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Isolation and molecular characterization of pathogenesis related *PR2* gene and its promoter from *Brassica juncea*

S. ALI[1,2], N. CHANDRASHEKAR[3], S. RAWAT[1], N.M.C NAYANAKANTHA[1], Z.A. MIR[1], A. MANOHARAN[4], M. SULTANA[2], and A. GROVER[1]*

National Research Centre on Plant Biotechnology, Pusa Campus, New Delhi, 110012, India[1]
Department of Advanced Zoology and Biotechnology, Presidency College, Chennai, 600005, India[2]
Division of Microbiology, CCUBGA, Indian Agricultural Research Institute, New Delhi, 110012, India[3]
Department of Plant Biology and Biotechnology, Presidency College, Chennai, 600005, India[4]

Abstract

Pathogenesis-related (PR) proteins play key roles in plant disease resistance. Here, we isolated and characterized pathogenesis-related *PR2* gene encoding β-1,3-glucanase from *Brassica juncea* and named it *BjPR2* (GenBank accession number DQ359125). The amino acid sequence of BjPR2 showed ~99 % similarity with β-1,3-glucanase of *Brassica rapa*, *B. napus*, and *B. oleracea*. *BjPR2* transcription was rapidly increased after *Alternaria brassicae* infection, salicylic acid application, and wounding, but the induction was delayed in response to jasmonic acid. To investigate the transcriptional regulation of *BjPR2* gene, its promoter was isolated. *In silico* analysis of *BjPR2* promoter showed *cis*-regulatory elements upstream of TATA and CAAT boxes responsive to defense, hormones, wounding, and plant developmental stage. Homozygous *Arabidopsis thaliana* lines were developed with plasmid construct having β-glucuronidase (*GUS*) reporter gene driven by *BjPR2* promoter. The analysis of GUS protein in *Arabidopsis* lines showed that *BjPR2* promoter drived distinct pattern of pathogen inducible expression after fungal infection (*Alternaria brassicae*, *Erysiphe orontii*), phytohormones, and wounding. It also showed age dependent and organ specific expressions. *BjPR2* promoter drove strong *GUS* activity in *Arabidopsis* seedlings and showed organ specific expression at the later growth stages (lateral organ junctions, leaf serrate, base of siliques, and receptacle). Due to stress-inducible and tissue specific nature, the *BjPR2* promoter can serve as a potential candidate in genetic engineering.

Additional key words: *Alternaria brassicae, Arabidopsis thaliana*, β-glucuronidase, Indian mustard, jasmonic acid, phylogenetic tree, salicylic acid, transgenic plant.

Introduction

Indian mustard (*Brassica juncea* L. Czern and Coss) is one of the major oilseed crops cultivated in India (Vinu *et al.* 2013) and the main factors limiting yield are insects and fungal diseases. Among various fungal diseases, *Alternaria brassicae* infection leads up to 70 % yield loss all over the world with no proven source of transferable resistance against the disease in any of the hosts (Vishwanath and Kolte 1997). Conventional breeding approach to develop *Alternaria* resistant cultivars is confounded mainly due to non-availability of suitable resistant sources within the germplasm and therefore, genetic engineering has become imperative approach for imposing resistance to this dreadful disease (Yadava and Singh 1999). Different strategies have been used in genetic engineering of plants to improve fungal disease resistance, such as expressing pathogenesis-related (PR) proteins or antimicrobial peptides, modifying the resistance signalling pathway and even pyramiding the cloned resistance (*R*) genes (Grover and Gowthaman 2003).

The PR proteins play key roles in plant disease resistance. The β-1,3-glucanase, which belongs to the PR2 family, catalyzes the hydrolysis of β-1,3-glucans found in the cell walls of many genera of fungi and so exhibits antifungal activity (Shi *et al.* 2006). The PR2 proteins are present at low amounts in healthy plants but

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Abbreviations: Bj - *Brassica juncea*; ET - ethylene; GUS - β-glucuronidase; HR - hypersensitive response; JA - jasmonic acid; ORF - open reading frame; PR - pathogenesis-related; SA - salicylic acid; SAR - systemic acquired resistance.
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* Corresponding author; e-mail: anitagrover@hotmail.com

increase significantly after biotrophic as well as necrotrophic fungal infections thus implying their role in disease resistance (Cheong *et al.* 2000, Shi *et al.* 2006, Aggarwal *et al.* 2011). The overexpression of β-1,3-glucanase alone or in conjunction with chitinase conferred increased resistance against fungal pathogens (Shi *et al.* 2006, Mondal *et al.* 2007, Gupta *et al.* 2013). The expression of *PR2* genes is regulated by various phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Liu *et al*. 2010). In addition to resistance to pathogens, *PR2* genes also play important role in responses to abiotic stresses as well as in various physiological and developmental processes (Cheong *et al.* 2000, Akiyama *et al.* 2009). In crop biotechnology a transgene is commonly driven by constitutive promoters, such as cauliflower mosaic virus (*CaMV* 35S), or its derivatives. Using of these promoters leads to number of problems, such as homology-dependent gene silencing, altered plant development or morphology, and high expression of genes in the absence of pathogen invasion, resulting in unexpected disease symptoms (Zheng *et al.* 2007). To overcome those problems, tissue or organ-specific and pathogen inducible promoters can be used to drive transgene expression. *Cis*-regulatory acting elements present in pathogen-inducible promoters are classified based on their interaction with defense signalling molecules such as SA, JA, and ET or some pathogen signals (Mazarei *et al.* 2008). In this work, we report the isolation and molecular characterization of *BjPR2* gene and its promoter from *B. juncea*.

Materials and methods

Plants and growth conditions: Indian mustard (*Brassica juncea* (L.) Czern and Coss) cv. Varuna was grown in pots containing a mixture of soil and organic manure (2:1) in a growth chamber at a temperature of 22 - 24 °C, a 12-h photoperiod, irradiance of 100 - 125 μmol m^{-2} s^{-1}, and a relative humidity of 80 %. *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds were surface sterilized using 70 % (v/v) ethanol for 2 min and 5 % (v/v) NaOCl for 10 min, and then were washed 3 - 5 times with sterile distilled water. After stratification, sterile seeds were germinated on Murashige and Skoog medium (1962) at a temperature of 22 ºC, a 16-h photoperiod, and an irradiance of 100 - 125 μmol m^{-2} s^{-1}. After 8 d, the seedlings were transferred into pots filled with *Soilrite* and grown under the same conditions.

