, http://www.ncbi.nlm.nih.gov/BLAST/

evolutionary relationship at the protein level. Among the three other defensin genes, the *Raphanus sativus* (P30230) gene conferred enhanced resistance to the foliar pathogen *Alternaria longipes* in transgenic tobacco (Terras et al. 1995) but the stress resistance related functions of three other species *Betv1* allergen family genes have not yet been examined.

Organ-specific and growth-stage expression analysis

Expression analysis was performed using specific primers with equal amounts of cDNA templates prepared from the mRNA of roots, stems, leaves,

and flower buds of Chinese cabbage by RT-PCR. *BrDLFP* showed lower level of expression when compared to the other genes in stems, leaves, and flower buds but no expression was observed in the roots. *BrBetv1AFP* showed high levels of expression in all the organs, except the root and the highest level of expression was observed in flower buds (Fig. 2a). These organs share characteristics that make them particularly prone to pathogen attack. This include the flower organs are largely devoid of mechanical barriers (e.g., cuticle) to allow reproductive functions such as pollen tube penetration. Thus, adequate protection of these organs against pathogen attack is critical, and the production of an antifungal molecule
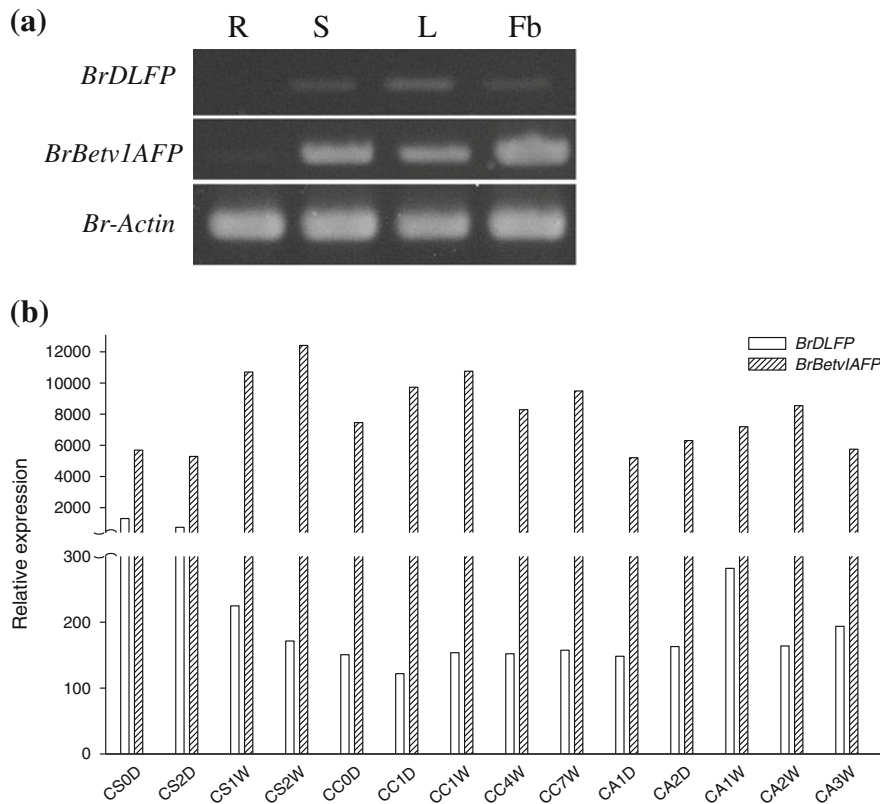

Fig. 2 Expression analysis of *BrDLFP* and *BrBetv1AFP* genes in healthy *B. rapa* subsp. *pekinensis*. **a** RT-PCR expression over different organs, lanes *1–4*, PCR products of roots (*R*), stems (*S*), leaves (*L*) and flower buds (*Fb*). **b** Microarray expression analysis at different growth stages: *CSOD* seed, mature; *CS2D* seedling, 2 days old; *CS1W* whole plant, 1 week old vegetative stage (7 days old); *CS2W* whole plant, 2 weeks old vegetative stage (14 days old); *CCOD* whole plant, 1 day after light-chilled at 4°C (21 days old); *CC1D* whole plant, 1 day after light-chilled at 4°C (22 days old); *CC1W* whole plant, 1 week after

light-chilled at 4°C (28 days old); *CC4W* whole plant, 4 weeks after light-chilled at 4°C (56 days old); *CC7W* whole plant, 7 weeks after light-chilled at 4°C (70 days old); *CA1D* whole plant, 1 day after greenhouse growth (71 days old); *CA2D* whole plant, 2 days after greenhouse growth (72 days old); *CA1W* whole plant, 1 week after greenhouse growth (77 days old); *CA2W* whole plant, 2 weeks after greenhouse growth (84 days old); *CA3W* whole plant, 3 weeks after greenhouse growth (91 days old)

