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ORIGINAL ARTICLE

Isolation and characterization of systemic acquired resistance marker gene *PR1* and its promoter from *Brassica juncea*

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Abstract

Systemic acquired resistance (SAR) is an inducible defense response in plants that provides enhanced resistance against a variety of pathogens. In this regard, SAR marker gene *PR1* (pathogenesis-related gene 1) was isolated from *Brassica juncea* and was named as *BjPR1*. The amino acid sequence of BjPR1 protein showed 99, 92, and 78% similarity with known *PR1* proteins of *Brassica rapa, Brassica napus,* and *Arabidopsis thaliana*, respectively. Quantitative real-time PCR (qRT-PCR) analysis showed increased expression of *BjPR1* gene both in local (infected) and distal (non-infected) leaves of *B. juncea* after *Alternaria brassicae* infection, whereas mechanical wounding showed expression only in local (wounded) leaves but not in distal (unwounded) leaves. Moreover, *BjPR1* gene was strongly induced by salicylic acid (SA), whereas no such induction was observed following jasmonic acid (JA) or abscisic acid (ABA) treatments. To further elucidate gene regulation pattern of *BjPR1*, 2 kb promoter region of *BjPR1* was isolated and subjected to in silico analysis which identified many potential *cis*-regulatory elements associated with plant defense as well as signaling pathways. The transient GUS expression analysis showed strong expression of GUS gene driven by *BjPR1* promoter after SA treatment, while as ABA and JA downregulates GUS gene expression compared to control. In addition, *BjPR1* promoter was significantly induced by wounding at local tissues. Hence, these results highlight the multiple role of *BjPR1* gene in response to biotic and abiotic stresses. In addition, the present study also reported *BjPR1* promoter as stress-specific inducible promoter that can be ideal candidate for controlling the expression of biotic stress response genes in transgenic plants.

Keywords Abscisic acid · *Alternaria brassicae* · *Brassica juncea* · Jasmonic acid · Pathogenesis-related proteins · Salicylic acid · Systemic acquired resistance

Introduction

Indian mustard (*B. juncea* L.) belonging to the family Brassicaeae is the second most important edible oilseed after groundnut in India. It contributes 28.6% in the total oilseeds production and sharing 27.8% in the India's oilseed economy (Shekhawat et al. 2012). There are various factors including both biotic and abiotic stresses that lower the productivity of this crop, among which *Alternaria* blight caused by *A. brassicae* is a major constraint (Kolte 1985). *Alternaria* blight appears every year (endemic) and causes up to 36.88% loss of yield in mustard due Alternaria blight (Bal and Kumar 2014). In addition to direct losses, *Alternaria* blight also affects the quality of the seed by reducing size, causing seed discolouration, and reduced oil content (Kaushik et al. 1984). Improving *Alternaria* disease resistance through the conventional breeding in *B. juncea* has been challenged by

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complex nature of disease resistance, costly and less precise phenotyping, low heritability, environment sensitiveness, and limited availability of resistant germplasm. Second, present protective measures rely heavily on fungicides, generating adverse environmental consequences (Chen and Zhou 2009). The advancement in proteomics, genomics, and transcriptomics techniques has impressively accelerated the research in plant–pathogen interactions. In this regard, transgenic technology and molecular-assisted breeding will provide an alternative approach to develop disease-resistant varieties, which can overcome the problems related to the conventional breeding and fungicides. Furthermore, a better understanding of molecular mechanism of plant–pathogen interactions and identification of novel disease-resistant genes such as pathogen-related (PRs) genes will be an essential tool for crop improvement.

Pathogenesis-related (PR) proteins are of great interest for engineering plants not only to disease resistance but also for developing pest resistant varieties. Currently, PRs have been classified into 17 families, based on their amino acid sequence similarities, enzymatic activities and other biological properties. They have been numbered in sequence of discovery (Sels et al. 2008; Sinha et al. 2014). During host–pathogen interactions, PRs do not only accumulate locally in the infected leaf but also induced Systemically (Hamamouch et al. 2011). Among the classes of *PR* proteins, *PR1* is one of the important pathogen-related proteins, which has been studied mostly in model plants (Arabidopsis and tobacco), but its molecular function remains unknown. A number of *PR1* like proteins have been identified in many plant species including both mono and dicotyledonous plants (Mitsuhara et al. 2008; Li et al. 2011). *PR1* group is found to be the most abundant group in *PR* gene families and is classified into two groups (acidic and basic proteins) based on the isoelectric point (Van Loon and Van Strien 1999). *PR1* is universally known as a molecular indicator of induced plant immune system such as hypersensitivity response (HR) and systemic acquired resistance (SAR) (Jung and Hwang 2000; Jung et al. 2009). SAR is an inducible defense response in plants that provides enhanced resistance against broad range of pathogens. The previous reports have shown that transcript levels of *PR1* gene increase significantly in plants exposed to biotic and abiotic stresses (Brederode et al. 1991; Mitsuhara et al. 2008), which suggests that they play an important role in combating these challenges. The *PR1* gene induction following pathogen infection has been well documented in a number of crop species, viz., *Paeonia suffruticosa*, *Nicotiana tobacum, Oryza sativa,* etc. (Agrawal et al. 2001; Sujon et al. 2005; Yang et al. 2013). Overexpression of PR1 proteins in different crop systems has generally resulted in enhancing disease resistance against many pathogens (Alexander et al. 1993; Lawton et al. 1993; Niderman et al. 1995; Sarowar et al. 2005).

Plant immunity strongly relies on two important defense signaling regulatory pathways like JA and SA which act synergistically or antagonistically (Glazebrook 2005). Interestingly, exogenous treatment with defense hormonal stimulators salicylic acid (SA), jasmonate (JA), and ethylene (ET) has been reported earlier to regulate *PR1* transcript accumulation which varies among plant species (Reymond and Farmer 1998; Kim and Hwang 2000; Zhang et al. 2010). JA- or an ethylene-dependent signaling pathway was found to induce the expression of basic *PR1* genes strongly, whereas an SA-dependent pathway was found to increase the expression of acidic *PR1* genes (Ward et al. 1991; Eyal et al. 1992; Niki et al. 1998).

One of the major challenges in plant genetic engineering is to find highly specific promoter which could drive the expression of target gene in transgenic crops (Hernandez-Garcia and Finer 2014). In general, constitutive promoters of both viral and plant origins have been commonly used to drive gene expression in many disease-resistant transgenic crops. These promoters cause a number of problems such as homology-dependent gene silencing, leading a fitness penalty in plant growth and development (Zheng et al. 2007). To solve this problem, spatially and temporally inducible promoters that are less exhaustive are needed to develop transgenic plants resistant to pathogens. Identification of ideal pathogen-inducible promoter mainly relies on the discovery of disease-resistant genes. The best feature of the pathogen-inducible promoter is the early and rapid activation in response to multiple phytopathogens. Till date, very few pathogen-inducible promoters have been isolated and characterized mainly from model plants. Therefore, it is very pertinent to isolate and characterize pathogen-inducible promoters for driving the expression of genes responsible for conferring disease resistance. Pathogen-inducible promoters usually possess many potential *cis*-regulatory elements based on their interaction with defense signaling molecules such as SA, JA, and ET or signals (Mazarei et al. 2008). Two important *cis*-acting elements, the GCC-like elements (Ohme-Tagaki et al. 2000) and the W-box (Eulgem et al. 2000) elements, have been well studied in pathogen-inducible promoters.

The aim of this study was to isolate and functionally characterize SAR marker gene *BjPR1* and its promoter after Alternaria infection, wounding, SA and JA treatment in *B. juncea*. These results will provide novel insights into the *Brassica-Alternaria* pathosystem and their signaling cascades which are largely unknown.