Construction of cDNA library and isolation of *BjPR2* clone: *B. juncea* plants (45-d-old) were sprayed with 2 mM SA. Leaves from control (sprayed with water) and SA-treated plants were harvested after 0, 2, 4, 8, 12, 24, 48, and 72 h and cDNA library was prepared from the tissue using *SMART* cDNA library construction kit (*Clontech,* CA, USA). First strand synthesis of cDNA, long-distance PCR for synthesis of full-length ds cDNA, *SfiI* digestion and size-fractionated ligation into the λTriplEx2 vector were conducted as described by Al-Taweel *et al*. (2011). The clones were sequenced (*ABI 3130 Genetic analyser, Applied Biosystems,* CA, USA) and full length sequence of *BjPR2* was submitted to GeneBank (accession No. DQ359125.1).

Sequence analysis of *BjPR2* gene: The nucleotide sequences were analyzed to assess their similarity with other sequences available in Genbank using *BLAST* program. A total of 19 homolog β-1,3-glucanase sequences were obtained from closely related plant species from NCBI to perform alignments. Phylogenetic relationships among β-1,3-glucanases were inferred using Maximum-Likelihood method implemented in *MEGA, v.7.0* (Kumar *et al.* 2016). Multiple alignment of β-1,3-glucanase proteins was done by *ClustalX* module of *BioEdit 7.2.3* package (Thompson *et al.* 1997). Conserved domain structure of BjPR2 protein were analyzed by *Pfam* database (http://pfam.xfam.org/).

Fungal infection of *B. juncea* plants: *Alternaria brassicae* strain was isolated from an infected leaf of *B. juncea* and cultured on radish dextrose agar (RDA) medium (Thakur and Kolte 1985) at a temperature of 22 °C in the dark. It was further confirmed as *A. brassicae* (I.D. No. 81651) by Indian Type Culture Collection. *B. juncea* (45-d-old) plants were inoculated by placing 4 - 6 drops of spore suspension (5×10^3 spores cm^{-3}) onto the upper surface of the first and second true leaves. The inoculated plants were maintained at 100 % relative humidity. Control *B. juncea* plants were mock inoculated with sterile distilled water and incubated separately to prevent cross contamination. The leaf samples were collected at 0, 2, 4, 8, 12, 24, 48, 72, and 96 h of post inoculation (hpi).

Phytohormone and wounding treatments in *B. juncea*: *B. juncea* plants (45-d-old) were sprayed with 2 mM SA or 100 μM JA and placed separately in dark chamber to prevent cross-talk signalling. Control plants were sprayed with sterile distilled water. Leaf samples for RNA isolation were harvested from control, SA, and JA treated plants after 0, 2, 4, 8, 12, 24, 48 and 72 h. *B. juncea* leaves were wounded with sterile needle at different places and samples were harvested after 0, 2, 4, 8, 12, 24, 48 and 72 h.

Expression analysis of *BjPR2* gene: The expression of *BjPR2* gene in response to *Alternaria* infection, SA, JA, and wounding treatments, were studied by real-time quantitative PCR using *BjPR2* gene specific primers. The first-strand cDNA was synthesized from 2 μg of *DNase*-treated total RNA by reverse transcriptase in 20 mm^3 of mixture containing oligo (dT) 18 primers, 10 mM deoxynucleotide (dNTPS), and water following the

manufacturer's instructions (*Fermentas*, Waltham, USA). The qPCR was done in a mixture containing 2 mm^3 of cDNA, 5 mm^3 of *SYBR Green Master Mix* (*Takara,* Tokyo, Japan) and 0.5 mm^3 of each primer (10 pmol). The reactions were performed in triplicates and the program was following: 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s. The *α-tubulin* gene (accession No. AK060893) was used as reference gene and the relative expression of *BjPR2* was quantified by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All primer sequences used in this study were synthesized using *Oligoanalyzer* software (Table 1 Suppl.).

Isolation and analysis of *BjPR2* promoter: The upstream region of *BjPR2* gene was isolated from the *B. juncea* genome by genome walking using universal genome walker kit (*Clontech,* CA, USA). *BjPR2* gene specific primers *GSP1*, *GSP2* (Table 1 Suppl.) and adaptor primers *AP1*, *AP2* (Siebert *et al.* 1995) were used to amplify the 1.8 kb *BjPR2* promoter. Upstream *cis*-elements in *BjPR2* promoter were analyzed by *PLACE* (Higo *et al.* 1999) and *PlantCARE* (Lescot *et al.* 2002) databases of plant *cis*- regulatory DNA elements.

Construction of binary vector and *Arabidopsis* transformation: To elucidate the function of *BjPR2* promoter, binary promoter less *GUS* vector pORER2 was used to clone the *BjPR2* promoter through blunt end cloning. To generate stable transformants, *A. thaliana* (Col-0) plants were transformed by *Agrobacterium tumefaciens* strain *GV3101* harbouring *BjPR2*-pORER2::*GUS* vector using floral dip method (Clough and Bent 1998). The T_2 homozygotic lines of transgenic *Arabidopsis* plants were used for *GUS* expression studies. The presence of the *GUS* reporter gene in *BjPR2* *Arabidopsis* transgenic plants was confirmed by PCR amplification, using *GUS*-specific primers.

Pathogen infection of *BjPR2* transgenic plants: To study the pathogen-inducibility of *BjPR2* promoter, leaves of one month old transgenic *Arabidopsis* plants carrying *BjPR2*promoter::*GUS* construct were inoculated with *A. brassicae* as mentioned above. Leaf samples were harvested at 48 h post inoculatiob (hpi) for histochemical *GUS* assays. For *E. orontii* infection, uninfected wild type *Arabidopsis* plants naturally acquired infection was used for inoculum preparation. The transgenic *Arabidopsis* plants (30-d-old) were infected by gently scraping the conidia from heavily infected leaves with a fine brush and maintained at 100 % humidity and 22 °C for disease development. Leaf samples were harvested at 5 dpi for *GUS* analysis.

