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ORIGINAL ARTICLE

Identification and comparative analysis of *Brassica juncea* pathogenesis-related genes in response to hormonal, biotic and abiotic stresses

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Abstract Pathogenesis-related proteins (PRs) are the antimicrobial proteins which are commonly used as signatures of defense signaling pathways and systemic acquired resistance. However, in *Brassica juncea* most of the PR proteins have not been fully characterized and remains largely enigmatic. In this study, full-length cDNA sequences of SA (*PR1*, *PR2*, *PR5*) and JA (*PR3*, *PR12* and *PR13*) marker genes were isolated from *B. juncea* and were named as BjPR proteins. BjPR proteins showed maximum identity with known PR proteins of *Brassica* species. Further, expression profiling of *BjPR* genes were investigated after hormonal, biotic and abiotic stresses. Pre-treatment with SA and JA stimulators downregulates each other signature genes suggesting an antagonistic relationship between SA and JA in *B. juncea*. After abscisic acid (ABA) treatment, SA signatures were downregulated while as JA signature genes were upregulated. During *Erysiphe cruciferarum* infection, SA- and JA-dependent *BjPR* genes showed distinct expression pattern both locally and systemically, thus suggesting the activation of SA- and JA-dependent signaling pathways. Further, expression of SA marker genes decreases while as JA-responsive genes increases during drought stress. Interestingly, both SA and JA signature genes were induced after salt stress. We also found that *BjPR* genes displayed ABA-independent gene expression pattern during abiotic stresses thus providing the evidence of SA/JA cross talk. Further, in silico analysis of the upstream regions (1.5 kb) of both SA and JA marker genes showed important *cis*-regulatory elements related to biotic, abiotic and hormonal stresses.

Keywords *Brassica juncea* · *Erysiphe cruciferarum* · PR genes · Abiotic stress · Abscisic acid · Jasmonic acid · Salicylic acid

Introduction

The genus *Brassica* comprises many diversified species that provides oil, vegetables, condiments, dietary fiber, and vitamin C (Talalay and Fahey 2001). *B. juncea* var. Varuna (Indian mustard) is one of the prominent members of *Brassica* genus, with great economic and agricultural importance across the globe. *B. juncea* is an amphidiploid species with a chromosome number of 18, derived from interspecific crosses between, *B. nigra* ($n = 8$) and *B. rapa* ($n = 10$). In India alone, mustard is cultivated around 6 million hectares and is projected that by 2020, 41% of total demand for oilseed will solely be met by this crop (Yadava and Singh 1999). Unfortunately, productivity of this crop is hampered by a variety of biotic (mainly fungal diseases) and abiotic (drought and salinity) stresses which lead to significant yield losses (Mathpal et al. 2011; Goel and Singh 2015). Improving stress tolerance in *B. juncea* through conventional breeding perspective is confounded mainly due to non-availability of suitable resistant sources within the germplasm

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and, therefore, genetic engineering has become imperative to compliment the conventional breeding approach for developing stress-tolerant varieties. However, the identity and role of potential genes or signaling cascades in *B. juncea* responses to different stresses (biotic and abiotic) are largely unknown. Therefore, it is necessary to characterize the stress-related gene families in *B. juncea* to develop resistant or tolerant varieties for sustainable and enhanced productivity of oilseeds.

Plants are constantly challenged by variety of stresses which includes both biotic and abiotic stresses (Ahmad et al. 2015). To protect themselves, plants use multidimensional approaches such as morphological, biochemical and molecular defense responses that help them to retain their fitness or survive under such circumstances. Furthermore, plants also combat biotic and/or abiotic stresses by synthesizing small heterogeneous group of proteins like PR proteins (Van Loon and Van Strien 1999). After first being identified in tobacco plant infected with TMV (tobacco mosaic virus), PR proteins have since been reported in many plants. PR proteins are known to be induced by a variety of biotic and abiotic stresses; hence, are generally considered to be part of multiple defense systems in plants. Presently, PR proteins are grouped into 17 families with diverse functions; some of these are PR1 (unknown), PR2 (β-1, 3-glucanase), PR3 (chitinases), PR5 (thaumatin like), PR9 (peroxidases), PR12 (plant defensins) and PR13 (thionins) (van Loon et al. 2006). Under non-stress state, most of the *PR* genes show basal level expression, but increases dramatically at infection site and also plays key role in systemic acquired resistance (SAR) pathway (Návarová et al. 2012). Generally, SAR is activated in the distal or uninfected tissues in response to a prior (primary or local) infection elsewhere in the plant. In plants, SAR is an inducible immune response that offers enhanced disease resistance against multiple pathogens (Sticher et al. 1997). Interestingly, *PR* genes have been frequently used as SAR signature genes in the model plant *Arabidopsis*. They are also induced by defense signaling inducers (SA or JA) and are widely used as molecular indicators of the activation of these pathways (Naidoo et al. 2013). Transcript profiling provides evidence that increased expression of *PR1*, *PR2* and *PR5* represents the activation of SA whereas increased expression of transcripts of *PR3* and *PR4* (endo-chitinases) represents the activation of JA pathway (Narusaka et al. 2015). Over-expression of *PR* genes in different crop systems showed enhanced disease resistance against biotrophic and necrotrophic pathogens (Kusajima et al. 2010; Jiang et al. 2015). Recently, SA and JA signaling cascades have also emerged as potential tools for improving plant stress tolerance to abiotic stresses (Khan et al. 2012; Khan and Khan 2014). ABA is a well-known regulator of abiotic stresses including drought, salinity, and cold and has been extensively studied (Shinozaki and Yamaguchi-Shinozaki 2007). In addition to abiotic stress, ABA has also gained the importance in plant defense signaling as a positive or a negative regulator based on the plant–pathogen interactions (Cao et al. 2011). Recently, another group of plant growth hormones such as auxin, cytokines (CKs) and gibberellins (GAs) have also emerged as important modulators of plant defense response but their function remains elusive (Pieterse et al. 2012). Hormonal crosstalk modulates and also optimizes plant fitness to biotic and abiotic stresses when they occur simultaneously. More studies are needed to study the dynamic roles of these versatile small molecules during individual or multiple stresses in plants.

PR genes are not only induced after pathogen attack but their involvement has also been shown to combat different abiotic stresses. In *Arabidopsis*, *PR* genes like *AtPR1*, *AtPR2* and *AtPR5* are induced by both drought and salinity stress (Seo et al. 2008; Singh et al. 2013). Transcripts of SAR marker gene *PR1* in pepper plants increased significantly after its exposure to a variety of abiotic stresses (Hong and Hwang 2005). In addition, transcript levels of JA marker gene *PR12* (*PDF.1*) was also increased during cold stress (Archambault and Strömvik 2011). Gaudet et al. (2003) reported that freezing increases the transcript accumulation of antifungal (*PR13*) thionin genes in wheat. Historically, known PR proteins like β-1,3-glucanase and chitinases possess antifreeze activity and protect cell damage due to cold stress (Janska et al. 2010). Recently, it was shown that transcript levels of *PR4* increased significantly during salinity, wounding and cold stress (Kim et al. 2014). Furthermore, maize *PR10* gene was also upregulated after various abiotic stresses (Fountain et al. 2010). Activation of transcription factors such as cup-shaped cotyledon (*CUC*), drought-induced protein 19 (*Di19*) and dehydration-responsive element binding proteins (*DREB*) by abiotic stresses leads to PR gene induction (Tsutsui et al. 2009). Availability of transcriptomic data from individual biotic and abiotic stress experiments in both model and crop plants has been utilized to identify mutual stress-responsive genes (Narsai et al. 2013). In field conditions, plants may be exposed to different stresses that may likely occur simultaneously, a greater effort must be made to imitate these conditions in lab studies (Mittler and Blumwald 2010). Considering the mutagenic natures of stress tolerance, identification of different stress-specific genes are required that can be transferred into crop systems through transgenic approach to confer resistance to multiple stresses. Therefore, the major goal of this study was to reveal the molecular mechanisms underlying *BjPR* gene response to hormonal, biotic and abiotic stress. Notably, in this work we have identified multiple stress inducible genes in *B. juncea*.

Materials and methods

Plant growth conditions and treatments

Brassica juncea var. Varuna plants were grown in pots containing a mixture of soil and organic manure (2:1) at 24 °C under a 16-h/8-h light–dark cycle with irradiance of 100–125 µmol m^{-2} s^{-1}, and a relative humidity of 80%. For cDNA library construction, *B. juncea* plants were sprayed with 2 mM SA (pH 7.0) and 100 µM JA, respectively. Control plants were similarly treated with sterile distilled water (SDW). Leaf samples were collected from control and hormone-treated plants at different time points.