Materials and methods

Plant material

Brassica juncea var. varuna and *Nicotinia benthamiana* plants were grown in a growth chamber at 22–24 °C under

12-h light and 12-h dark cycle. 40-day-old *B. juncea* plants were used for constructing a cDNA library and for endogenous gene expression assays. For transient assay, 1-month-old *N. benthamiana* plants were used.

Isolation and phylogenetic analysis of *BjPR1*

Brassica juncea cDNA library was constructed from total RNA of SA-treated leaf samples using BD SMART cDNA library construction kit following the manufacturer's instructions (Clontech Inc., USA). The full-length sequence of *BjPR1* gene was isolated from SA library as described by (Taweel et al. 2011) and sequenced. The bioinformatic tool GENSCAN (http://genes.mit.edu/GENSCAN.html accessed 17 June 2016) was used to predict the open reading frame of BjPR1 and its deduced amino acid sequence, respectively. Protein sequence similarity analysis of *B. juncea* PR1 protein was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast), and for multiple sequence alignment, ClustalX was used. Phylogenetic relationships of *B. juncea PR1* homologs were constructed using the neighbour-joining method with bootstrapping (1000 replicates) using MEGA 7.0 program (Kumar et al. 2016). The structural features of BjPR1 protein were analysed using the expert protein analysis system (http://www.expasy.org/). Conserved domain structure of BjPR1 protein was analysed by Pfam database (http://pfam.xfam.org/).

Culture of *A. brassicae* and inoculation

Alternaria brassicae stain was obtained from Indian Type Culture Collection (I.D. No. 81651) Division of Plant Pathology, IARI, New Delhi and cultured on radish dextrose agar at 22 °C for 20 days. The conidia of well-grown *A. brassicae* were suspended in sterile distilled water, filtered with two layers of muslin cloth, and diluted to 5×10^3 conidia/ml. Forty-five-day-old *B. juncea* plants were inoculated with 4–6 drops of spore suspension of *A. brassicae* (5×10^3 spores cm^{-3}) on four different selected spots of the leaf surface and then incubated in a chamber at 25 °C, with 100% relative humidity. Control *B. juncea* plants were inoculated with 10 µl sterile distilled water. The leaf samples were collected from both local (infected) and distal (non-infected) leaves of *B. juncea* plants at 0-, 2-, 4-, 8-, 12-, 24-, 48-, 72-, and 96-h post-inoculation (hpi), stored at − 80 °C after being flash frozen in liquid nitrogen. Three different *B. juncea* plants were infected with *A. brassicae* on separate occasions to provide biological replicates for qRT-PCR analysis.

Wounding and hormonal treatments

Leaves of 40-day-old *B. juncea* plants were effectively wounded using sterile syringe needle and samples were harvested at different time intervals. For hormonal treatments, 40-day-old *B. juncea* plants were sprayed with 2-mM salicylic acid pH 7.0 (Chengguo et al. 2012), 100-µM jasmonic acid (Zhao and Chye 1999), and 50-µM ABA individually, kept separately in dark chamber to prevent cross talk signaling, evaporation, and light-induced degradation. Control plants were sprayed with sterile distilled water. Leaf samples for RNA isolation were harvested from control, SA, JA, and ABA-treated plants after 0-, 2-, 4-, 8-, 12-, 24-, 48-, and 72-h post-treatment.

RNA isolation and qRT-PCR

Total RNA was isolated from the control and treated leaf samples using the protocol of PureLink RNA Mini Kit (Ambion Life Technologies, USA). The purity and concentration of total RNA was determined by Nanodrop spectrophotometer (NanoDrop 2000c; Thermo Scientific, Wilmington, DE). First-strand cDNA was generated from 2-µg of DNase-treated total RNA by reverse transcriptase in 20-µl reaction volume containing oligo (dT) 18 primers, 10-mM dNTPS, and water following the manufacturer's protocol (Fermentas, Canada). cDNA sample was 10 times diluted and kept at − 80 °C for further expression studies. qRT-PCR reaction mixture contains 2 µl of cDNA, 5 µl of SYBR green qRT-PCR master mix (Takara, Japan), and 0.5 µl (10 picomol) of *PR1* forward and reverse primers (Table 1). The reactions were performed in triplicates and program of the qRT-PCR was; 95 °C for 5 min; followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. The relative levels of *BjPR1* mRNA were evaluated against the *A. thaliana* housekeeping gene α-tubulin (GenBank accession no-NM_100360.4) amplified with specific primer pairs (Table 1). The relative expression levels of *BjPR1* mRNA in all the treated samples were quantified by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 1 List of primers used in this study

Gene	Sequence
PR1	F-5′GAACACGTGCAATGGAGAATG3′ R- 5′CCATTGTTACACCTCGCTTTG3′
Alpha tubulin	F-5′TGC TTT CGT TCA CTG GTA TGT3′ R-5′ CAG CAC CGA CCT CTT CAT AAT C3′
AP1	F-5′ GTAATACGACTCACTATAGGGC3′ R-5‘GTA ATA CGA CTC ACT ATA GGG C 3
AP2-F	F-5′-ACTATAGGGCACGCGTGGT-3′ R-5′ACT ATA GGG CAC GCG TGG 3′
GSP1	5′TATTTTTGTGTGTTCCCCGGCCGTAATGG3
GSP2	5′CAAGAGCTCCCACAAGGGCAGCCAAAA TTA3′

Isolation of *BjPR1* promoter by genome walking

BjPR1 promoter was isolated from the *B. juncea* genome by PCR walking using Universal Genome Walker kit (Clontech, CA, USA). Briefly, high-quality genomic DNA was extracted from *B. juncea* using a DNeasy Plant Midi Kit (Qiagen, Valencia, CA). Purified DNA (2.5 µg) was digested at 37 °C with *EcoR*V, *Dra*I, *Pvu*II, and *Stu*I restriction enzymes supplied with the GenomeWalker™ kit (Clontech, CA, USA). These restriction digestions generate blunt ends of the genomic DNA. Short adaptor DNA sequence provided with genome walker kit (Clontech laboratories Inc. http://www.clontech.com) was ligated to blunt end digested genomic DNA fragments, thus generating four Genome walker libraries (Table 1). These libraries were used as template for the isolation of promoters by two-step PCR (primary and secondary PCR) using adaptor-specific and gene-specific primers. The adaptor-specific primers provided in the kit and *BjPR1* gene-specific primers for primary PCR and GSP2 for secondary PCR were designed within the 5′end of the *BjPR1* sequence deposited in Genbank (accession no DQ359128) using primer 3.0 software (Table 1). The PCR purified product (*BjPR1* promoter) approximately 2 kb was isolated from *Stu1* library and cloned into pGEMT Easy vector for sequencing. To investigate the presence of *cis*-regulatory elements such as TATA box, CAAT box, and stress regulatory *cis*-acting element in *BjPR1* promoter, sequence was analysed by PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002) databases of plant *cis*- regulatory DNA elements. The *BjPR1* promoter sequence was submitted to the GenBank nucleotide sequence databases with accession number KC865598.