Hormonal and wounding treatments of *BjPR2* transgenic plants: To examine the *GUS* gene expression, leaf samples of 30-d-old transgenic *Arabidopsis* plants were treated with 2 mM SA or 100 μM JA, and control with water. After 24 h, leaf samples were stained for *GUS* activity. Further, leaves of 30-d-old *BjPR2* transgenic *Arabidopsis* plants were effectively wounded with sterile needle and analysed after 24 h for *GUS* analysis. The *GUS* reporter gene expression in transgenic plants was performed by histochemical assay as described by Jefferson *et al.* (1987).

Statistics: For all experiments, three biological replicates were used and each repeated three times. A student's *t*-test was carried out to determine significant differences in gene expression in *A. brassicae*, SA, JA, and wound treated samples in comparison with respective controls.

Results

A full-length cDNA encoding β-1,3-glucanase protein, designated as PR2, was isolated from a cDNA library prepared from SA-treated *B. juncea* plants. *BjPR2* cDNA is 1 130 bp in length, including the 1 020 bp open reading frame (ORF), 17 bp of the 5' untranslated region (UTR) and 93 bp of the 3' UTR. The ORF encodes a protein of 339 amino acids, with molecular mass of 37,61 kDa and a pI 9.13. After *BLASTP* analysis, sequence similarity of deduced amino acid of *B. juncea* β-1,3-glucanase (ABC94638) revealed 99 % similarity with known β-1,3-glucanase of *B. rapa* (XP009104047) and *B. napus* (XP013707055), 67 % with that of *A. thaliana* (NP191283), 59 % with *Vitis vinifera* (NP001268153), and 58 % with *Glycine max* (XP003521159) and *Nicotiana tabacum* (XP016491598) (Fig. 1). A phylo-genetic tree based on the deduced sequence of *B. juncea* β-1,3-glucanase with other homologs reported from different plants, indicates that they may share a common ancestor and performed similar functions (Fig. 2).

Disease development in *B. juncea* after *A. brassicae* infection was analyzed. The necrotic lesions appeared as grey circular areas at the site of inoculation on the infected leaves after 48 h while no symptoms appeared on non-infected leaves (Fig. 3*A*,*B*). These results showed the susceptibility of *B. juncea* with respect to *Alternaria* infection and leaf samples were further used for *BjPR2* gene expression studies. A time course-study was carried out to study the expression profiling of *BjPR2* gene after *Alternaria* infection, using qPCR. The induction of *BjPR2* gene was increased as early as 2 h (59-fold induction) after fungal inoculation and reached a maximum at 24 h (149-fold induction) followed by a decline at 72 h (14-fold induction (Fig. 4*A*). Little or no expression was seen in uninfected or mock treated leaf samples. Our findings clearly demonstrate that the *BjPR2* gene was induced by *A. brassicae*, and induction seems to be time dependent.

Upon SA treatment, the increase in *BjPR2*

transcription was evident already 2 h after SA treatment, reached the peak at 24 h (110-fold induction) but declined at 48 and 72 h (Fig. 4*B*). In JA treated *B. juncea*, *BjPR2* expression increased after 24 h (22 fold induction) and

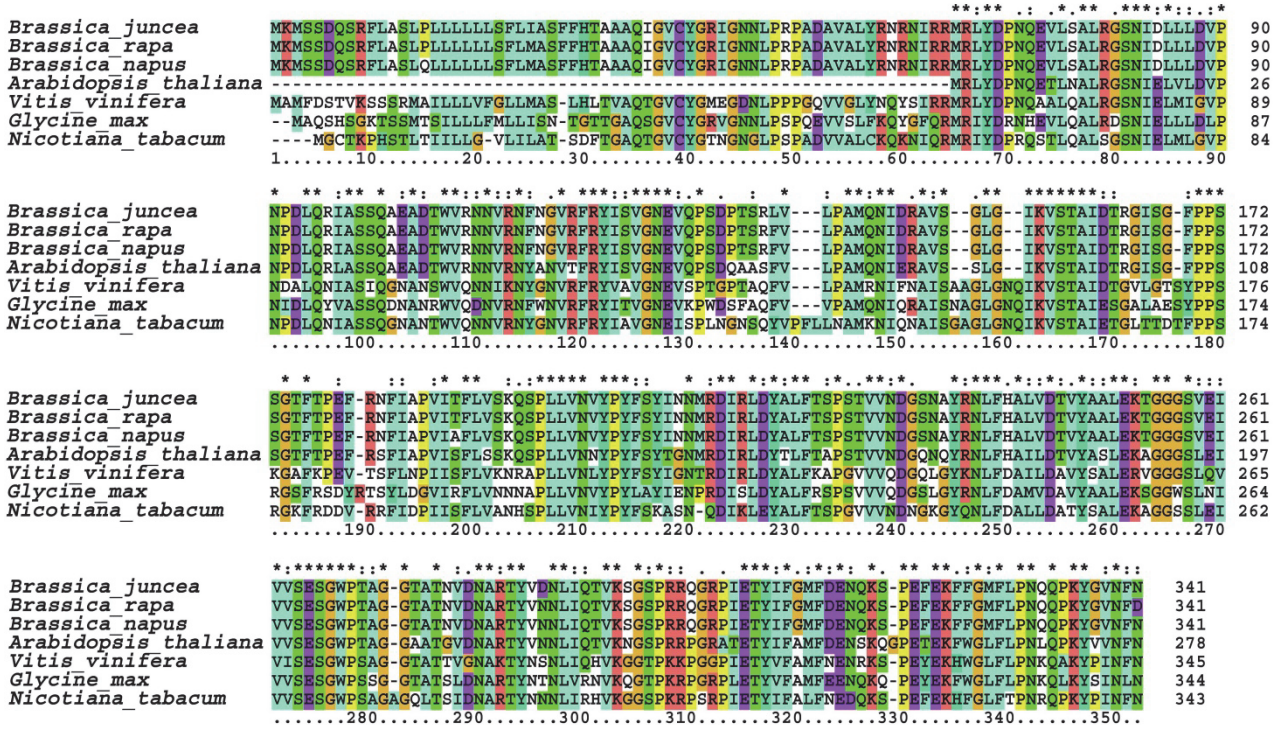

Fig. 1. Alignments of the deduced amino acid sequences of the BjPR2 with other plant PR2 proteins from *B. juncea* β-1,3-glucanase (ABC94638) with known β-1,3-glucanase of *B. rapa* (XP009104047), *B. napus* (XP013707055), *A. thaliana* (NP191283), *Vitis vinifera* (NP001268153), *Glycine max* (XP003521159), and *Nicotiana tabacum* (XP016491598) using *ClustalX* program.