Construction of *B. juncea* cDNA library

Total RNA was isolated from the SA-, JA- and water (control)-treated *B. juncea* leaf samples using the protocol of PureLink RNA Mini Kit (Ambion, Carlsbad, CA, USA). *B. juncea* cDNA libraries were constructed from total RNA of SA- or JA-treated leaf samples using BD SMART cDNA library synthesis kit (Clontech Inc., USA). For cDNA amplification long-distance PCR (LD-PCR) was performed and the reaction mixture contains 11 µL first-strand cDNA, Advantage 2 PCR buffer, dNTP Mix, CDS 1II/3′ PCR primer (5′ATTCTAGAGGCCGAGGCGGCCGACATG3′, 5′ PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) Advantage 2 polymerase mix and deionised H_2O. The program of LD-PCR was: 72 °C for 10 min; 95 °C for 20 s (3 cycles) and 68 °C for 8 min. The LD-PCR product was treated with 2 µL of proteinase K (20 µg $µl^{-1}$) to inactivate DNA polymerase activity and was further purified. Purified cDNA was digested with *SfiI* enzyme to generate cohesive ends for directional cloning into λTriplEx2 vector (Clontech, Inc., USA). cDNA was ligated into the λTriplEx2 vector and the reaction mixture contains 0.5 µl 10 × ligation buffer, 0.5 µl 10 mM ATP, 0.5 µl cDNA, 1.0 µl vector (500 ng $µl^{-1}$), 0.5 µl T4 DNA ligase and 2 µl deionised H_2O incubated at 16 °C overnight. Furthermore, bacterial plate culturing, tittering the unamplified library as well as the percentage of recombinant clones were determined according to the protocol SMART cDNA synthesis kit. Prior to sequencing, conversion of a recombinant λTriplEx2 to the pTriplEx2 vector was carried out into the *E. coli* BM25.8.

Screening of cDNA libraries for *BjPR* genes

For screening, the unamplified libraries were used. SA-induced library was used for screening of *BjPR1*, *BjPR2* and *BjPR5* clones while as JA-induced library was used for screening of *BjPR3*, *BjPR12* and *BjPR13* clones using probes derived from *Arabidopsis* homolog *PR* genes. All the *BjPR* clones were further sequenced and analysed using different bioinformatic tools.

Phylogenetic and structural analysis of *BjPR* genes

Protein sequence similarity analysis of BjPR proteins (BjPR1, BjPR2, BjPR3, BjPR5, BjPR12 and BjPR13) were carried out using BLAST tool (http://www.ncbi.nlm.nih.gov/blast). To elucidate the evolutionary relationship of BjPR proteins, additional homologs of PR proteins were retrieved from *Brassica* database (BRAD) and NCBI databases, respectively. Phylogenetic trees of BjPR proteins were constructed using the neighbour-joining method with bootstrapping (1000 replicates) using MEGA.7 program. In silico protein structure of BjPR proteins was analysed using EXPASY software (http://www.expasy.org/). In addition, the isoelectric point and molecular weight of BjPR proteins were determined by Compute PI/MW tool of Expasy. In silico subcellular localizations of BjPR proteins were determined by Cell-PLoc 2.0 program (Chou and Shen 2010).

Hormonal treatment of *B. juncea* plants

For hormonal treatments, 10-day-old *B. juncea* plants were sprayed with 50 µM ABA, 100 µM JA and 2 mM SA individually and kept separately to prevent hormonal cross talk. Control plants were treated with sterile distilled water (SDW) containing equal amount of solvent used for hormone preparation. Leaf samples for RNA isolation were collected from both hormone-treated and control plants after 1, 4 and 6 h post-treatment.

Erysiphe cruciferarum infection in *B. juncea* plants

The pure culture of *E. cruciferarum* pathogen was isolated from *B. juncea*-infected leaves collected from the fields of Indian Agricultural Research Institute (IARI) New Delhi India. It was further confirmed as *E. cruciferarum* by Herbarium Cryptogamae Indiae Orientalis (H.C.I.O-ID: no. 52067) IARI, New Delhi, India. To investigate the distal and local expression of *BjPR* genes after *E. cruciferarum*, 1-month-old *B. juncea* plants were infected with *E. cruciferarum* as described by (Meur et al. 2006). For control, plants were sprayed with SDW. The inoculated plants were kept in a growth chamber at 100% RH and 22 °C. For RNA isolation, samples were harvested from both local (*E. cruciferarum* infected) and distal non-infected leaves of *B. juncea* plants.

Abiotic stress treatments in *B. juncea* plants

For abiotic stress treatments, *B. juncea* seeds were first treated with triton × 100 for 5 min followed by treatment with 0.1% $HgCl_2$ for 10 min. After $HgCL_2$ treatment, seeds of *B. juncea* were washed with SDW and grown on half-strength MS medium (Murashige and Skoog 1962) in Magenta boxes (Magenta vessel Corp, USA) at 24 ± 2 °C under cool white florescent light (90–150 µmol photons m^{-2} s^{-1}) in a 16-/8-h (light/dark) photoperiod. Ten-day-old *B. juncea* seedlings were aseptically transferred from MS solid agar medium to ½ liquid MS medium and stabilized for 4 h before stress. Drought stress was performed by transferring the *B. juncea* seedlings from MS medium to sterile Whatman filter paper (3MM) in petri dishes, while control plants were kept on sterile 3MM Whatman filter paper in petri dishes supplied with ½ liquid MS. For imposing salt stress, 10-day-old *B. juncea* seedlings were transferred to ½ MS liquid media supplemented with 150 mM NaCl and control plants were kept in normal ½ MS liquid media incubated at room temperature. All control and treated plants were maintained under white light conditions. Only leaf samples were collected from both control and treated *B. juncea* seedlings at 1, 4 and 6 h for RNA isolation. Three biological replicates were used for each treatment.

cDNA synthesis and RT-qPCR analysis

For cDNA synthesis, total RNA was extracted from 100 mg leaf samples of both control and treated *B. juncea* seedlings using PureLink RNA Mini Kit (Ambion Life Technologies, USA). Purity and concentration of RNA was measured by Nanodrop spectrophotometer (NanoDrop 2000 Thermo Scientific, Wilmington, DE). First-strand cDNA was generated from 2 µg of DNase-treated total RNA using Superscript III cDNA synthesis kit following the manufacturer's protocol (Invitrogen, Canada). Primers of *BjPR* genes and alpha-tubulin were designed using Oligoanalyzer software (Table 1). qRT-PCR mixture contained 2 µl of cDNA, 5 µl of SYBR green qRT-PCR master mix (Takara, Japan) and 0.5 µl (10 picomol) of each primer and was run at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The reactions were performed in triplicates and repeated using three biological replicates. Housekeeping gene, alpha-tubulin, was used in all the experiments as an internal control and the relative expression levels of each gene were analysed by delta C_T method (Livak and Schmittgen 2001). Fold changes with *p* values less than 0.05 were considered significant.

Table 1 List of primers used in this study

Gene	Primer
RT-PR1	F-5′ GAACACGTGCAATGGAGAATG 3′ R-5′ CCATTGTTACACCTCGCTTTG 3′
RT-PR2	F-5′ CGTCTCTCTACAATTCGCTCTG 3′ R-5′ CGATATTGGCGTCGAATAGGT 3′
RT-PR3	F-5′ AAGACCAGGTTCTTGCCTTC 3′ R-5′ TCCGGTACACTCCCTACTATTC 3′
RT-PR5	F-5′ GCAGAACAATTGCCCTTACAC 3′ R-5′ R-GCGCCTGGATTCAGTTGATA
RT-PR12	F-5′ CAATGGTGAAAGCGCAGAAG3′ R-5′ AGGTTGATGCACTGGTTCTT 3′
RTPR13	F-5′ GAGAAGCAATGGCAGGTTCTA 3′ R-5′ CGCACTCCGTGTTGTAGTT 3′
Alpha-tubulin	F-5′ GCCTCGTCTCTCAGGTTATTTC 3′ R-5′ TGAAGTGGATTCTTGGGTATGG 3′

In silico analysis of *BjPR* gene promoters

The upstream regions of *BjPR* genes were isolated from the *B. juncea* by genome walking using universal genome walker kit (Clontech, CA). 1.5 kb promoter region of *BjPR* genes was scanned by PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002), promoter databases for finding biotic, abiotic stresses and hormonal responsive *cis*-regulatory elements.