Vector construction and transient expression assays

The binary vector pORE-R2 (promoter less GUS reporter vector) was used in this study. The *BjPR1* promoter was cloned in this binary vector at *Pst*1 and *BamH*1 site, respectively. The cloning of the *BjPR1* promoter was confirmed by colony PCR and restriction analysis. The recombinant (*BjPR1*promoter-pORER2) GUS vector was further transformed into *Agrobacterium tumefaciens* strain (*EHA105*) by freeze thaw method and confirmed by colony PCR using promoter-specific primers. To investigate the *BjPR1* promoter activity, transient assays were carried out in tobacco leaves. *Agrobacterium* strain *EHA105* containing *BjPR1*-pORER2:GUS construct was grown in Luria–Bertani broth (LB) containing antibiotics (Rifampcin 25 µg $mL^{-1}$and Kanamycin 50 µg mL^{-1}) at 28 °C until the culture reached OD600 = 0.8. The culture was centrifuged at 7000 g for 10 min and resuspended in infiltration media containing (10-mM MES (pH 5.5), 10-mM $MgCl_2$, and 100-µM acetosyringone) incubated at 28 °C for 3 h before agroinfiltration. The two young expanded leaves were infiltrated gently with the bacterial culture using 1-ml needleless syringe and kept in a growth chamber at 22 °C for 24 h. To examine the inducibility of *PR1* promoter, 24 h of initial agroinfiltrated tobacco leaves were further infiltrated on the same spot with 2-mM SA, 100-µM MeJA, and 50-µM ABA samples were harvested after 24 h for GUS staining. For control, leaves were infiltrated with sterile distilled water. Leaf discs from both control- (negative and positive) and hormonal-treated samples were collected in small petridishes (Himedia), and immersed in GUS staining solution containing (0.1-M NaHPO4 pH 7.0, 0.5-mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 0.001-M EDTA pH 8.0, 20% methanol, 0.1% Triton X-100, and 0.5-mM X-gluc), and incubated at 37 °C for 16 h. After staining, samples were bleached by 75% ethanol 2–3 times and photographed. *A. tumefaciens* strain with promoter less GUS reporter vector was used as a negative control.

Results

Cloning and sequencing of *BjPR1* gene

The full-length cDNA sequence of *PR1* gene was isolated from SA-treated *B. juncea* library. The clone designated as *BjPR1* (GenBank accession no. KM506762) was structurally and functionally characterised. Sequence analysis of *BjPR1* revealed that it is comprised of 672 bp with an open reading frame of 486 nucleotides, encoding a protein of 161 amino acids with a calculated molecular mass of 17.53 kDa and a theoretical PI of 7.07. The nucleotide sequence of this gene also showed a 5′ untranslated region (5′ UTR) of 63 nucleotides and a 3′ UTR of 126 nucleotides. In silico subcellular localisation revealed that *BjPR1* is expressed in Vacuole. The software NetPhos (http://www.cbs.dtu.dk/services/NetPhos) predicted that BjPR1 had six serines and one threonine as potential phosphorylation sites (Fig. 1a). The 3D structure of the BjPR1 protein is shown in (Fig. 1b). The predicted BjPR1 protein contained a conserved motif at residues 30–161 aa that belonged to the SCP-like super family (Fig. 1c). Its deduced amino acid sequence revealed highest similarity with PR1 proteins of its close relative *B. rapa* (99%) followed by *A. thaliana* (78%), *Eutrema japonicum* (78%), and *Heve brassiliensis* (66%), respectively (Fig. 1d). The phylogenetic relationships of *BjPR1* with its homologs from both monocots and dicot plants were constructed through the neighbour-joining method using MEGA 7.1 program, which resulted four major clusters viz., I, II, III, and IV. The *BjPR1* as well as *PR1* genes of all Cruciferae members are grouped together in cluster I, and *BjPR1* was nearest to *PR1* of *B. rapa* followed by *B. napus, B. oleracea,* and *S. parvula*. However, *BjPR1* was most diverged from

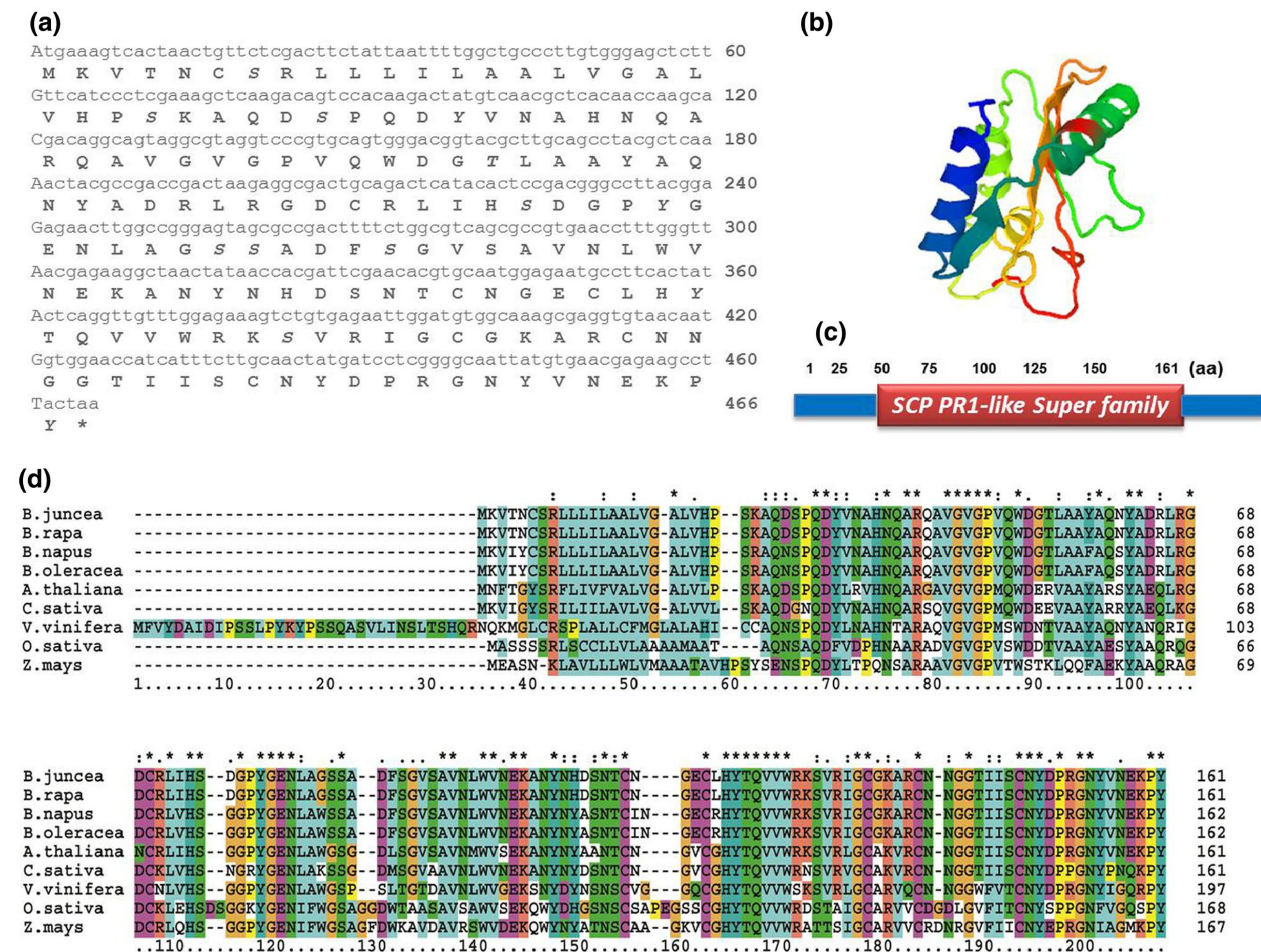

Fig. 1 Structural analysis of *BjPR1* nucleotide and amino acid sequences **a** Nucleotide and amino acid sequences of *BjPR1* with putative phosphorylation sites like serine and tyrosine are shown in bold italics **b** 3D structure of BjPR1 protein **c** Conserved domain of the BjPR1 protein. The predicted BjPR1 protein contained a conserved motif at residues 30–161 aa that belonged to the SCP-PR1 like super family **d** Multiple sequence alignment of the BjPR1 protein sequence with other plant PR1 proteins. Comparison of deduced amino acid sequence of BjPR1 with other plant PR1 s from *B. rapa, B.napus, B. oleracea, A. thaliana, C. sativa, O. sativa,* and *Z. mays.* Conserved residues are shown with shaded colours

PR1 of Gramineae family that are clustered in separate and distinct cluster IV (Fig. 2).

