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

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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 drove 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 sarrate, 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...
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 °C, a 16-h photoperiod, 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 brassicaceae 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. brassicaceae (I.D. No. 81651) by Indian Type Culture Collection. B. juncea (45-d-old) plants were inoculated with 4 - 6 drops of spore suspension (5 × 10⁷ spores cm⁻²) 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 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$^3$C DNA, 5 mm$^3$C SYBR Green Master Mix (Takara, Tokyo, Japan) and 0.5 mm$^3$C 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 $\alpha$-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

**Results**

A full-length cDNA encoding $\beta$-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 $\beta$-1,3-glucanase (ABCG96438) revealed 99 % similarity with known $\beta$-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 phylogenetic tree based on the deduced sequence of B. juncea $\beta$-1,3-glucanase with other homologs reported from different plants, indicates that they may share a common ancestor and performed similar functions (Fig. 2).

**Pathogen infection of BjPR2 transgenic plants:** To study the pathogen-inducibility of BjPR2 promoter, leaves of one month old transgenic Arabidopsis plants carrying BjPR2promoter::GUS construct were inoculated with A. brassicaceae as mentioned above. Leaf samples were harvested at 48 h post inoculation (hpi) for histochemical GUS assays. For E. orontii infection, uninjected 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. brassicaceae, SA, JA, and wound treated samples in comparison with respective controls.

Disease development in B. juncea after A. brassicaceae 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. 3A,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. 4A)). Little or no expression was seen in uninjected or mock treated leaf samples. Our findings clearly demonstrate that the BjPR2 gene was induced by A. brassicaceae, 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. 4B). 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 (CAC68130), A. thaliana 2 (AA22756), Glycine soja (KHN14941), G. max (XP003521159), Populus euphratica (XP001039449), Malus hupehensis (ADR71671), Citrus sinensis (XP006482033), 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. 4C). 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. 4D).

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. 5A and Table 2 Suppl. BjPR2 promoter was cloned into pORE-R2 promoter less GUS reporter vector by blunt end cloning (Fig. 5B). 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. 5C).

To examine the pathogen inducibility 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. 6A,B). Alternaria infection in transgenic Arabidopsis plants significantly increased transcription of GUS gene in infected leaves when compared to uninfected leaves (Fig. 7A,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. 7C,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. 8A,B). Upon JA treatment, moderate GUS activity was observed in the leaf sample of transgenic plants (Fig. 8C). 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
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. 8D). 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 T3 transgenic plants, at different development stages. BjPR2 promoter drove strong GUS gene expression in roots and young leaves of 10-d-old Arabidopsis seedlings (Fig. 9A), and later decreased in mature tissues of fully expanded leaves (3-week-old) (Fig. 9B). GUS analysis showed, that BjPR2 promoter was expressed in various organs including leaf serrations (Fig. 9C), base of siliques (Fig. 9D), and base of flower receptacle (Fig. 9E). 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. brassicaceae and E. orontii pathogens. A - spores of A. brassicaceae. B - spores and hyphae of E. orontii seen under (40× magnification) bright field microscope.
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. brassicaceae* 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. brassicaceae* at 24 hpi as has been reported in *Arabidopsis - A. brassicola* interaction, where 80 % germination of fungal spores 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. brassicaceae* 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. brassicaceae. 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 transcriptional abundance 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. brassicaceae 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 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 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

Fig. 9. Histochemical detection of GUS activity in T2 transgenic plants, carrying BjPR2 promoter-GUS construct at different development stages. A - GUS activity observed in 14-d-old T2 seedlings grown in MS medium; B - in mature leaf; C - in leaf serrate; D - in base of silique; and E - receptacle parts of flowers.

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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. brassicaceae 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 ethylene (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 G TGA, 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 drove 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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