Statistical analysis

For all the experiments, three biological replicates were used. Student's *t* test was used to determine significant differences of expression data of *BjPR* genes between treated and control *B. juncea* plants which are shown as statistically significant ($*p < 0.05$) or extremely significant ($**p < 0.01$).

Results

Isolation, phylogenetic and structural analysis of *BjPR* genes from *B. juncea*

PR gene families constitute a large and important group of defense proteins in plants. In this study, two cDNA libraries (SA and JA cDNA library) were constructed from *B. juncea* plants after SA and JA treatment. The cDNA sequences of *BjPR1*, *BjPR2* and *BjPR5* were isolated from SA cDNA library while as *BjPR3*, *PR12* and *PR13* were isolated from JA cDNA library using probes derived from *A. thaliana* homologous *PR* gene families. The sizes of cDNA sequences of *BjPR* genes range between 137 and 1041 nucleotides. A homology search against the NCBI database was carried out to confirm whether the obtained sequences from *B. juncea*

encoded *PR* gene family. BLASTP analysis revealed that the deduced amino acids of *BjPR* genes were closely related to PR proteins of *Brassicaceae* family, respectively. The phylogenetic relationship of BjPR1 protein with its homologs from other plants revealed that they were closely related to PR1 proteins of *B. rapa*, *B. napus*, *B. nigra* and *Schrenkiella parvula* (Fig. 1a). Similarly, BjPR2, BjPR3, BjPR5 and BjPR12 clustered within the clade containing their respective homologs from *B. napus*, *B. oleracea* and *B. rapa* (Fig. 1b, e). Phylogenetic analysis also showed that BjPR13 protein was closely related to PR13 proteins of *B. napus* and *B. oleracea* and *Arabidopsis* (Fig. 1f). Phylogenetic analysis of BjPR proteins with other PR homologs of different plant species suggests that they may share a common ancestor. The accession numbers, molecular weight, isoelectric point, predicted coding sequences, protein size and in silico subcellular localisation of BjPR1, BjPR2, BjPR3, BjPR5, BjPR12 and BjPR13 proteins are mentioned in Table 2.

Expression profiling of *BjPR* genes after hormonal treatments reveals unique interactions

Hormonal regulations of *PR* genes are well documented in model plants but not in crop plants. This study investigates the effect of various phytohormones (ABA, SA and JA) on the expression of *BjPR* genes. Here, after ABA treatment, distinct expression pattern of *BjPR* genes was observed. The transcript levels of *PR1*, *PR2* and *PR5* significantly decreased after ABA treatment as compared to control. In contrast to SA, JA-responsive genes *PR3* (17.33-fold) and *PR13* (5.12-fold) were significantly increased at 6 h after ABA treatment while *PR12* was marginally induced (Fig. 2a). As expected, the higher accumulation of *PR3* and *PR13* further supports previous findings of synergistic relation between JA and ABA. After SA treatment the expression of *PR1*, *PR2*, and *PR5* increases at 1 h and reaches a maximum at 6 h of post-treatment. In contrast, to JA signature genes *PR3*, *PR12* and *PR13* were not induced by SA treatment (Fig. 2b). Following the JA treatment, *PR3*, *PR12* and *PR13* exhibited increased transcript accumulation at 1 h and reached maximum at 6 h. SA signatures (*PR1*, *PR2* and *PR5*) were not induced after JA treatment in *B. juncea* (Fig. 2c).

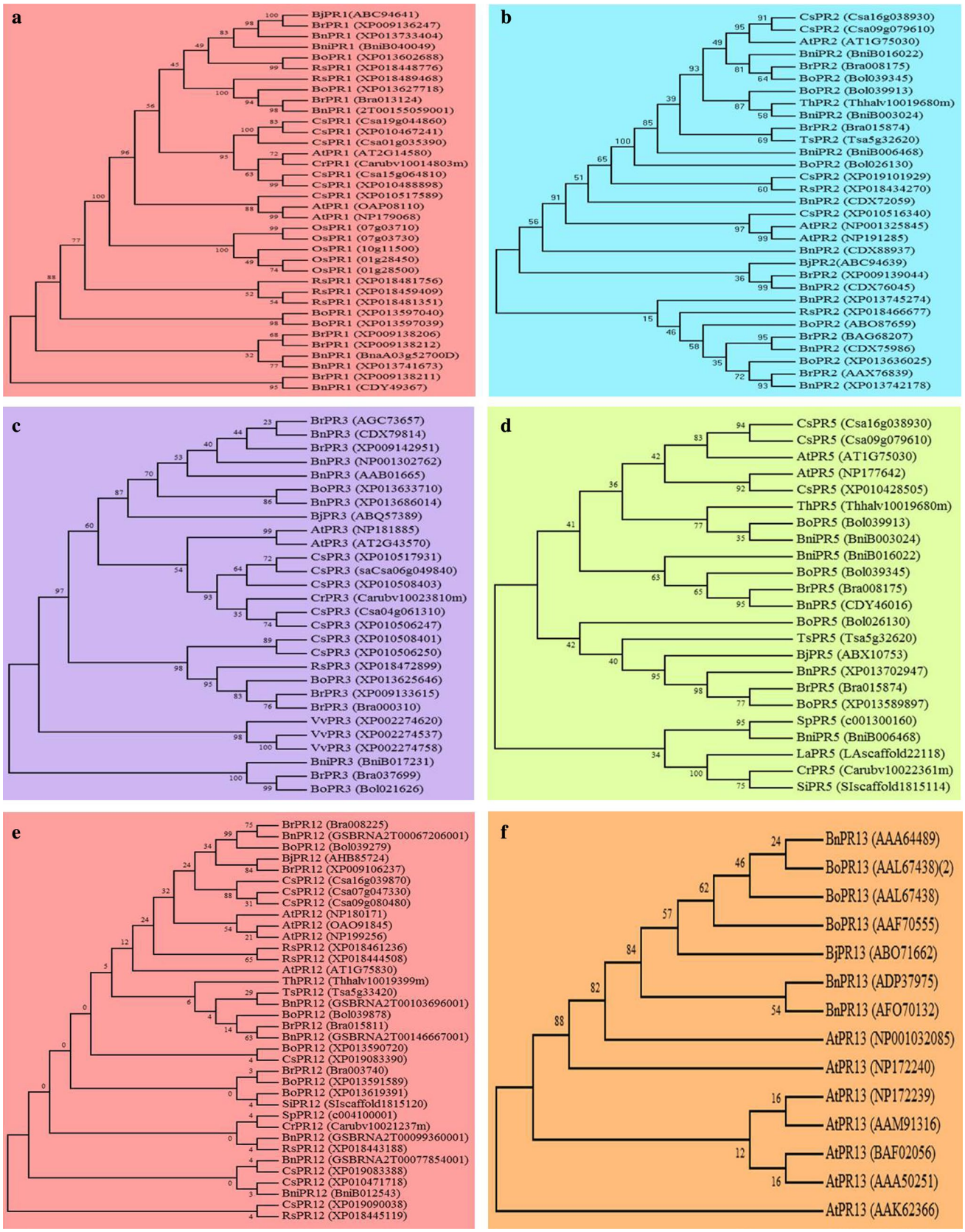
Disease progression and defense expression (local and systemic) of *BjPR* genes

Erysiphe cruciferarum is one of the important pathogens of rapeseed mustard crops. However, the molecular mechanism of powdery mildew and Brassica pathosystem as well as disease resistance is not fully understood. In this study, we isolate and identified this obligate ascomycete from naturally infected *B. juncea* plants. Typical powdery mildew symptoms like white, star-shaped colonies of mycelia were visually observed with naked eye on the naturally infected leaves of *B. juncea* (Fig. 3a). Further, microscopic observations showed ovoid to cylindrical hyaline conidia (70–115 × 8–10 µm) of *E. cruciferarum* (Fig. 3b). The appearance of spores of our isolate (H.C.I.O-ID: no. 52067) was similar to previous records of *E. cruciferarum* HUST-WUH1 (GenBank no. KP730001) isolated from infected rapeseed plants by Alkooranee et al. (2015). We further investigated the disease progression in healthy *B. juncea* leaves after *E. cruciferarum* inoculation. Our results revealed that after *E. cruciferarum* inoculation, spores germinated on 30-day-old *B. juncea* leaf surface and produced the same disease symptoms thus satisfying Koch's postulates (Fig. 3c). However, no symptoms were seen on uninfected leaves of *B. juncea* (Fig. 3d). These results further reveal that *B. juncea* is highly susceptible to powdery mildew disease.