BjPR1 expression in response to *Alternaria* infection

An accurate monitoring of disease progression in crop plants is very important to evaluate the role of pathogen-related genes. Therefore, we first studied the disease development in *B. juncea* during *Alternaria* infection. Pure culture of *A. brassicae* and its spore morphology are shown in (Fig. 3a–b). After *Alternaria* infection, necrotic lesions appeared as grey circular areas at the site of inoculation on the infected leaves of *B. juncea,* while as no symptoms appeared on non-infected leaves (Fig. 3c–d). These results showed the compatible interaction between *Alternaria* and *B. juncea* pathosystem. In the present study, we examine the transcriptional changes of *BjPR1* gene in local (infected) and distal (non-infected) leaf sample of *B. juncea* in response to *Alternaria* infection. The qRT-PCR results showed that transcript levels of *BjPR1* gene increase significantly in local infected tissue at 4 h (2.3 fold) and reached maximum at 24 h (3.8-fold) of post-inoculation as compared to control, but decreased sharply to a relatively low expression from 48 to 96 h (Fig. 4a). In comparison, the expression of *BjPR1* gene in distal leaves (non-infected) was significantly lower at early stages but was detected after 48 hpi and reached to peak at 72 h (Fig. 4b). In general, the *BjPR1* genes showed higher expression in local leaves (infected) than that of distal leaves (non-infected). These results, therefore, revealed

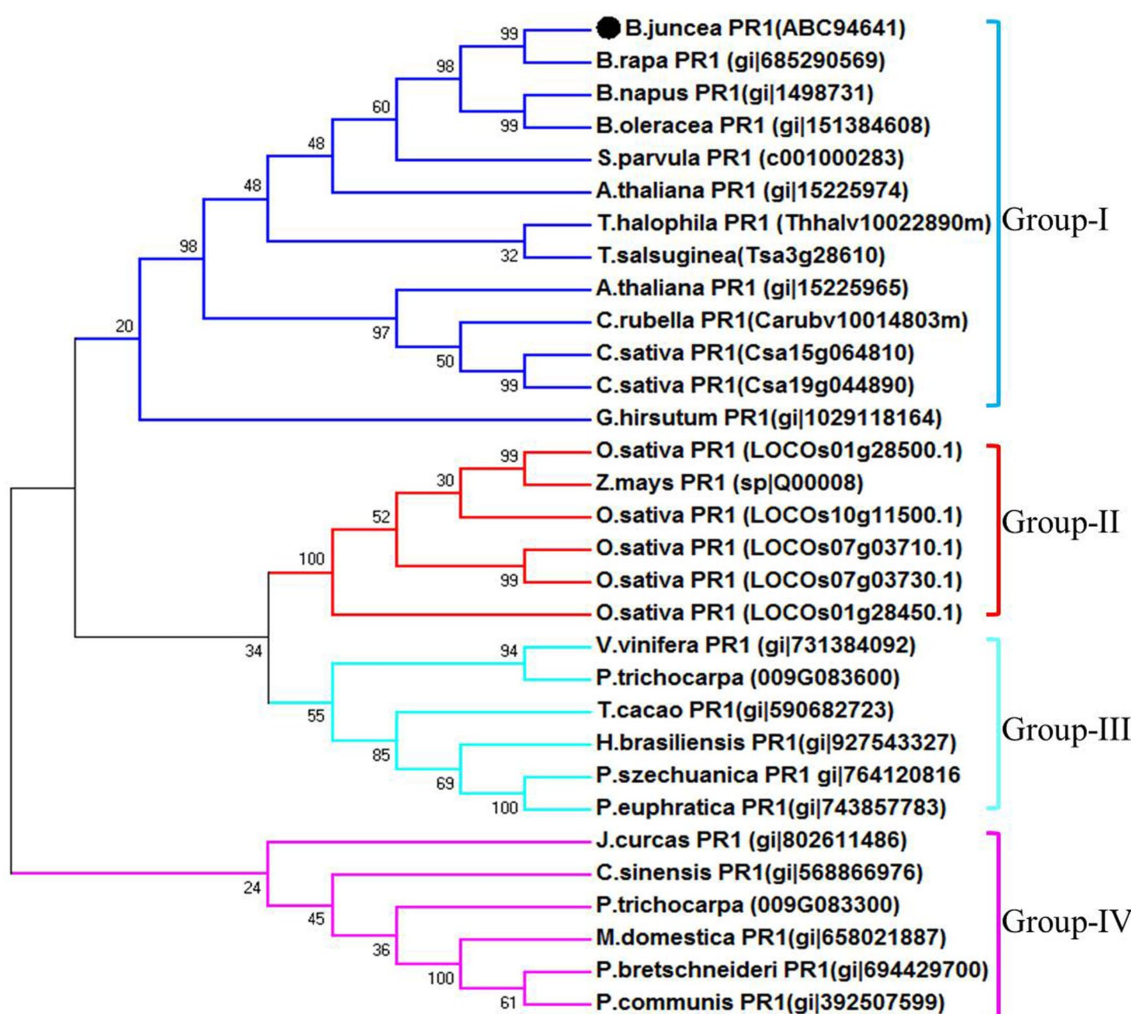

Fig. 2 Phylogenetic relationship of *BjPR1* with homologs of other plant species, constructed using the MEGA 7.0 program. Bootstrap values denote the divergence of each branch and the scale indicates branch length. *BjPR1* is highlighted as black colour circular marker

that *BjPR1* gene is induced both locally as well as systematically in *B. juncea* followed *A. brassicae* infection.

BjPR1 expression in response to wounding

Plants respond to wounding through induction of variety of genes both locally or systematically that contribute in healing of damaged tissues and further invasion of pathogens (Durrant et al. 2000). Therefore, in the present study, we examined the local and systemic expression of *BjPR1* gene after wounding at early and late time intervals. Our results revealed that transcript levels of *BjPR1* gene increases at 2 h and reached maximum at 4 h (threefold induction) followed by decline at later 8 h and 12 h in local tissues (Fig. 4c). In contrast, no systemic induction of *BjPR1* was observed in leaves (unwound) when compared to control (Fig. 4d).

BjPR1 expression in response to hormonal treatments

SA and JA are the plant hormones that are well-known modulators for plant defense system in plants. However, role of ABA in plant defense is not fully understood. To further examine the expression of *BjPR1* gene in response to above hormones, *B. juncea* plants were treated with 2-mM SA, 100-µM JA, and 50-µM ABA, and are profiled over various time points. The transcript levels of *BjPR1* gene were significantly increased at 2 h, reached the peak at 12 hpi rapidly after SA treatment, and then declined at later time points (Fig. 5a). In contrast, no significant transcript accumulation of *BjPR1* was reported after JA treatment or ABA treatment at any time points (Fig. 5b–c). SA-induced expression of *BjPR1* further provides the evidence that it can be used as SA or SAR marker in *B. juncea*.