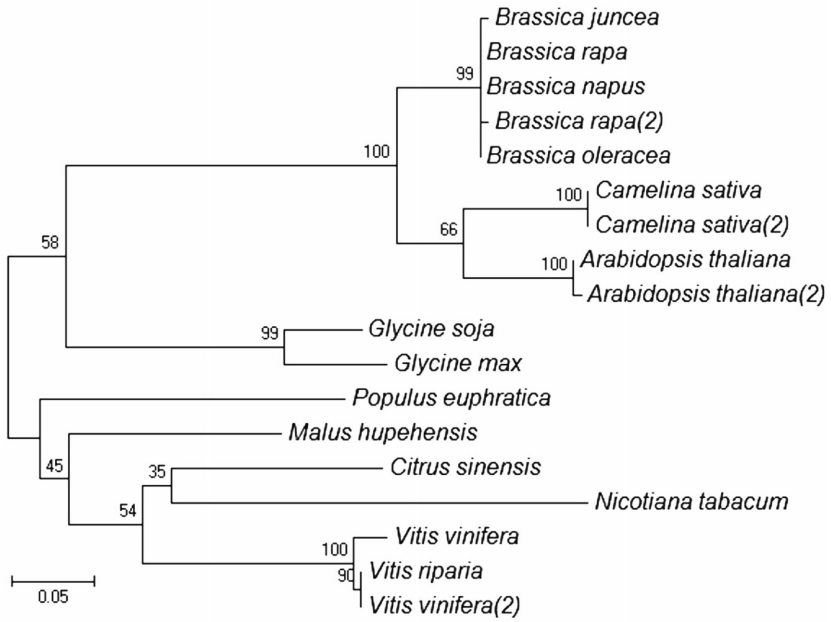

Fig. 2. Phylogenetic relationships of BjPR2 (ABC94638) with other PR2 proteins from, *B. rapa* (XP009104047), *B. napus* (XP013707055), *B. rapa* 2(AFN85666), *B. oleracea* (XP013588400), *Camelina sativa* (XP010427593), *C. sativa* 2 (XP010427594), *Arabidopsis thaliana* (CAB68130), *A. thaliana* 2 (AAA32756), *Glycine soja* (KHN14941), *G. max* (XP003521159), *Populus euphratica* (XP011039449), *Malus hupehensis* (ADR71671), *Citrus sinensis* (XP006482037), *Nicotiana tabacum* (XP016491598), *Vitis vinifera* (NP001268153), *V. riparia* (ACD45060), and *V. vinifera* 2 (ACD45061) constructed using the program *MEGA 7.1*.

reached maximum after 48 h (47 fold induction) (Fig. 4*C*). In *B. juncea,* wounding increased the expression of *BjPR2* after 2 h (57-fold induction), declined after 4, 8, and 12 h, but significantly increased at 24 and 48 h (47-fold). The highest wound induction of *BjPR2* was observed only at 2 h (Fig. 4*D*).

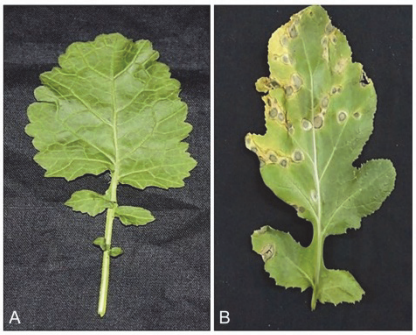

Fig. 3. Disease development in *B. juncea* after *A. brassicae* infection. Inoculation was done by placing 0.01 cm^3 of 5×10^3 spores cm^{-3} on different selected spots of the leaf surface. Control plants were inoculated with 10 cm^3 of sterile distilled water. *A* - No symptoms appeared on control leaves. *B* - After 24 h of inoculation, tissue surrounding the inoculation spore droplet appeared yellowish and showed the necrotic lesions.

Gene expression observations suggest that the promoter of *BjPR2* gene might contain specific *cis*-acting elements for defense and wound responses. The *BjPR2* promoter (1.8 kb) was isolated from the *B. juncea* genomic DNA by PCR walking. The promoter of *BjPR2* was sequenced and deposited in Gene Bank *NCBI* with accession No. KC865599. *In silico* analysis of *BjPR2* promoter revealed important *cis*-acting regulatory DNA elements related to defense, wounding, developmental and tissue specific and signalling pathways are shown in Fig. 5*A* and Table 2 Suppl. *BjPR2* promoter was cloned into pORE-R2 promoter less *GUS* reporter vector by blunt end cloning (Fig. 5*B*). Stable transgenic *Arabidopsis* plants were developed, and T-DNA integration in eight transgenic lines was confirmed by PCR amplification, using *GUS*-specific primers. No amplification was found in untransformed plants (Fig. 5*C*).

To examine the pathogen inducbility of *BjPR2* promoter, we performed infections with both necrotrophic (*A. brassicae*) and biotrophic (*E. orontii*) fungal pathogens in *BjPR2* transgenic *Arabidopsis* plants. These pathogens were identified based on their spore morphology (Fig. 6*A,B*). *Alternaria* infection in transgenic *Arabidopsis* plants significantly increased transcription of *GUS* gene in infected leaves when compared to uninfected leaves (Fig. 7*A,B*). Results showed that *GUS* accumulation was predominantly present in the petiole, veins, and midribs of *Alternaria* infected leaves with no preferential expression around the tissue zone showing the characteristic hypersensitivity response (HR). The GUS activity significantly increased as the distance from the site of *Alternaria* infection increased. On the other hand, transgenic plants infected with *E. orontii* also showed a strong *GUS* gene expression driven by *BjPR2* promoter as compared to non infected plants (Fig. 7*C,D*). The accumulation of *GUS* gene was relatively higher at the site of infection and was not found in petiole or veins. The results obtained here provide further evidence that *BjPR2* promoter was not only induced by necrotrophic pathogen but also by biotrophic pathogen. However, the *GUS* activity was relatively higher in *E. orontii* infected leaves than in *Alternaria* infected transgenic leaves.