An overview of typical SAR in distal leaves after infection is shown in (Fig. 3e). To investigate the transcriptional changes of *BjPR* genes in local (inoculated) and distal (non-inoculated) leaves, *B. juncea* plants were infected with *E. cruciferarum*. Based on the results transcript levels of SA signature genes (*BjPR1*, *BjPR2*, and *BjPR5*) increased significantly both locally as well as systematically after fungal infection. However, among SA-dependent genes the transcript levels of *BjPR1* (55.48-fold in local and 30.33-fold in distal tissues) gene was comparatively higher than *BjPR2* (25.29-fold in local and 20.87-fold in distal tissues) and *BjPR5* (7.67-fold in local and 6.45-fold in distal tissues) after infection. In contrast to JA signatures, *BjPR3* (15.67-fold) gene was significantly induced in local-infected leaves while very low transcript levels of *BjPR12* and *BjPR13* were detected in local or distal tissues after infection (Fig. 3f). In *B. juncea*, SAR was accompanied by a systemic accumulation of transcripts of most of the *PR* genes which further reveals that role of PR genes in SAR is associated with activation of these genes.

Transcriptional profiling of *BjPR* genes during abiotic stresses

Plants are very often exposed to drought stress which stimulates the accumulation of various stress signaling molecules especially ABA which in turn regulates a number of genes. This study reveals the effect of drought stress on biotic-responsive genes in *B. juncea*. Compared to control, transcript levels of SA marker genes *PR1* (3.16-fold), *PR2* (10.85-fold) and *PR5* (4.63-fold) showed upregulation at 1 h but downregulates at 4 and 6 h after drought stress. On the other hand, *PR3* (17.93-fold) and *PR13* (9.06-fold) showed a significant increase at 4 h post-treatment. However, the JA marker gene *PR12* (2.83-fold) was marginally induced when compared to control by drought stress in *B. juncea* (Fig. 4a).


a
BjPR1(ABC94641)
BrPR1 (XP009136247)
BnPR1 (XP013733404)
BniPR1 (BniB040049)
BoPR1 (XP013602688)
RsPR1 (XP018448776)
RsPR1 (XP018489468)
BoPR1 (XP013627718)
BrPR1 (Bra013124)
BnPR1 (2T00155059001)
CsPR1 (Csa19g044860)
CsPR1 (XP010467241)
CsPR1 (Csa01g035390)
AtPR1 (AT2G14580)
CrPR1 (Carubv10014803m)
CsPR1 (Csa15g064810)
CsPR1 (XP010488898)
CsPR1 (XP010517589)
AtPR1 (OAP08110)
AtPR1 (NP179068)
OsPR1 (07g03710)
OsPR1 (07g03730)
OsPR1 (10g11500)
OsPR1 (01g28450)
OsPR1 (01g28500)
RsPR1 (XP018481756)
RsPR1 (XP018459409)
RsPR1 (XP018481351)
BoPR1 (XP013597040)
BoPR1 (XP013597039)
BrPR1 (XP009138206)
BrPR1 (XP009138212)
BnPR1 (BnaA03g52700D)
BnPR1 (XP013741673)
BrPR1 (XP009138211)
BnPR1 (CDY49367)
b
CsPR2 (Csa16g038930)
CsPR2 (Csa09g079610)
AtPR2 (AT1G75030)
BniPR2 (BniB016022)
BrPR2 (Bra008175)
BoPR2 (Bol039345)
BoPR2 (Bol039913)
ThPR2 (Thhalv10019680m)
BniPR2 (BniB003024)
BrPR2 (Bra015874)
TsPR2 (Tsa5g32620)
BniPR2 (BniB006468)
BoPR2 (Bol026130)
CsPR2 (XP019101929)
RsPR2 (XP018434270)
BnPR2 (CDX72059)
CsPR2 (XP010516340)
AtPR2 (NP001325845)
AtPR2 (NP191285)
BnPR2 (CDX88937)
BjPR2(ABC94639)
BrPR2 (XP009139044)
BnPR2 (CDX76045)
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RsPR2 (XP018466677)
BoPR2 (ABO87659)
BrPR2 (BAG68207)
BnPR2 (CDX75986)
BoPR2 (XP013636025)
BrPR2 (AAX76839)
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c
BrPR3 (AGC73657)
BnPR3 (CDX79814)
BrPR3 (XP009142951)
BnPR3 (NP001302762)
BnPR3 (AAB01665)
BoPR3 (XP013633710)
BnPR3 (XP013686014)
BjPR3 (ABQ57389)
AtPR3 (NP181885)
AtPR3 (AT2G43570)
CsPR3 (XP010517931)
CsPR3 (saCsa06g049840)
CsPR3 (XP010508403)
CrPR3 (Carubv10023810m)
CsPR3 (Csa04g061310)
CsPR3 (XP010506247)
CsPR3 (XP010508401)
CsPR3 (XP010506250)
RsPR3 (XP018472899)
BoPR3 (XP013625646)
BrPR3 (XP009133615)
BrPR3 (Bra000310)
VvPR3 (XP002274620)
VvPR3 (XP002274537)
VvPR3 (XP002274758)
BniPR3 (BniB017231)
BrPR3 (Bra037699)
BoPR3 (Bol021626)
d
CsPR5 (Csa16g038930)
CsPR5 (Csa09g079610)
AtPR5 (AT1G75030)
AtPR5 (NP177642)
CsPR5 (XP010428505)
ThPR5 (Thhalv10019680m)
BoPR5 (Bol039913)
BniPR5 (BniB003024)
BniPR5 (BniB016022)
BoPR5 (Bol039345)
BrPR5 (Bra008175)
BnPR5 (CDY46016)
BoPR5 (Bol026130)
TsPR5 (Tsa5g32620)
BjPR5 (ABX10753)
BnPR5 (XP013702947)
BrPR5 (Bra015874)
BoPR5 (XP013589897)
SpPR5 (c001300160)
BniPR5 (BniB006468)
LaPR5 (LAscaffold22118)
CrPR5 (Carubv10022361m)
SiPR5 (SIscaffold1815114)
e
BrPR12 (Bra008225)
BnPR12 (GSBRNA2T00067206001)
BoPR12 (Bol039279)
BjPR12 (AHB85724)
BrPR12 (XP009106237)
CsPR12 (Csa16g039870)
CsPR12 (Csa07g047330)
CsPR12 (Csa09g080480)
AtPR12 (NP180171)
AtPR12 (OAO91845)
AtPR12 (NP199256)
RsPR12 (XP018461236)
RsPR12 (XP018444508)
AtPR12 (AT1G75830)
ThPR12 (Thhalv10019399m)
TsPR12 (Tsa5g33420)
BnPR12 (GSBRNA2T00103696001)
BoPR12 (Bol039878)
BrPR12 (Bra015811)
BnPR12 (GSBRNA2T00146667001)
BoPR12 (XP013590720)
CsPR12 (XP019083390)
BrPR12 (Bra003740)
BoPR12 (XP013591589)
BoPR12 (XP013619391)
SiPR12 (SIscaffold1815120)
SpPR12 (c004100001)
CrPR12 (Carubv10021237m)
BnPR12 (GSBRNA2T00099360001)
RsPR12 (XP018443188)
BnPR12 (GSBRNA2T00077854001)
CsPR12 (XP019083388)
CsPR12 (XP010471718)
BniPR12 (BniB012543)
CsPR12 (XP019090038)
RsPR12 (XP018445119)
f
BnPR13 (AAA64489)
BoPR13 (AAL67438)(2)
BoPR13 (AAL67438)
BoPR13 (AAF70555)
BjPR13 (ABO71662)
BnPR13 (ADP37975)
BnPR13 (AFO70132)
AtPR13 (NP001032085)
AtPR13 (NP172240)
AtPR13 (NP172239)
AtPR13 (AAM91316)
AtPR13 (BAF02056)
AtPR13 (AAA50251)
AtPR13 (AAK62366)