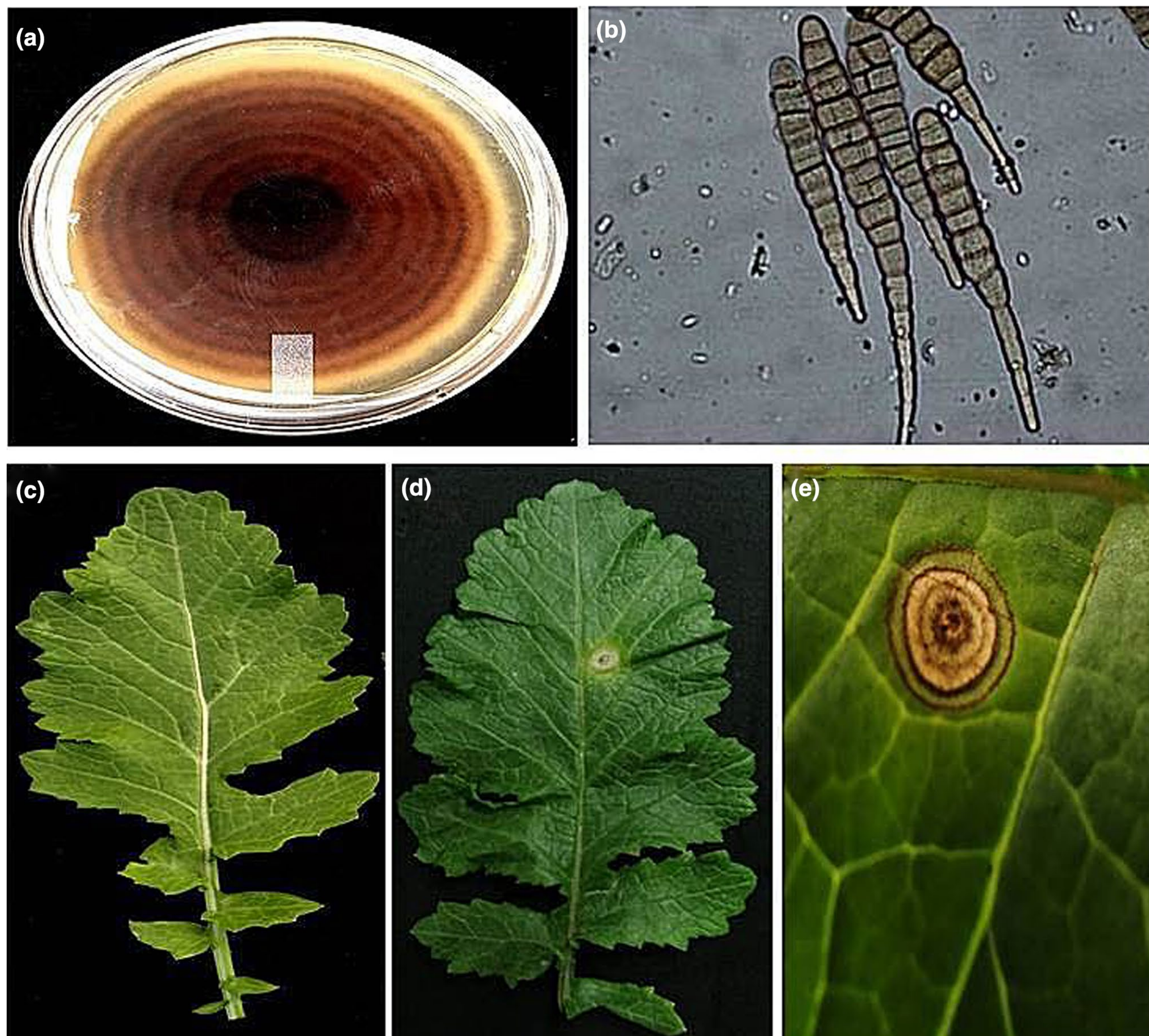

Fig. 3 *In vivo* infection of *B. juncea* with *A. brassiace* **a** *A. brassiace* culture grown on root radish medium **b** Microscopic identification of *A. brassicae* fungus (Conidia under 100X microscope) **c** Uninfected *B. juncea* leaf as control **d–e** *B. juncea* leaves after *Alternaria* infection

Isolation and *In silico* analysis of *BjPR1* promoter

The 2 kb upstream sequence of *BjPR1* gene was isolated from *B. juncea* using genome walking approach and submitted into gene bank (accession no. KC865598.1). *BjPR1* promoter sequence was scanned using PlantCARE and PLACE promoter databases for identifying putative *cis*-acting regulatory elements and are classified into three groups based on their function. The first group corresponds to basal regulatory elements that consist of two copies of TATA box and 7 copies of CAAT box (PlantCARE). The second group is related to pathogen and defense signaling-responsive *cis*-elements and comprise of GT-1 element (GAAAAA) required for rapid response to pathogen attack and salicylic acid inducible gene expression, WBOXATN*PR-1* element required for salicylic acid response (PLACE), TCA element (CAGAAAAGGA) for SA response, TGACG motif for MeJA response, TC-rich motifs (ATTTTCTTCA) for defense response, and AT-rich sequence (TAAAATACT) for maximal elicitor-mediated activation (PlantCARE). Third group includes abiotic stress-related *cis*-elements such as ABREs motif (ACGT) for ABA-dependent expression, MYC-motif (CACATG), and MYB (GGATA) involved in early response to drought inducible gene induction, HSE element (AGAAAATTCG) involved in heat stress, Circadian motif (CAANNNNATC) involved in circadian control,