The treatment with SA strongly induced *GUS* activity in leaves of *BjPR2* transgenic plants and no *GUS* activity was observed in untreated leaf sample under normal conditions (Fig. 8*A,B*). Upon JA treatment, moderate *GUS* activity was observed in the leaf sample of transgenic plants (Fig. 8*C*). These findings revealed that *BjPR2* promoter was not only induced by SA but also by JA, however the transcription of *GUS* gene in SA treated

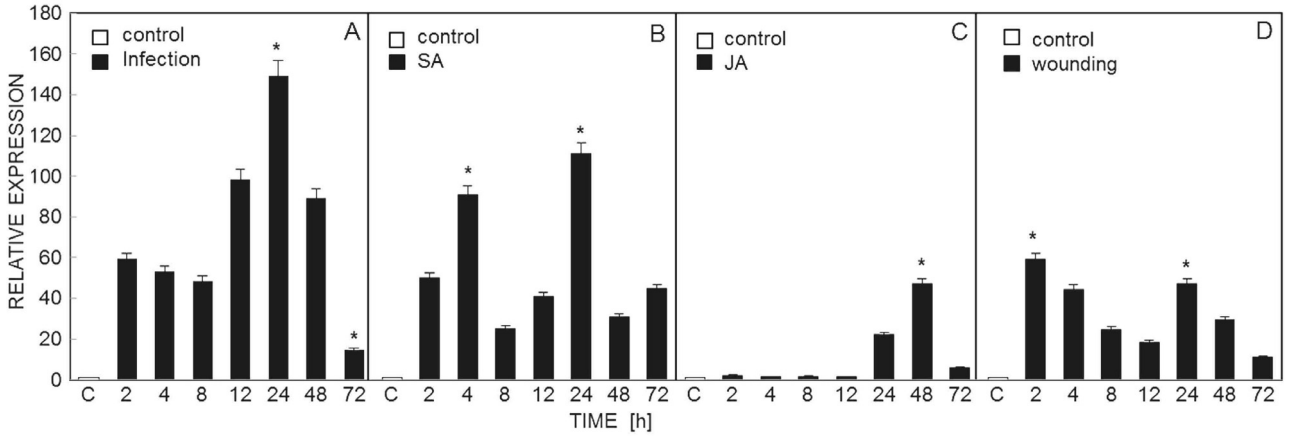

Fig. 4. Expression profiling of *BjPR2* gene by RT-qPCR. *A* - the expression of *BjPR2* gene after *A. brassicae* post inoculation; *B* - the expression of *BjPR2* gene after SA treatment; *C* - effect of JA on *BjPR2* gene expression; *D* - wound induced expression of *BjPR2* gene. The α-tubulin gene was used as an internal control. Means ± SEs of three replicates. A significant difference ($P < 0.05$) between control and treated samples is denoted by an *asterisk*s.

leaf samples was much higher than in JA treated leaves. To investigate whether *BjPR2* is responsive to wounding, leaf samples of transgenic *Arabidopsis* were effectively wounded and analyzed for *GUS* expression after 24 h. Wounding increased transcript abundance of *GUS* gene in transgenic leaf sample, however, there was no GUS activity observed in unwounded (control) plants (Fig. 8*D*). In addition, *GUS* activity was found to be relatively higher near the wounded sites in *BjPR2* transgenic leaves.

To examine the developmental regulation of *BjPR2* promoter in different tissues at different stages of plant development, the histochemical detection of *GUS* activity was done in homozygous T_2 transgenic plants, at different development stages. *BjPR2* promoter drived strong *GUS* gene expression in roots and young leaves of 10-d-old *Arabidopsis* seedlings (Fig. 9*A*), and later decreased in mature tissues of fully expanded leaves (3-week-old) (Fig. 9*B*). *GUS* analysis showed, that *BjPR2* promoter was expressed in various organs including leaf serrations (Fig. 9*C*), base of siliques (Fig. 9*D*), and base of flower receptacle (Fig. 9*E*). The intensity of *GUS* staining was clearly affected by the age of the plant. *In silico* analysis of *BjPR2* promoter revealed various developmentally regulated and tissue specific *cis*-regulatory elements that might be involved in organ or tissue specific expression.