◂**Fig. 1** Phylogenetic relationship of BjPR proteins of *B. juncea* with other PR proteins from plant species. *A. thaliana* (At), *B. napus* (Bn), *B. nigra* (Bni), *B. oleracea* (Bo), *B. rapa* (Br), *Camelina sativa* (Cs), *Capsella rubella* (Cr), *Leavenworthia alabamica* (La), *Oryza sativa* (Os), *Raphanus sativus* (Rs), *Sisymbrium irio* (Si), *Schrenkiella parvula* (Sp), *Thellungiella halophila* (Th), *Thellungiella salsuginea* (Ts), *Vitis Vinifera* (Vv). **a** Phylogenetic analysis of BjPR1, **b** BjPR2, **c** BjPR3, **d** BjPR5, **e** BjPR12 and **f** BjPR13 protein sequences with its homologs from other plant species obtained from *Brassica* and NCBI databases. The phylogenetic trees of BjPR proteins were constructed by neighbour-joining method using MEGA7.0 software with 1000 bootstrap

A few reports have explored the effect of salt stress on *PR* genes (Seo et al. 2008). To examine whether salinity regulates the expression of *PR* genes in *B. juncea*, we treated plants with 150 mM NaCl and expression analysis were carried out at different time points. In this study, significant upregulation of *PR1* (4.92-fold), *PR2* (13.36-fold) and *PR5* (6.58-fold) was observed at 4 h after salt treatment in *B. juncea*. On the other hand, *PR3* (26.49), *PR12* (2.6-fold) and *PR13* (9.25-fold) showed maximum expression at 6 h (Fig. 4b). These observations suggest that salt stress modulates key immune genes in *B. juncea* largely through SA-/JA-dependent signaling pathways.

We have summarized the detailed expression analysis data of *BjPR* genes in *B. juncea* after hormonal, biotic and abiotic stress which clearly show the kinetics of gene expression (upregulation or downregulation) during these stresses (Table 3).

In silico analysis of SA- and JA-dependent *BjPR* gene promoters reveals *cis*-elements responsive to multiple stresses

To further investigate the regulation aspect of *BjPR* genes in response to hormonal, biotic and abiotic stress, we analysed the upstream sequences of BjPR genes to identify the *cis*-elements involved in multiple stresses. In silico analysis of both SA and JA marker *BjPR* genes showed many biotic stress-related *cis*-regulatory elements such as TC-rich repeats (ATTTTC), SARE (SA-responsive element) or (JA-responsive element) (TGACG) motifs, W BOX [(T) TGAC (C/T)] and GT1GMSCAM4 motif (GAAAAA). Many potential abiotic-responsive elements such as ABREs motif (ACGT) for ABA-dependent expression, DREs motif (TAC CGACAT) for ABA-independent expression during salt and drought stress, (LTRE) motif TGG/ACCGAC for low-temperature response, MYB motif (TAACTG) for drought stress, MYC motif CATGTG, CACATG) for early response to drought and ABA induction, Wbox (TTGAC, TGACT) for the activation of defense and wounding-related genes, GT1 motif (GAAAAA, GGTTAA) for pathogen and salt response and HSE (CNNGAANNTTCNNG) involved in heat stress were also found in single or multiple copies in *BjPR* gene promoters (Fig. 5). The presence of these cis-regulatory elements in the upstream regions of *B. juncea PR* genes reveals that they might be regulated by multiple stresses.

Discussion

Plants are very often exposed to multiple stresses resulting in substantial agricultural losses. Global climate change will possibly increase the emergence of virulent strains of phytopathogens with broad host range. Therefore, understanding the mechanisms underlying plant resistance or tolerance to above stresses will help us to genetically engineer crops for multiple stress tolerance. Plant responses to multiple stresses are generally complicated process and involve a network of genes and signaling cascades. However, the regulation and molecular function of most of these genes or signaling cascades to these stresses are largely unknown. Therefore, we carried out transcriptional analysis of one of the important stress-related gene families (*PRs*) in *B. juncea* after multiple stresses. We identified the *BjPR* genes in *B. juncea* to understand *Brassica* immune response and their signaling pathways. Based on BLAST algorithms, domain prediction and phylogenetic analysis, we found that *BjPR* genes share similar protein sequence identities as well as conserved domains of known PR proteins from other crucifers.

Phytohormones are essential not only for plant growth and development but also play a vital role in the stress tolerance (Wani et al. 2016). Plants respond to stress through a multifaceted group of signaling cascades which are mainly regulated by small molecules called hormones such as ABA, ET, JA and SA that interact synergistically and/or antagonistically to each other. *PR* genes are generally considered as the molecular indicators of SA and JA defense signaling pathways in model plants. Recently, their role in various abiotic stresses has gained importance to study hormonal cross talk (Khan and Khan 2013). In *Arabidopsis*, SA and JA exert antagonistic interactions with each other (Van-der-Does et al. 2013); however, there are reports that these pathways also act synergistically (Lazniewska et al. 2010). To further investigate the hypothesis of synergistic or antagonistic relationship in *B. juncea*, expression profiling of SA and JA signature genes were studied after hormonal treatment. This work also aimed to find the signatures of SA and JA signaling in *B. juncea*. Upon SA treatment, transcripts of *PR1*, *PR2*, *PR5* increases dramatically while as *PR3*, *PR12* and *PR13* decreases which were similar to the findings observed in *Arabidopsis* (Seo et al. 2008). In contrast, JA upregulates *PR3*, *PR12* and *PR13* but downregulates SA marker genes *PR1*, *PR2* and *PR5*; thus, our results are consistent with previous reports observed in *Arabidopsis* (Thomma et al. 1998). Our findings suggest

Table 2 BjPR proteins along with their accession numbers, molecular weight (M.wt.), isoelectric point (PI), CDS and protein length, and subcellular localization

Protein	Accession numbers	M. wt. (kDa)	PI	CDS length (bp)	Protein length (a.a)	Subcellular localization
BjPR1 (unknown)	ABC94641	17.53	7.07	661	161	Vacuole
Bj-PR2 (β,1-3 glucanase)	ABC94639	38.08	9.13	1041	346	Vacuole
BjPR3 (chitinase)	ABQ57389	17.03	4.82	468	155	Vacuole
BjPR5 (thaumatin)	ABX10753	11.65	5.98	341	114	Cell wall Cytoplasm
BjPR12 (defensin)	AHB85724	8.93	8.47	243	80	Vacuole
BjPR13 (thionin)	ABO71662	4.44	4.10	137	45	Unknown