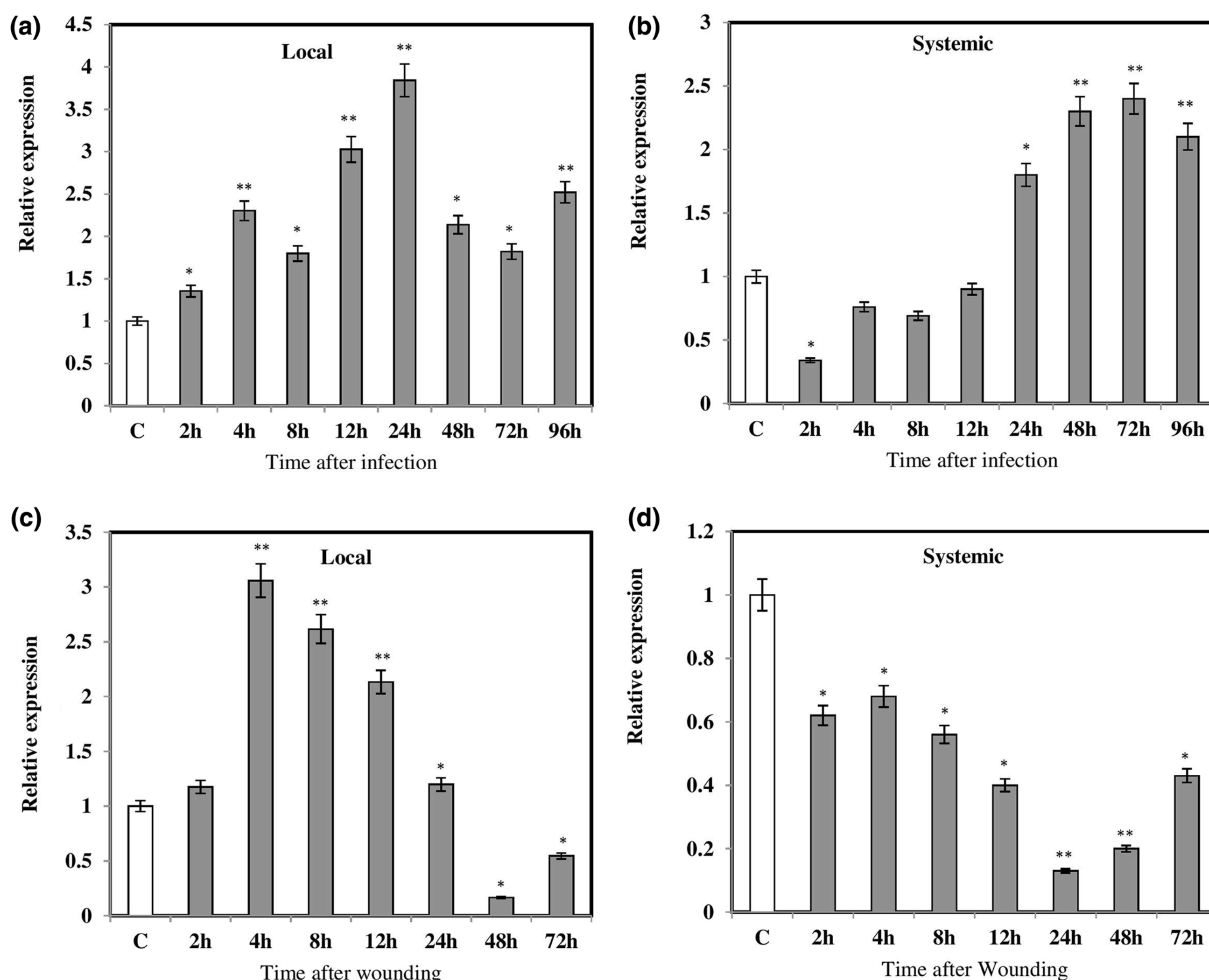

Fig. 4 Local and systemic expression of *BjPR1* gene after *Alternaria* infection and wounding: **a** expression of *BjPR1* in local (infected) leaves at various time points; **b** expression of *BjPR1* in distal (non-infected) leaves; **c** relative expression of *BjPR1* in local (wounded) leaves; **d** relative expression of *BjPR1* in distal (unwounded) leaves. SE for each bar is shown. The relative expression was calculated using ΔΔCt method. The asterisks indicate statistically significant differences between the control and treated *B. juncea* plants (*$P < 0.05$; **$P < 0.01$)

AE-box (AGAAACT), G-box (CACGTT) involved in light response, and RY-element (CATGCATG) seed-specific regulation, (Fig. S1). A complete list of all predicted *cis*-elements present in the *BjPR1* promoter was shown in Table 2. Hence, in silico analysis of *BjPR1* promoter revealed that it could be induced by both biotic as well as abiotic stresses.

Hormonal response of *BjPR1* promoter

To examine the inducibility of the *BjPR1* promoter, we generated a construct containing the whole promoter region (1800-bp) fused with the GUS reporter gene in pORER2 vector (Fig. 6a). We further studied GUS expression levels driven by *BjPR1* promoter using agrobacterium-mediated transient expression assay in *N. benthamiana* leaves (Fig. 6b). This technique is simple and widely used for the quantitative analysis of plant promoters in vivo (Li et al. 2011). We examined the GUS activity upon hormonal treatments such as (ABA, JA, and SA) after the *Agrobacterium* strains were infiltrated into *N. benthamiana* leaves. *BjPR1* promoter showed basal expression under control conditions, while as no GUS activity was observed in negative control (Fig. 6c–d). Interestingly, histochemical GUS analysis revealed that *BjPR1* promoter showed weak expression after ABA or JA treatment as compared to untreated tobacco leaves

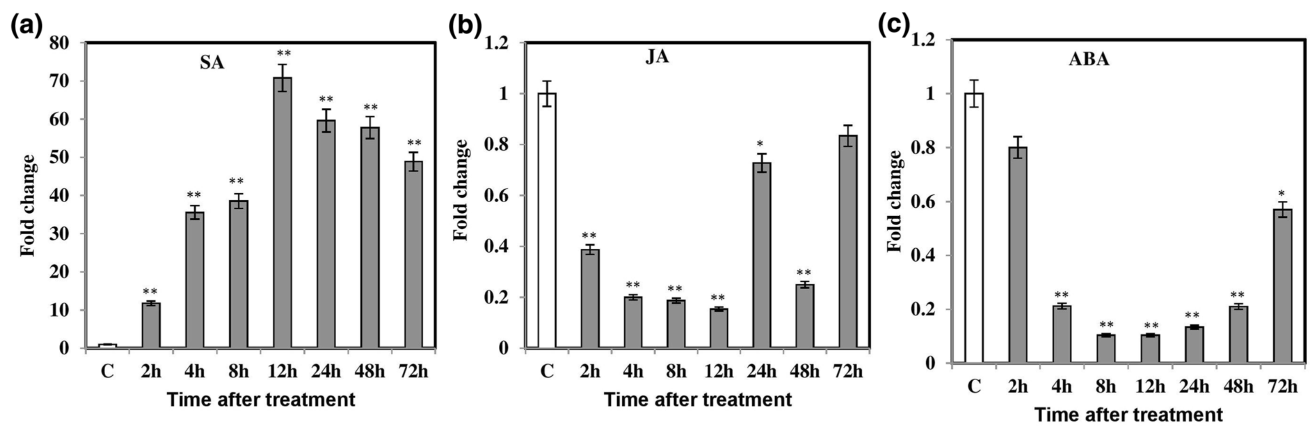

Fig. 5 Transcriptional studies of *BjPR1* under various hormonal stresses by qRT-PCR analysis. *B. juncea* plants were treated with 2-mM SA, 100-μM MeJA, and 50-μM ABA, respectively. House-keeping gene alpha tubulin was used as internal control. All data are represented as means of three replicates (n = 3) ± SE. The asterisks indicate statistically significant differences between the control and hormone treated *B. juncea* plants (*P < 0.05; **P < 0.01)

Table 2 Putative *cis*-regulatory elements in *BjPR1* promoter sequence identified by PlantCARE and PLACE promoter databases

Motif	Copies	sequence	Function
TATA box	2	TTATA	Core promoter element
CAAT box	10	CAAT	*Cis*-acting regulatory element related to meristem (mesophyll) expression
TCA element	1	CAGAAAAGGA	*Cis*-acting element involved in salicylic acid responsiveness
GT-1	2	GAAAAA	*Cis*-acting regulatory element required for rapid response to pathogen attack, salinity, and salicylic acid inducible gene expression
I-box	1	GATAGGG	Part of a light responsive element
MYC	1	CACATG	*Cis*-acting regulatory element involved in early response to drought and abscisic acid induction
HSE	1	AGAAAATTCG	*Cis*-acting element involved in heat stress responsiveness
Pollen specific	4	AGAAA	Required for pollen expression
MeJA motif	1	TGACG	*Cis*-acting regulatory element involved in methyl jasmonates responsiveness
RY-element	1	CATGCATG	*Cis*-acting regulatory element involved in seed-specific regulation
W-box	3	TTGAC, TGAC	*Cis*-acting regulatory element involved in direct fungal elicitor stimulated transcription of defense genes and activation of genes involved in response to wounding
MYB	1	GGATA	Involved in regulation of drought inducible gene expression
G-box	2	CACGTT, CACATGG	*Cis*-acting regulatory element involved in light responsiveness
AE-box	1	AGAAACTT	Part of a module for light response
Erd1	1	ACGT	*Cis*-acting regulatory element required for early response to dehydration
TC-rich repeats	2	ATTTTCTTCA,	*Cis*-acting element involved in defense and stress responsiveness
GATA	7	GATA	*Cis*-acting regulatory element required for high level light regulated and tissue specific expression
AT-rich sequence	1	TAAAATACT	Element for maximal elicitor-mediated activation
ERE-motif	1	AATTCAAA	Ethylene-responsive element

(Fig. 6e–f). As expected, *BjPR1* promoter drives strong GUS gene expression in tobacco leaves after SA treatment, suggesting that the promoter is strongly and rapidly induced (Fig. 6g). We also observed moderate GUS activity driven by *BjPR1* promoter in wounded *B. juncea* leaves (Fig. 6h).