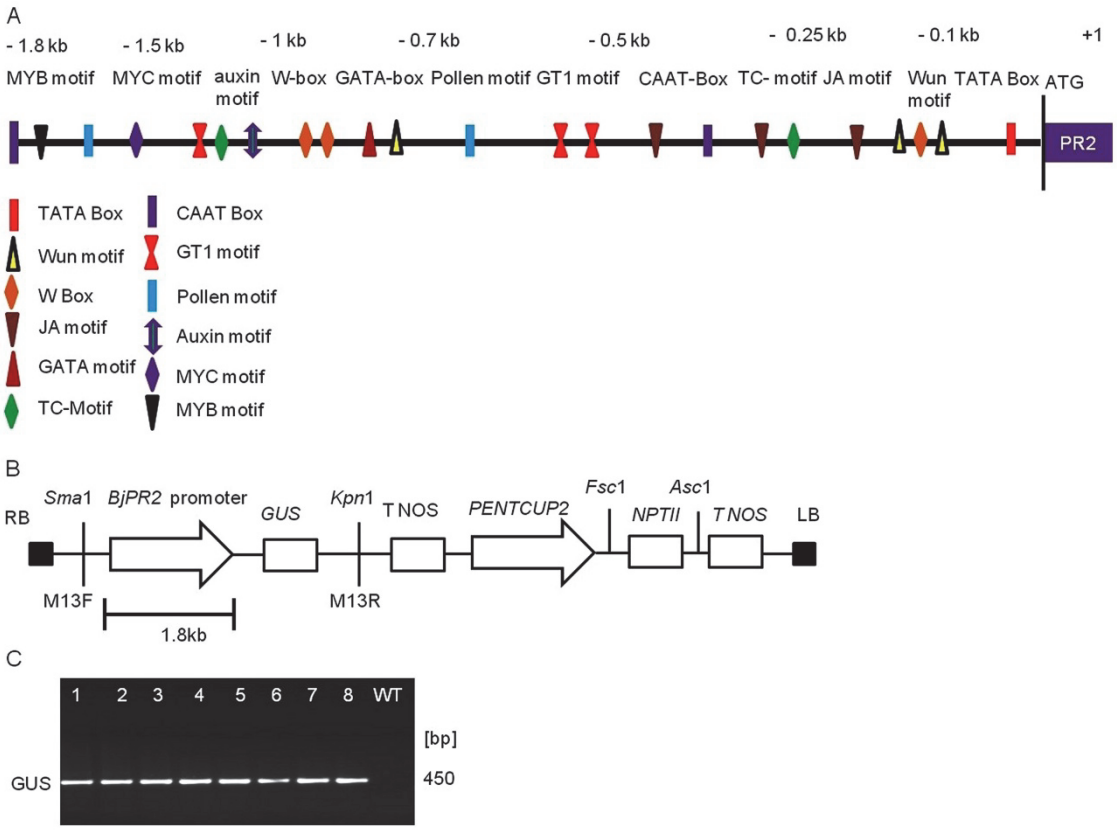

Fig. 5. *A* - *BjPR2* promoter *cis*- elements are shown in different shapes and colors along with their respective positions from the start codon ATG on the both sides of the horizontal solid bar, which represents the -1800 bp. Putative *cis*-elements identified in *BjPR2* promoter are also described in Table 2 Suppl. *B* - Schematic representation of *BjPR2*-pORER2::*GUS* construct and T-DNA map of pORE-R2 binary vector. *C* - T-DNA integration in 8 transgenic lines was confirmed by PCR amplification, using *GUS* gene specific primers.

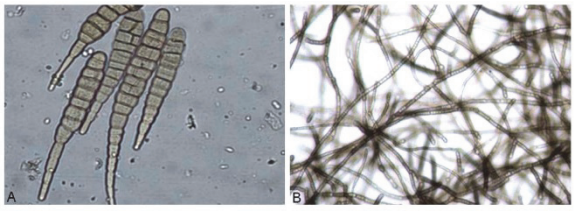

Fig. 6. Identification of *A. brassicae* and *E. orontii* pathogens. *A* - spores of *A. brassicae*. *B* - spores and hyphae of *E. orontii* seen under (40× magnification) bright field microscope.

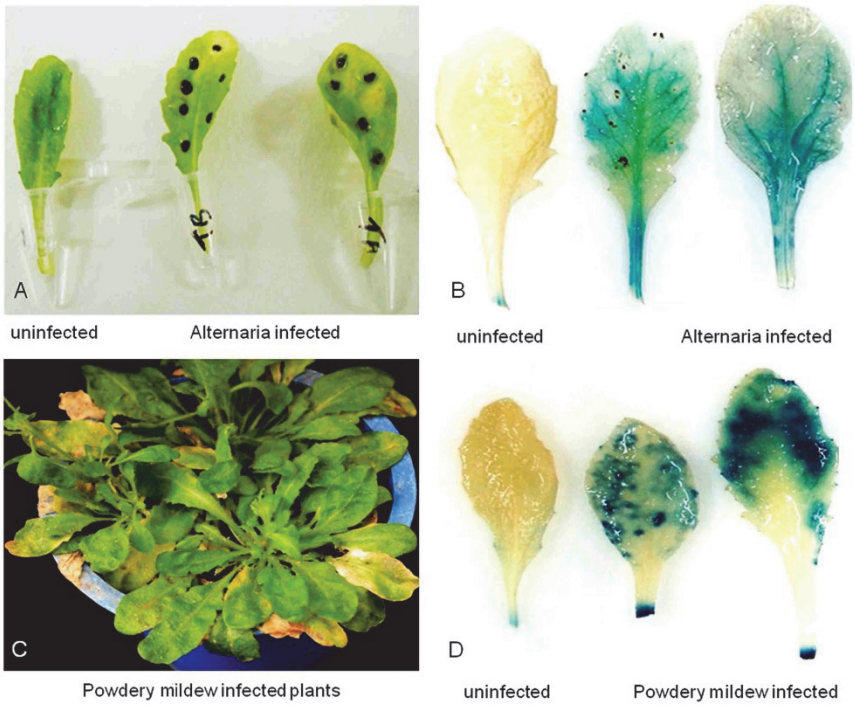

Fig. 7. Pathogen inducible expression of *BjPR2* promoter in response to necrotrophic and biotrophic fungal infection. *A* - Non-infected (control) and *BjPR2* T_2 *Arabidopsis* leaves after 72 h of *Alternaria* infection. *B* - Uninfected (mock) *BjPR2* transgenic leaves show no *GUS* expression but it increased significantly after *A. brassicae* infection. *C* - 5-d-old *E. orontii* infected *BjPR2* T_2 plants. *D* - Histochemical *GUS* analysis of uninfected and *E. orontii* infected *BjPR2* transgenic *Arabidopsis* leaves.