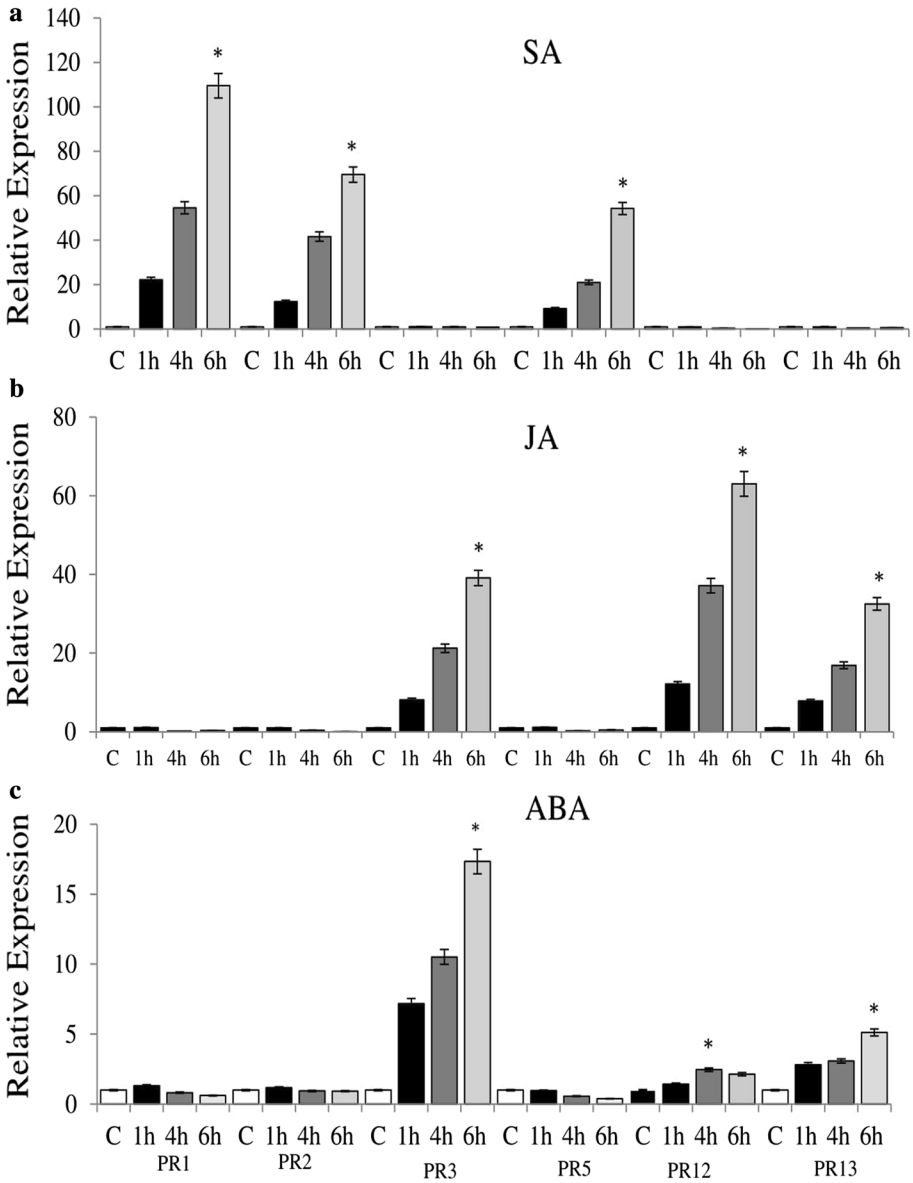

Fig. 2 Expression analysis of *BjPR* genes after hormonal treatments. 10-day-old *B. juncea* plants were treated with **a** SA (1 mM), **b** JA (100 µM) and **c** ABA (50 µM). Leaf samples were harvested at different time points for RNA isolation. Control plants for each treatment were treated with sterile distilled water containing equal amount of solvent used for hormone preparation. SE for each bar is shown. Treatment bars marked by an asterisk are significantly greater than untreated (controls) ($p < 0.05$)

that the known SA/JA antagonistic relationship in the model plant *Arabidopsis* was also observed in *B. juncea* during early hours of hormonal treatments. Furthermore, *BjPR* genes studied in this work can be used as suitable markers or molecular indicators for SA and MeJA signaling pathways in *B. juncea*.

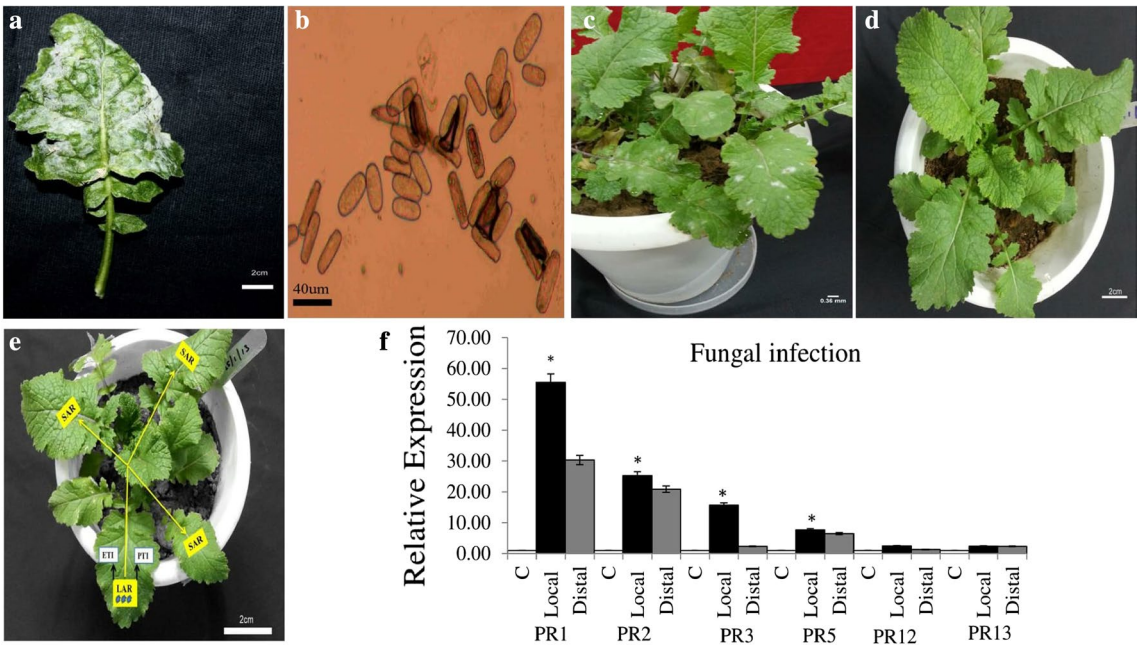

Fig. 3 *In planta* infection of *B. juncea* with *E. cruciferarum* and expression profiling of *BjPR* genes in local (inoculated) and distal (non inoculated) leaves. **a** *B. juncea*-infected leaf with *E. cruciferarum* in IARI fields, bar = 2 cm. Pure culture of *E. cruciferarum* (H.C.I.O-ID: no. 52067) was isolated from above-infected leaf of *B. juncea* and used as inoculum. **b** Microscopic identification of *E. cruciferarum* fungus (Conidia under 40X microscope), bar = 40 µm. **c** *E. cruciferarum*-inoculated *B. juncea* plants after seven days of post-inoculation, bar = 0.36 mm. **d** Uninfected or control *B. juncea* plants, bar = 2 cm. **e** Schematic diagram of SAR in plants. After infection, plants show PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), in the infected leaves, bar = 2 cm. **f** Transcriptional profiling of the *BjPR1*, *BjPR2*, *BjPR3*, *BjPR5*, *BjPR12* and *BjPR13* genes were investigated in the uninfected (control = C) and *E. cruciferarum*-infected leaves. The expression levels of *BjPR* genes in control seedlings were normalized to a value of 1. SE for each bar is shown. The α-tubulin gene was used as an internal control. A significant difference ($p < 0.05$) between control and treated samples is denoted by an asterisk above the bar

Generally, ABA not only plays a central role in abiotic stresses, but also plays a critical role in plant–pathogen interactions (De-Torres-Zabala et al. 2007; Fan et al. 2009; Lim et al. 2015). To further understand the role of ABA in *B. juncea* defense response, we studied the expression of key immune genes (*BjPR* genes) after ABA treatment. Our results revealed that ABA downregulates the expression of SA marker genes *PR1*, *PR2* and *PR5* in *B. juncea* at all time points which suggest that ABA and SA interact antagonistically in *B. juncea*. There are reports which have shown that ABA paralyzes the plant defense responses by suppressing the SA pathway, thereby acting as negative regulator of SA-mediated immunity (De Vleesschauwer et al. 2013). In addition, many reports have shown ABA suppresses the SA pathway which fails to establish SAR both in *Arabidopsis* as well as in tobacco plants (Yasuda et al. 2008; Kusajima et al. 2010). Our results are in agreement with the above findings that ABA inhibits the expression of SAR marker genes *PR1*, *PR2* and *PR5* in *B. juncea*, therefore, might increase susceptibility to biotrophic pathogens. A significant finding of our study was that SA marker genes were downregulated by ABA but showed upregulation during various abiotic stresses. These results further provide the affirmation of the participation of other signaling pathways like SA/JA or the occurrence of interrelated pathways which could trigger the expression of these genes in ABA-independent manner. Interestingly, we observed the synergistic interaction between ABA and JA in *B. juncea* as JA marker genes *PR3* and *PR13* were significantly induced. Similar expression pattern of *PR3* and *PR13* genes was reported in *Arabidopsis* and rice after ABA treatment (Yazaki et al. 2003; Seo et al. 2008). Furthermore, JA signature genes were also induced by various abiotic stresses; therefore, there induction would be either JA or ABA dependent in *B. juncea*. In *Arabidopsis*, exogenous application of ABA increases disease resistance to necrotrophic pathogens *Alternaria brassicicola* and *Pythium irregulare* (Adie et al. 2007). Therefore, our results suggest that induction of JA marker genes by ABA might have a role in combating biotic and abiotic stresses in *B. juncea*.