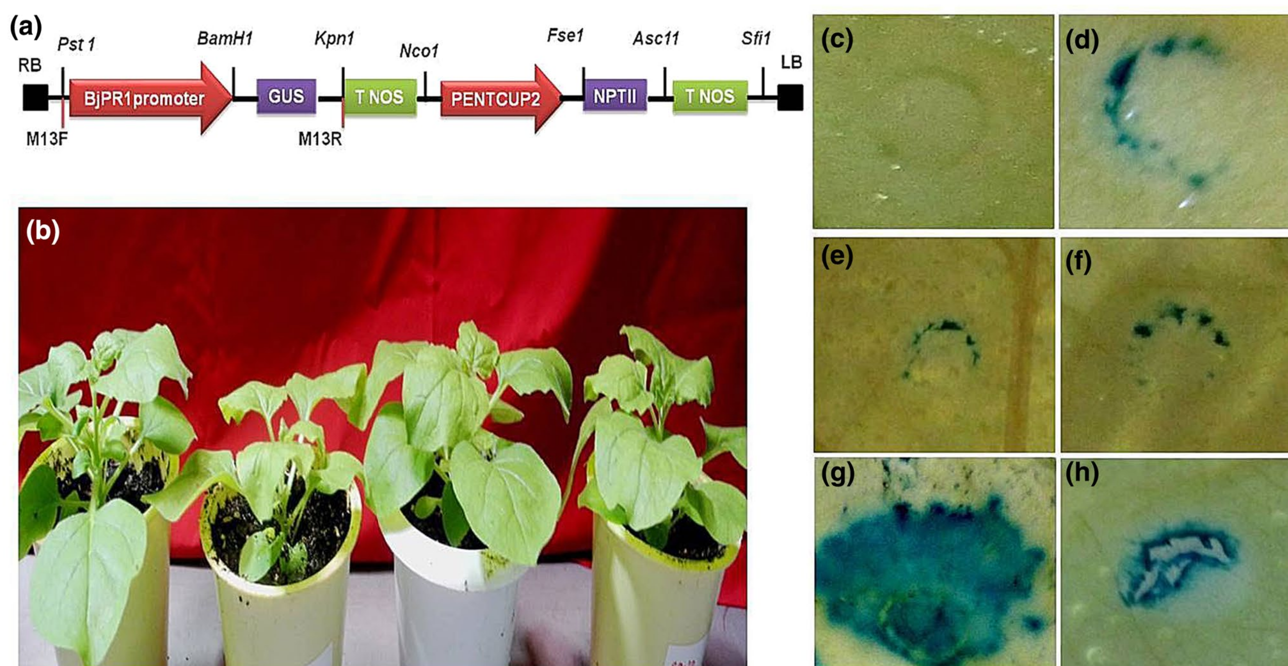

Fig. 6 Transient expression analysis of *BjPR1* promoter in tobacco leaves: **a** schematic representation of *BjPR1* promoter cloned in pORER2 vector (promoter less GUS reporter vector) at *Pst*1 and *BamH*1 sites for studying promoter inducibility; **b** healthy *N. benthamiana* plants for transient expression analysis. After 24 h of agroinfiltration, plants were treated with sterile water (control), ABA, JA, and SA, respectively. Wounding was carried out with sterile needle. Leaf samples were harvested from control and treated plants after 24 h of treatment for GUS staining; **c** Promoter less GUS reporter vector as negative control; **d** GUS gene expression driven by *BjPR1* promoter without treatment; **e** effect of ABA on the expression of GUS gene driven by *BjPR1* promoter; **f** effect of JA on the expression of GUS gene driven by *BjPR1* promoter in tobacco leaf; **g** Effect of SA on the expression of GUS gene driven by *BjPR1* promoter; **h** wound-induced GUS accumulation in tobacco leaf

Discussion

Plant pathogens are unquestionably the most versatile for ecological adaption and in the devastation of plant growth. However, different strategies have been carried out to increase disease resistance in plants like overexpression of pathogenesis-related (PR) proteins or antimicrobial peptides, modifying the resistance signaling pathway and even pyramiding the cloned resistance (*R*) genes (Grover and Gowthaman 2005). Genetic engineering has become an imperative approach to develop new varieties with high disease resistance. To date, a limited number of disease-resistant genes have been identified and characterized in *B. juncea*. Therefore, in the present study, one of the key genes of plant disease resistance *PR1* and its promoter was studied for its expression pattern in response to *Alternaria* infection, wounding, and defense stimulators (SA and JA). The gene predicted (*BjPR1*) belongs to SCF family that is known to be antifungal and cell wall degrading proteins. Phylogenetic analysis of the predicted *BjPR1* protein with other known PR1-like sequences revealed that they are grouped into distinct clades. However, *BjPR1* fall within the same clade as other *Brassica* genus PR1 proteins (Fig. 2). Many studies have shown that *PR1* plays multifaceted roles in plant defense and also are considered as signaling indicators of SA pathways, but the molecular function is still unknown.

Plant defense system is modulated by different *PR* proteins, which are the key players of plant immune system. The previous studies showed that *PR1* expression is activated by various biotic and abiotic stresses (Mitsuhara et al. 2008; Thierry et al. 1995). Hou et al. (2012) reported that *V. vinifera PR1* gene was highly induced after *Plasmopara viticola* inoculation and expression level of *VvPR1* reached the highest peak at 24 hpi, suggesting that *VvPR1* might be related to disease resistance. Similarly, transcript levels of *PR1* gene isolated from *Paeonia suffruticos* increases significantly after *Cylindrocladium canadense* inoculation, and its expression peaked in 24 hpi, which implicated that *PsPR1* might be involved in the disease defense (Yang et al. 2013). Our results showed that the expression of *BjPR1* gene was significantly induced by *A. brassiace* (compatible interaction) in local as well as distal leaves; however, we also observed the expression of *PR1* reached peak at 24 h after inoculation (Fig. 4a-b). The expression profiles of *BjPR1* were similar to the previous studies, though transcript levels were relatively different,

which may be due to nature or type of plant–pathogen interactions. These findings are supported by the similar expression pattern of *PR1* gene in *Arabidopsis* and *Solanum lycopersicoides* inoculated with *A. brassicola* and *Botrytis cinerea*, respectively (Schenk et al. 2003; Smith et al. 2014). Interestingly, *BjPR1* was induced both locally and systemically in *B. juncea,* which further provides the evidence for the role of *BjPR1* in SAR. Furthermore, upregulation of SA marker gene *PR1* upon SAR activation might directly contribute to resistance execution following fungal and oomycete pathogen assault, because *PR1* proteins isolated from tobacco and tomato possess in vitro antifungal activity (Niderman et al. 1995). SA signaling generally regulates plant resistance against biotrophic pathogens, whereas JA/ET pathways are commonly associated with resistance to necrotrophic pathogens, and to herbivorous pests (Glazebrook 2005). The present study revealed that *BjPR1* is induced by necrotrophic pathogen (*A. brassicae*) which suggests hormonal crosstalk in *B. juncea*. Mazumder et al. (2013) also reported the similar expression of *PR1* gene and higher accumulation of salicylic acid (SA) after *A. brassicicola* inoculation in *B. juncea*. Therefore, it seems that *Alternaria* infection increases SA accumulation which acts as mobile signal that are transported to distal leaves from the local infected leaves to participate in SAR by activating defenses systemically (Hammond-Kosack and Jones 1996). SAR results in a heightened state of preparedness in the uninfected organs against subsequent infections. The induction of the SA and JA pathways is highly coordinated; with induction of one pathway occur at the expense of the other.

Wounding triggers the activation of many *PR* genes, thus understanding the expression pattern of *BjPR1* under wounding treatment in *B. juncea* will help us to uncover its detailed role under different stresses. In the present study, *BjPR1* was upregulated in local (wounded) leaves and reaches to maximum fold change at 4 h followed by decline at latter time points (Fig. 4c). Thus, *BjPR1* seems to be early wound inducible gene as there are reports of early and late wounding responsive genes in plants (Scranton et al. 2013). Moreover, the defense response and wounding have been reported to share a number of components in their signaling pathways which includes SA, JA and ET (Maleck and Dietrich 1999). It was interesting to note that wounding did not increase the systemic expression of *BjPR1* in distal (unwounded) leaves. This could be because of high accumulation of JA and ABA which might suppress the SA signaling pathways responsible for the establishment of SAR. Second, wound-induced expression of *PR1* seems to be SA or JA independent in *B. juncea* as we could not observe the induction of *BjPR1* in distal leaves (SAR) or after JA treatment. Therefore, further studies needs to be carried out to investigate the role of other hormones (especially ethylene) which might regulate *PR* genes during wounding.