Discussion

Model plants such as *Arabidopsis* have been used to study various pathogen interactions but very little information is available with respect to *Alternaria* infection and *Brassica* species. We studied the disease development in *B. juncea* after *A. brassicae* infection in detail. The necrotic lesions appeared 2 d after inoculation and older leaves were more susceptible than young leaves. These results further provide evidence that *B. juncea* is highly susceptible to *Alternaria* infection that causes significant yield losses. Till to date, a limited number of disease resistance genes have been characterized in *B. juncea*. Therefore, uncovering the role of *BjPR2* in *B. juncea* could provide novel insights into the *Brassica* - *Alternaria* interaction. The β-1,3-glucanases are well known enzymes because they not only hydrolyze the glucan, but also release oligosaccharides from fungal cell walls which elicit the host defence response and systemic acquired resistance (SAR; Shi *et al.* 2006). Previous reports have noted an increase in the expression of various *β-1,3-glucanase* genes in plants during infection (Shi *et al*. 2006, Liu *et al*. 2010). In this study, *A. brassicae* inoculation induced *BjPR2* gene at an early stage and reached maximum at 24 hpi. Increased *BjPR2* transcription at 12 and 24 h could be connected with maximum spore germination of *A. brassicae* at 24 hpi as has been reported in *Arabidopsis* - *A. brassicola* interaction, where 80 % germination of fungal spores

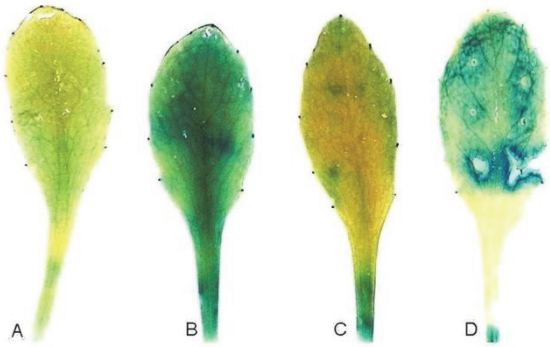

Fig. 8. Inducible expression of *GUS* driven by *BjPR2* promoter. Analysis of T_2 plants after 24 h of treatment with (2 mM SA and 100 µM JA) and wounding. *A* - Untreated or mock treated leaf used as control; *B* - SA increases *GUS* activity; *C* - effect of JA treatment; *D* - wounding induced *GUS* activity in transgenic leaves.

occurred after 12 h and 100 % of germination occurred at 24 hpi (Otani *et al.* 1998). This is also supported by the results observed in strawberry (Shi *et al.* 2006), and *Eruca sativa* (Gupta *et al.* 2013). To further understand the transcriptional regulation of *BjPR2* gene, its promoter was isolated and functionally validated in *A. thaliana*. The *Arabidopsis* - *A. brassicae* pathosystem model shows compatible interaction initially as the leaf blight symptoms appeared on the infected leaves. After 4 d of

incubation the symptoms did not spread on the infected leaves thus providing evidence that *Arabidopsis* displayed incompatible interaction with *A. brassicae*. Our studies revealed that *GUS* activity driven by *BjPR2* promoter was restricted to the veins, midribs, and petiole of the inoculated plant. In addition, *GUS* gene expression in the infected leaves took place far away from the necrotic lesions that originated from the hypersensitive response (HR) during the incompatible interaction. Similar findings are also observed for two PR genes during incompatible pathogen interactions in *Arabidopsis* (Jorda and Vera 2000). On the other hand, a study of transgenic plants infected with *E. orontii* revealed that *BjPR2* drives strong *GUS* expression mostly at the infection sites on leaf lamina and not in the veins or midribs. Previous reports have shown that powdery mildew infection increases the accumulation of *PR2* and other *PR* genes in *A. thaliana* (Reuber *et al.* 1998) and *Vitis vinifera (*Fung *et al.* 2008). *BjPR2* promoter showed increased transcription not only after biotrophic infection but also after necrotrophic infection. Previous reports have also shown the induction of β-1,3-glucanases as a defence against both biotrophic and necrotrophic fungal pathogens in many plants as reviewed by Zemanek *et al.* (2002), Liu *et al.* (2010), and Zamora *et al.* (2012). GUS accumulation was relatively higher in powdery mildew infected leaves than *A. brassicae* leaves possibly because *PR2* has been known to be induced by biotrophic pathogen through SA signalling pathway (Thomma *et al.* 1998). Interestingly, GUS activity was observed in petiole of the *Alternaria* infected leaf and was not seen in the leaves after powdery mildew infection or wounding. Many potential pathogen responsive elements are found in pathogen inducible promoters such as GCC Box (AGCCGCC) (Ohme-Takagi and Shinshi 1995), W Box (TTGACC) (Eulgem *et al.* 2000), P Box, L Box, G box, H Box, and SARE (Rushton and Somssich 1998). *In silico* analysis of the *BjPR2* promoter revealed two copies of TC-rich repeats (ATTTTC), 3 copies of GT1GMSCAM4 motif (GAAAAA) and 3 copies of W Box [(T) TGAC (C/T)] *cis*-regulatory elements required for defense response.

Phytohormones SA and JA are important players in

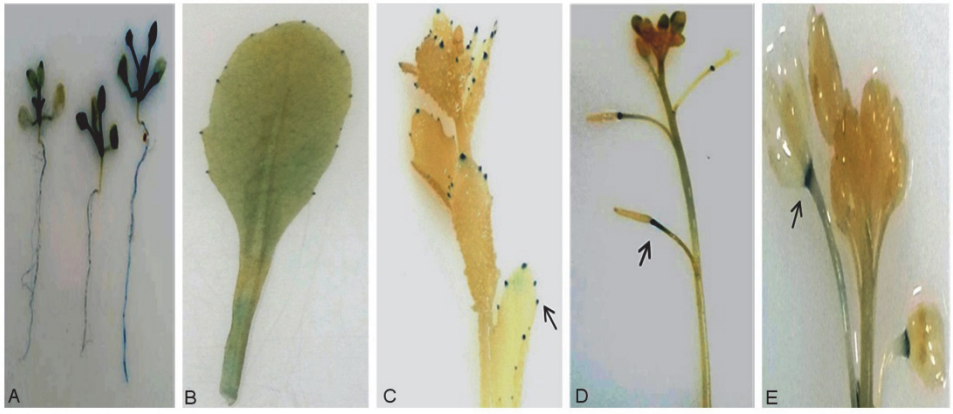

Fig. 9. Histochemical detection of GUS activity in T_2 transgenic plants, carrying *BjPR2* promoter-GUS construct at different development stages. *A* - GUS activity observed in 14-d-old T_2 seedlings grown in MS medium; *B* - in mature leaf; *C* - in leaf serrate; *D* - in base of silique; and *E* - receptacle parts of flowers.