Brassica juncea crop production is adversely affected by biotic stresses. Among them fungal diseases are rated as the most important factor for significant yield losses with no proven source of disease resistance. One of the important biotrophic fungal pathogens of *B. juncea* and other crucifers is *E. cruciferarum* which causes severe damage and

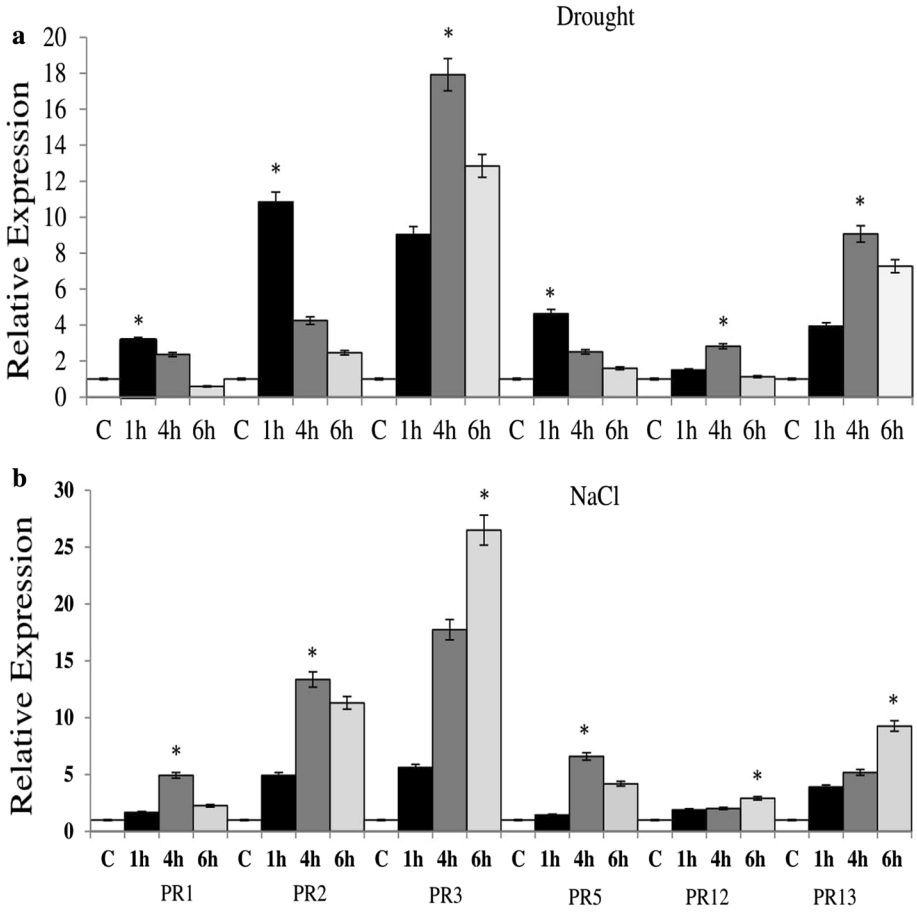

Fig. 4 Relative expression analysis of *BjPR* genes in *B. juncea* after abiotic stresses at different time points. Expression profiling of *BjPR* genes in drought (**a**), salt (**b**) and control (C = untreated) leaves of *B. juncea* at various time points. The expression levels of *BjPR* genes in control seedlings were normalized to a value of 1. Expression of *BjPR1*, *BjPR2*, *BjPR3*, *BjPR5*, *BjPR12* and *BjPR13* at 1, 4 and 6 h after different stresses are represented in different colours and control (C) is represented with black colour bar. The relative expression levels of *BjPR* genes are compared with that of a control alpha-*tubulin* gene. The data are the mean ± SE of three biological replicates. SE for each bar is shown. A significant difference ($p < 0.05$) between control and treated samples is denoted by an asterisk above the bar

Table 3 Differential gene expression profiling of *BjPR* genes in response to hormonal, biotic and abiotic stress treatments

Gene name	SA	JA	ABA	*E. cruciferarum*		Drought	Salt
				Local	Distal		
BjPR1	+++	−−	−	+++	++	+	+
BjPR2	++	−−	−	++	++	+	+
BjPR3	−−	+++	++	++	−	++	++
BjPR5	++	−−	−	+	+	+	+
BjPR12	−−	+++	+	+−	+−	+	−
BjPR13	−−	++	+	+−	+−	+	+

'+' to '+++' strong upregulation, '+−' low expression '−' weak to '−−' strong down-regulation

yield losses. The defense response of the *B. juncea* to this pathogen has not been fully deciphered at molecular level. Plants generally involve LAR and SAR immune responses to counterpart pathogen attack (Fu and Dong 2013). Both these resistance pathways are associated with activation of array of genes like *PR* genes. Till date, limited studies have been carried out on the defense mechanisms and signaling pathways involved in LAR or SAR in *B. juncea* after pathogen attack. Therefore, we studied the transcriptional regulation of *BjPR* genes in local and distal tissues of *B. juncea* in response to *E. cruciferarum* pathogen. Among *PR* genes, *PR1* has been universally known as molecular indicator of induced plant immune system and exhibits antifungal activity (Zhu et al. 2012). Similarly, during our study on the interaction of *B. juncea* and *E. cruciferarum* pathogen, *PR1* was strongly upregulated to a greater level both locally and systematically and can be used as SAR marker in *B. juncea*. Interestingly, higher accumulation of *PR2* gene was also seen in *B. juncea* leaves after *E. cruciferarum* infection. Generally, transcript levels of *PR2* genes are comparatively low in healthy or non-infected plants but increases dramatically after biotrophic or necrotophic fungal pathogen attack, thus implying its role in disease resistance (Cheong et al. 2000; Shi et al. 2006; Zhu et al. 2013). Another important member of the SA-dependent

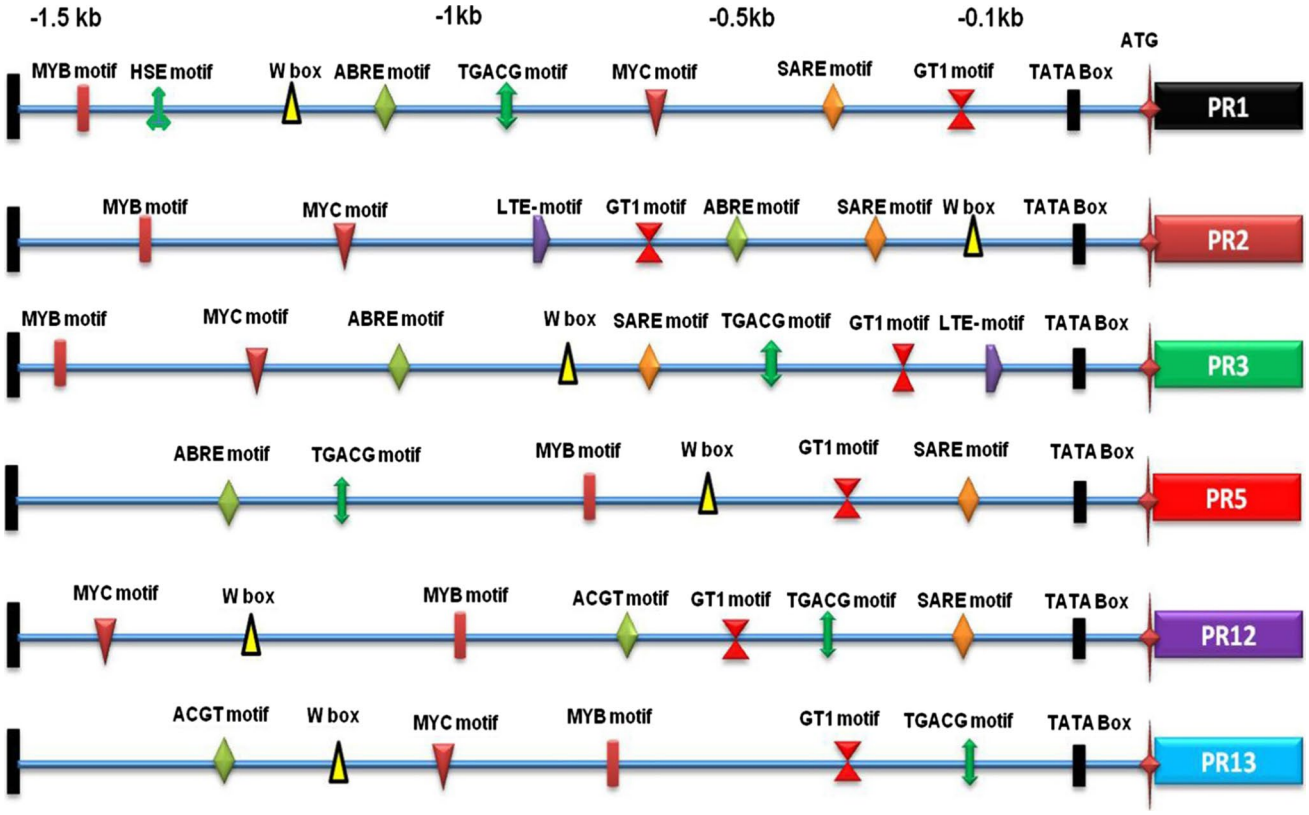

Fig. 5 In silico analysis of PR gene promoters of *B. juncea.* Promoter *cis*-elements of SA (*BjPR*, *BjPR2*, *BjPR5*) and JA signature (*BjPR3*, *BjPR12* and *BjPR13*) genes in response to biotic, abiotic and hormonal stresses are shown in different shapes and colours along with their respective positions from the start codon ATG