Two important key players in plant defense response are the classical defense hormones SA and JA, both of which have been well described in model plant (Arabidopsis). However, the role of ABA is largely unknown. SA has received particular attention, because it activates many *PR* genes and SAR in plants (Yin and Hou 2007). Our results showed that SA leads rapid and strong induction of *BjPR1* gene in *B. juncea* and can be used as SA signaling marker gene in *B. juncea* (Fig. 5a). In contrast, transcript levels of *BjPR1* gene were not increased by JA in *B. juncea* at any time points which was upregulated in rice and tobacco plants (Xu et al. 1994; Agarwal 2000) (Fig. 5b). Interestingly, *A. thaliana* belonging to the same family as *B. juncea* (Crucifeciae) also displays similar results, as *ATPR1* has been shown to be induced only by SA but not by JA (Thomma et al. 1998; Durrant and Dong 2004). Hence, increased SA levels in plants lead to the onset of systemic acquired resistance (SAR), an inducible defense response against broad spectrum of pathogens, and also promote *PR* gene induction (Durrant and Dong, 2004). ABA is a positive regulator mostly involved in abiotic stress responses, but has been also known to play positive or negative roles in plant defense through cross-interaction with SA, JA, and ET signaling transduction pathway. Our studies showed that *BjPR1* was significantly downregulated by ABA (Fig. 5c), which was different with the previous studies (Hou et al. 2012; Gao et al. 2015). However, our results are consistent with the previous reports, indicating that ABA downregulates SAR marker gene (*PR1*) in *Arabidopsis*. These findings further provide the evidence that ABA plays negative role in *B. juncea* plant defense response by suppressing SAR marker gene (*PR1*). Collectively, ABA and JA showed antagonistic interaction with SA signaling pathway in *B. juncea*.

Despite the emergence of new techniques and years of study, one of the greatest challenges in the development of transgenic disease-resistant crop plants was the identification of inducible promoters which should replace exhaustive constitutive promoter (35S promoter). The use of constitutive promoters in plant genetic engineering is not always desirable, because constitutive overexpression of transgenes may compete for the building blocks that are required for plant growth under normal conditions. Therefore, stress or pathogen-inducible promoters are expected to be optimal for driving transgenes. In many crop species, variety of potential resistant genes for both biotic and abiotic stresses has been isolated, but using them in practical transgenic breeding has failed due to lack of stress-specific inducible promoters. The best stress-inducible promoter is the one induced by a wide array of stresses and must be inactive under stress-free conditions. In this regard, *BjPR1* promoter regulating the expression of *BjPR1* gene in *B. juncea* was isolated and

subjected to in silico and GUS analysis to know whether it is an inducible or constitutive promoter. Expression of pathogen-related genes usually occurs either by SA- or JA-dependent pathway which is conferred by the presence of single or multiple copies of salicylic acid-responsive elements (SARE-motifs) or jasmonic acid-responsive elements (JAR motifs), respectively. *Insilco* analysis showed that *BjPR1* promoter contains many SA-responsive *cis*-regulatory DNA elements such as TCA element (2 copies), GT1 motifs (3 copies), and W-box which might be responsible for the induction of *BjPR1* after SA treatments. TCA, a *cis*-acting regulatory element involved in salicylic acid responsiveness, is known to be present in the non-translated regions of many monocot and dicot plant genes which are induced by one or more forms of stress (Goldsbrough et al. 1993). Similar location of TCA element was also observed in the untranslated region of *BjPR1* promoter. On the other hand, GT1-elements are *cis*-acting regulatory elements required for rapid response to pathogen attack, salinity, and salicylic acid inducible gene expression. Our results have also identified the sequence element similar to GT1 motif in the promoter region of *BjPR1* gene which further reveals that *BjPR1* could be induced by SA. In addition, *BjPR1* promoter also contains 2 copies of W-box (T)TGAC (C/T), another important *cis*-acting DNA element have been found in the promoters of a number of SA-inducible genes and have been shown to be essential for the full expression of SA-responsive gene *SFR2*. A well-known pathogen-related motif TC-rich repeats was also found in *BjPR1* promoter which mediates biotic stress responses in plants. In addition to pathogen-responsive motifs, *PR1* promoter also contains abiotic stress-related motifs, viz., drought (MYB-motif GGATA; MYC-motif-CACATG), heat (HSF-motif AGA AAATTCG), salt (GT1-motif GAAAAA, DRE-motif A/GCCGAC), and also W-box which mediates abiotic stress responses in plants like freezing, wounding, oxidative stress, drought, salinity, cold, and heat by binding with various WRKY TFs (Fig S1). Collectively, these findings indicated that transcription of *BjPR1* promoter might be complex and regulated by a variety of biotic and abiotic stresses.

To further investigate the *BjPR1* promoter activity, a qualitative GUS activity assay was conducted by Agrobacterium-mediated transient assay in tobacco leaves (Fig. 6c–h). Agrobacterium-mediated transient expression has been widely used for both qualitative and quantitative analysis of plant promoter and also for *cis*-element/trans-factor interactions. Moreover, the use of this technique is growing at an accelerated rate being fast as well as has no environmental risks associated with the production of stable transgenic plants, and hence have opened up new strategies for transgenic studies (Omidvar et al. 2008). Since *PR* gene expression has been reported to be induced by various factors such as pathogen attack, SA, JA, and wounding treatments. In addition, *PR* genes are considered as molecular indicators of the activation of SA and JA signaling pathways, and can be termed as signatures of these pathways. In this study, *BjPR1* induction was monitored after treatment with defense stimulators (SA, JA, and ABA) as well as wounding. Transient assay revealed that *BjPR1* promoter driven GUS gene activity shows basal expression under control conditions but increases significantly after SA treatment. On the other hand, *BjPR1* driven GUS activity was decreased after ABA or JA treatments compared to control. In general, SA and JA predominantly have an antagonistic relationship (Pieterse et al. 2009), similar to that observed in our study. This further confirms that *BPR1* is predominantly SA-dependent and can be used as a molecular indicator of SA signaling in *B. juncea*. The presence of potential SA-responsive *cis*-elements in *BjPR1* promoter might be responsible for rapid and strong GUS gene expression in tobacco leaves after SA treatment. Mechanical wounding as a result of abiotic or biotic factors not only physically damages plant tissue, but it also provides entry for microbial and fungal pathogen invasion. Interestingly, *BjPR1* promoter was also induced by wounding. Therefore, wound-induced expression of pathogenesis-related gene (*BjPR1* and its promoter) further provides the evidence that they might play an important role in combating the detrimental effects of pathogen attack as well as wounding in *B. juncea*. Our results also identified many wound-responsive elements in the *BjPR1* promoter such as W-box, G-box, and ethylene-responsive element which might be responsible for wound-induced expression of *BjPR1* promoter.

Conclusion

In this study, we have isolated and functionally characterised SAR marker gene *PR1* after *Alternaria* infection, wounding, and defense hormonal treatments in *B. juncea*. Hence, from the *BjPR1* gene expression study, it is evident that *BjPR1* gene is predominantly SA-dependent. However, the induction of *BjPR1* gene by *A. brassicae* not by JA further provides the evidence of hormonal crosstalk in *B. juncea*. The present study also identified *BjPR1* promoter as stress-inducible promoter which can be successfully and effectively used for the development of transgenic crops for fungal resistance.

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AG has given the final shape of manuscript and all authors aproved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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