pathogen signalling pathways that regulate the expression of pathogenesis-related proteins (Balbi and Devoto 2008). As previously stated, in *A. thaliana* SA induces expression of *PR1*, *PR2*, and *PR5* gene and MeJA induces several SA independent PR proteins such as PR3 and PR4 (Thomma *et al.* 1998, Seo *et al.* 2008). In *B. juncea* there is limited information available on the role of SA and JA on *PR* gene expression or in plant defence. Therefore, the present study explored the roles of SA and JA on the expression of *BjPR2* gene and promoter in *B. juncea.* SA treatment induced the expression of *BjPR2* gene distinctly at various time points. The distinct expression of *BjPR2* gene after SA treatment could be dependent on circadian rhythms. A previous study have revealed that tomato plants are more susceptible in the evening then during night to infection with *Pseudomonas syringae.* This diurnal effect is accompanied with increased SA accumulation and defence-related gene transcription (Yang *et al*. 2015). In this study, we observed the similar diurnal changes of *PR2* gene in *B. juncea* after SA treatment. Pathogen-inducible genes have been identified to have diurnal and/or circadian rhythms of expression (Wang *et al.* 2011). Previous reports have also shown the SA induces expression of *PR2* in barley and strawberry respectively (Li *et al.* 2005, Zamora *et al.* 2012). Histochemical GUS analysis also revealed that *BjPR2* promoter was induced after SA treatment. This inducible promoter shows the presence of SA responsive motif GT1 box which was reported to function as a transcriptional enhancer conferring SA inducibility to reporter genes in transgenic plants (Sa *et al.* 2003). SA is a key regulatory molecule that accumulates following the pathogen recognition, participates in SAR activation and induction of many *PR* genes (Yin and Hou 2007). Interestingly, in *B. juncea* transcriptional abundance of *BjPR2* gene and its promoter

was also significantly induced by methyljasmonate which is in seeming contrast to previous reports (Thomma *et al.* 1998) but goes in line with observation in other plants which show the expression of *PR2* after JA treatment (Jayaraj *et al.* 2004, Akiyama *et al.* 2009). The presence of JA responsive *cis*-elements (TGACG) further confirms the statement that this gene might play a role in SA independent plant defence signalling. Our results also suggest that there is a SA/JA crosstalk that regulates *BjPR2* gene in *B. juncea*. However, the two hormones induce *BjPR2* at different times: up-regulation of *BjPR2* gene was observed as early as 2 h after SA treatment whereas JA induced transcript accumulation only after 24 h of treatment and may not be involved in early defence. Previous studies have shown that MeJA signal transduction pathways play important role in resistance to *A. brassicola* in *Arabidopsis* (Thomma *et al.* 1998). We suggest that the late induction of *BjPR2* gene and its promoter by MeJA could lead to susceptibility to *A. brassicae* in *B. juncea.*

Several reports indicate that wounding regulates number of genes that play important roles in plant defence (Durrant *et al.* 2000). Interestingly, we observed early and late wound responses of *BjPR2* gene but maximum transcript abundance only at early stages. Previous reports have shown that the transcriptions of *β-1,3-glucanases* increase after wounding (Akiyama *et al.* 2009). As expected *BjPR2* promoter activity increased by mechanical wounding consistent with previous reports (Cheong *et al.* 2000). Defence response and wounding also share a number of components in their signalling pathways, which include SA, JA, and ethylen (Maleck and Dietrich 1999). It is well known that JA is a key component of wound signalling that is also essential for many pathogen responses in plants (Thomma *et al.* 1998, Rojo *et al.* 1999). Our earlier results showed that JA do not induce β-1,3-glucanase at early stages but after 24 h. Therefore, our results suggest that in *B. juncea* SA may play important role in wound signalling at early stages. The presence of wound responsive elements (TGACT) further confirms the fact that this gene might play a role in wound signalling in *B. juncea.*

The developmentally regulated and organ-specific expressions of *β-1,3-glucanase* genes have been extensively studied in many plants (Ko *et al.* 2003, Wan *et al.* 2011). To obtain better understanding age dependent and organ specific expressions of *BjPR2* promoter, transcript accumulation of *GUS* reporter gene driven by *BjPR2* promoter was analyzed in transgenic plants at various growth stages. It was observed that *GUS* gene driven by *BjPR2* promoter was highly expressed in *Arabidopsis* seedlings in contrast to previous reports (Seo *et al.* 2008) but was similar to findings observed in *Prunus persica* (Ko *et al.* 2003). However, the accumulation of *GUS* gene was relatively low in the leaves of one month old transgenic plants, indicating that *BjPR2* shows spatial and temporal regulation as previously reported (Cheong *et al.* 2000). Organ- specific GUS activity was also observed at the base of siliques, flowers, leaf serrations, and lateral organ junctions. During the reproductive phase, all floral tissues undergo rapid cell expansion, thus *BjPR2* is possibly involved in cell wall loosening to facilitate cell elongation. These results also imply that *BjPR2* gene may play a defensive role against various fungal pathogens attacking sensitive reproductive and other parts of *B. juncea*. The 5'upstream region of *BjPR2* promoter contains anther and pollen specific regulatory elements, including 4 copies of GTGA, 12 copies of AGAAA, and 3 copies of ACTTTA motifs required for pollen specific expression (Rogers *et al.* 2001, Filichkin *et al.* 2004). Thus, our result suggests that presence of these elements in the upstream region of *BjPR2* gene might govern the tissue or organ specific expression in *B. juncea.*

In conclusion, we characterized a *PR2* gene and its promoter from *B. juncea* after fungal infection, hormonal treatment, and wounding. The *BjPR2* gene was induced by both SA and JA suggesting that there was a SA/JA cross talk in *B. juncea*. The *BjPR2* promoter is stress inducible promoter and drived strong *GUS* gene expression in response to necrotrophic and biotrophic fungal pathogens as well as wounding. The *BjPR2* promoter also showed developmental and tissue specific expressions in *B. juncea.* This inducible promoter can be used to develop fungus resistant transgenic plants in order to avoid gene silencing that often occurs due to constitutive promoters. Further deletion analysis will be carried out to identify and functionally characterize pathogen regulatory *cis*-elements in *BjPR2* promoter.

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