could be part of a local defense strategy (Hamel and Bellemare 1995). Using microarrays data, expression of these two genes were analyzed at different growth stages and both were found to be expressed at all growth stages from mature seed to 91 days old plants. Very low levels of *BrDLFP* expression were observed at all growth stages except at the mature seed and 2 days old seedlings stage (CS0D and CS2D). On the other hand, high levels of *BrBetvIAFP* expression were observed at all growth stages (Fig. 2b). *BrDLFP* showed the highest level of expression at the mature seed stage (CS0D) and 2 days old seedlings stage (CS2D), which decreased gradually towards the mature stages. Organ-specific expression of this gene was low because the cDNA was extracted from the mature plant samples for RT-PCR. Most of the plants defensin proteins are isolated from seed-derived but the expression of several plants defensin isoforms has been observed in leaves (Kragh et al. 1995; Terras et al. 1995) and flowers (Gu et al. 1992; Moreno et al. 1994). This suggests that these genes are differentially regulated in different tissues or organs, thereby providing more efficient protection against pathogen infection.

Expression analysis after applying stresses

Expression in *B. rapa* was also analyzed by microarray data after infection with *Pectobacterium carotovorum* subsp. *carotovorum*, which is a serious pathogen for *Brassica* and causes soft rot disease. Both genes responded to infection: *BrDLFP* was up-regulated at 48 h post-infection (hpi) and *BrBetv1AFP* was down-regulated from 6 hpi (Fig. 3a, b). *BrBetv1AFP* was highly responsive to bacterial infection and its expression decreased by about fourfold at 48 hpi relative to the non-infected plants. This gene therefore remains active in the healthy plants and becomes inactive after pathogen infection; this is then an important association in resistance against biotic stresses.

The results of previous studies also support the association of these types of genes with disease resistance. The expression of some defensin genes is developmentally regulated (Epple et al. 1997), whereas expression of other defensin genes is greatly elevated in response to biotic and abiotic external stimuli (Kragh et al. 1995; Broekaert et al. 1997). Defensin genes were induced upon pathogen infection

Fig. 3 Microarray expression analysis of **a** *BrDLFP* and ▶ **b** *BrBetv1AFP* genes in *B. rapa* subsp. *pekinensis* after infection with *P. carotovorum* subsp. *carotovorum*. **c** RT-PCR and real-time quantitative PCR expression of **d** *BrDLFP* and **e** *BrBetv1AFP* genes after application of cold, salt, drought and ABA stresses. The *error bars* represent the standard error of the means of three independent replicates. Values denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

in peas (Chiang and Hadwiger 1991), tobacco (Gu et al. 1992), and *Arabidopsis* (Epple et al. 1997). Again, the PR-10 class of proteins, first identified as a major pollen allergen (*Betv1*) from white birch (Breiteneder et al. 1989), are induced by pathogen attack in a wide variety of plant species, including parsley (Somssich et al. 1986), potato (Matton and Brisson 1989), pea (Barratt and Clark 1991), soybean (Crowell et al. 1992), asparagus (Warner et al. 1993), sorghum (Lo et al. 1999) and rice (Midoh and Iwata 1996; McGee et al. 2001). However, there has been no direct evidence of their physiological activity and function in plant-defense mechanisms.

Some genes respond significantly when exposed to various environmental and pathogenic stresses and associated with broad spectrum resistance (Lee et al. 2008). To further explore such resistance, we analyzed the expression of three genes using the same primers after applying abiotic stress treatments including cold, salt, drought, and ABA. Expression of both genes changed compared to control plant after cold, salt, drought, and ABA stress treatments. In case of cold stress, *BrDLFP* and *BrBetvIAFP* genes were up-regulated and highest expression was observed after 24 h (Fig. 3c, d, e). In the salt stress, expression of *BrDLFP* was significantly changed with stress treatment but not directional, and *BrBetvIAFP* expression was increased from 0 to 1 h and gradually decreased afterwards (Fig. 3c, d, e). After drought stress treatment, *BrDLFP* and *BrBetvIAFP* both genes showed the highest expression after 1 h and gradually decreased from 1 to 24 h treatments (Fig. 3c, d, e). Again, expression of *BrDLFP* and *BrBetvIAFP* were increased from 0 to 1 h and decreased afterwards while treated with ABA stress (Fig. 3c, d, e). Different types of changes of expression of these genes with abiotic stress treatments indicate their probable association with abiotic stress resistance and we can exploit these genes for overexpression or down-regulation to enhance resistance against abiotic