PR gene family is PR5 or thaumatin-like genes which have been reported to be induced by diverse pathogens. Our results also showed that *PR5* gene of *B. juncea* was induced by fungal pathogen both in local and distal leaves. Among SA marker genes, transcript level of *PR1* gene was relatively higher than *PR2* and *PR5* after infection. On the onset of SAR, SA is transported from the infected leaf to the distal leaves, which leads to the activation of SAR downstream genes such as *PR1*, *PR2* and *PR5* in the pathogen-free tissues (Dempse and Klessig 2012). Therefore, these genes might play crucial part in LAR and SAR in *B. juncea*. Interestingly, among JA signature genes, *PR3* (chitinase) was significantly induced by biotrophic pathogen (*E. cruciferarum*) but the expression was restricted to local-infected tissues. Previous study has also shown that powdery mildew increases transcript levels of *PR3* gene in grapevine (Jacobs et al. 1999). Plant defensins (*PR12*) and thionins (*PR13*) are known be strongly induced by fungal pathogens (Kong et al. 2005). In this study, we found less induction of *BjPR12* and *BjPR13* after *E. cruciferarum* infection in *B. juncea.* It is generally well established, with some exceptions, that SA pathway provides resistance to biotrophic pathogens while as JA/ET pathways show resistance to necrotrophs and herbivorous pests, respectively (Glazebrook 2005). Earlier reports in *A. thaliana* suggested that many of the powdery mildew-regulated genes are unlikely to be directed by SA signaling, but are also regulated by other signals like hydrogen peroxide, ET, JA or by fungal elicitors (Chandran et al. 2009). Therefore, our results suggests that *E. cruciferarum* mediated expression of *BjPR3*, *BjPR12* and *BjP13* genes is SA independent, as they were downregulated by SA. Altogether, these results revealed that increased pathogen inducible expression of *BjPR* genes might directly contribute to disease resistance because most of the *PR* genes isolated from different plants have shown antifungal activity in vitro and enhanced resistance to pathogenic fungi when constitutively overexpressed in planta.

Generally, *PR* genes are universally known as marker genes of plant defense responses. However, few reports have shown the activation of *PR* genes by various abiotic stresses and have gained importance. Abiotic stress factors (salinity, heat and drought) possess huge threat to modern agriculture and decreases yields in most of the major crops. *B. juncea* is greatly affected by drought stress causing significant yield losses (Chauhan et al. 2007; Khan et al. 2017; Raza et al. 2017). Plants exposed to drought stress have shown

to modulate plant defense response which further leads to susceptibility or disease resistance. To further investigate how drought stress regulates defense responses in *B. juncea*, we examined the expression profiling of SA and JA signature genes after drought stress. Our findings showed that SA-responsive genes increased at 1 h and decline at later time points whereas transcript levels of JA-responsive genes were relatively higher after 1 h of drought stress. But if we look at the timing of induction of *PR* genes by SA and JA, it is different from drought stress but ABA induced genes to follow the same pattern. It seems that ABA is involved in regulating the expression of *PR* genes during drought stress. In addition, decreased expression of SA marker genes like *PR1*, *PR2* and *PR5* might be due to high accumulation of ABA which may suppress SA-responsive genes in *B. juncea* during drought stress. Similar reports were also observed in *Arabidopsis* where high accumulation of ABA due to drought stress or exogenous treatment antagonizes the SA signaling pathway (De-Torres-Zabala et al. 2007). Our findings suggest the occurrence of so-called crosstalk between biotic and abiotic stresses in *B. juncea*. Interestingly, drought-induced expression of JA signature genes may provide disease resistance to pathogens if both stresses occur simultaneously in *B. juncea*, as there are reports which have shown that drought stress reduces fungal biomass in tomato plants during gray mold disease caused by *B. cinerea* and also prevents powdery mildew infection (*Oidium neolycopersici*) (Achuo et al. 2006). On the other hand, it has been shown that pathogens also improve plant tolerance to drought stress which might be due to the activation of *PR* genes.

Nowadays, interest for studying salinity stress is rising rapidly because it is not only inhibiting plant growth but also interferes with other plant responses to environmental stimuli such as disease response. Recent, studies have also reported that salinity stress severely impairs *B. juncea* productivity and, therefore, molecular biology intervention is essential for the betterment of sustainable mustard cultivation particularly in northwestern agroclimatic region of India (Yousuf et al. 2016). It has been demonstrated that SA and JA defense hormones play important role in combating salt tolerance in many plants (Khan and Khan 2014). However, the molecular mechanism underlying how SA and JA combating salt tolerance in plants is poorly understood. Therefore, we evaluated the expression analysis of *BjPR* genes which are known as molecular indicators of SA and JA signaling pathways in *B. juncea* after salt stress. We observed the salt-mediated expression of both SA- and JA-dependent *PR* genes in *B. juncea.* However, the expression of *BjPR3* and *BjPR2* was relatively higher than other *PR* genes. These results provide the evidence that SA- and JA-mediated salt tolerance in plants could be because of the coordinated expression of *PR* genes or other SA/JA pathway genes. Moreover, earlier studies have also shown the positive impact of salt stress on disease resistance to *B. cinerea* and *Oidium neolycopersici* in tomato (Achuo et al. 2006). Here, we found that the universally known antifungal genes such as *PR2* (glucanase), *PR3* (chitinase) and *PR13* (thionin) were induced by salt stress that may lead to disease resistance against fungal pathogens. Together, these results indicate that *PR* genes are not only induced by pathogens but also by salinity stress in *B. juncea.*

To further investigate the stress-related expression of *PR* genes in *B. juncea*, 1.5 kb promoter regions were scanned for *cis*-elements involved in multiple stresses. Based on in silico analysis, *BjPR* promoters showed many stress-related *cis*-acting regulatory elements and were present in single or multiple copies. Among biotic stress-related *cis*-regulatory elements are TC-rich repeats (ATTTTC), SARE (TCAGAA GAGG, TCATCTTCTT), JA (TGACG) motifs, W BOX [(T) TGAC (C/T)], GT1GMSCAM4 motif (GAAAAA) found in *BjPR* gene promoters which further confirms the fact that *BjPR* genes might play an important role in biotic stress. Activation of abiotic stress-related genes usually occurs either by ABA-dependent pathway which is conferred by the presence of single or multiple copies of ABREs motifs, or independently which possess DRE motifs (TACCGACAT) to which different groups of DREBPs bind (Roychoudhury et al. 2013). MYB and MYC motifs have also been known to regulate genes during abiotic stresses. In addition, LTRE a low-temperature-responsive element motif is commonly found in cold-responsive genes (Brown et al. 2001). A well-known pathogen-related motif W box mediates abiotic stress responses in plants to wounding, oxidative stress, drought, heat, cold and salinity by binding various WRKY transcription factors (TFs). The presence of SA- and JA-responsive motifs in stress-related genes is known to increase stress tolerance to wide range of stresses. These motifs were also found in *B. juncea PR* genes in single or multiple copies that further confirm the fact that these genes might play a role in abiotic stress.

Conclusion

This is the first report of a comparative analysis of *B. juncea PR* genes after hormonal, biotic and abiotic stresses. Our results showed that *E. cruciferarum* induces both LAR and SAR pathways in *B. juncea* which will help us to select the potential candidate gene like *PR1*, *PR2* and *PR3* for developing disease-resistant plants. This study also revealed that besides ABA, SA and JA are also involved in abiotic stress signaling in *B. juncea* and there is a hormonal crosstalk. In this work, SA marker genes were downregulated by ABA but showed upregulation during abiotic stresses which further provides the evidence that SA could trigger the expression

of these genes in ABA-independent manner. These identified *PR* genes can serve as potential candidates for developing transgenic crops resistant to multiple stresses which are the theme of future research in plant genetic engineering and molecular breeding.

Author contribution statement AG conceived and designed research. SA has performed all the experiments and wrote the manuscript. ZAM and PKP contributed to data analysis. AT has contributed to bioinformatic analysis. NC and SR contributed to RNA isolation. AG contributed to manuscript proofreading. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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