(a)

(b)

(c)

(d)

(e)
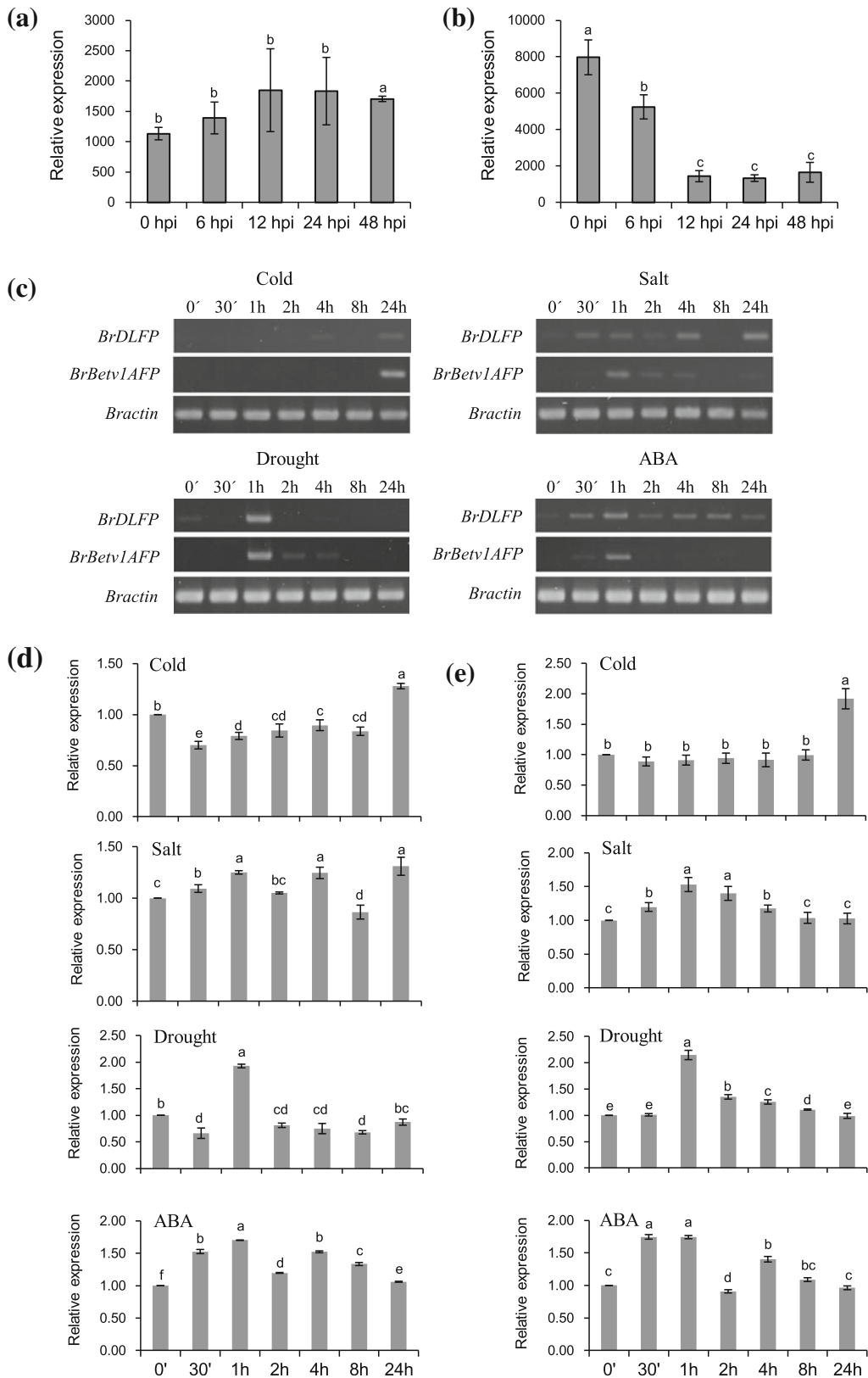
stresses in *Brassica*. In earlier studies, down-regulation of two genes, homologous to aspartic proteinase 2, and homolog of Jumonji class of transcription factor showed relative drought tolerant phenotypes in *Nicotiana benthamiana* (Senthil-Kumar et al. 2007). Induction of novel rice *Betv1* (PR-10) protein was also observed after salt, drought, and ABA stress treatments (Hashimoto et al. 2004; Moons et al. 1997) but defensin genes had no abiotic stress responding report so far we studied.

In conclusion, *BrDLFP* and *BrBetv1AFP* genes in *Brassica* may be useful sources for engineering transgenic plants to be resistant to biotic and abiotic stresses.

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