Expression pattern of the synthetic pathogen-inducible promoter (SynP-FF) in the transgenic canola in response to *Sclerotinia sclerotiorum*

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Abstract
Sclerotinia sclerotiorum is a phytopathogenic fungus which causes serious yield losses in canola. A pathogen inducible-promoter can facilitate the production of *Sclerotinia*-resistant transgenic canola plants. In this study, the "gain of function approach" was adopted for the construction of a pathogen-inducible promoter. The synthetic promoter technique was used, which involved the insertion of the dimerized form of the *cis*-acting element (F) upstream of the minimal CaMV 35S promoter, which drives the expression of the β-glucronidase (GUS) gene. The pGFF construct containing this synthetic promoter (SynP-FF) was used for stable transformation of the canola plant. Fluorometric GUS expression analysis indicated that the SynP-FF promoter is responsive to methyl jasmonate and *S. sclerotiorum* treatments. The results of histochemical GUS expression patterns showed strong reporter expression in leaf, flower and stem tissues of canola. Hence, the SynP-FF synthetic promoter, carrying fungal pathogen-inducible features, could be considered as a valuable tool for controlling the expression of transgenes to improve resistance against the same lifestyle pathogens.
Keywords**:** *cis*-acting element; Synthetic promoter; Pathogen-inducible; *Brassica napus*; Fungal elicitor; Reporter gene; *Sclerotinia sclerotiorum*

INTRODUCTION

Canola (*Brassica napus* L.) is one of the most important source of edible vegetable oil, industrial oil and protein-rich products in the world. Like many other crops, the production of this crop is challenged by phytopathogenic fungi. *Sclerotinia sclerotiorum* is distributed worldwide and is pathogenic to oilseed crops (Hemmati *et al*., 2009). Infection of oilseed plants can occur any time after emergence of seedlings. This fungus is a causal agent of stem rot disease that causes serious yield losses in oilseed crops including canola (Hind *et al*., 2003; Lu 2003). It is thus desirable to develop fungal-resistant plants through the introduction of foreign fungal-resistant genes into commercially important crops (Gurr and Rushton 2005a). Many early attempts to boost disease resistance have used constitutive promoters for the over expression of transgenes, but this has frequently resulted in poor quality plants due to reduced growth and altered development (Hammond-Kosack and Parker, 2003). Synthetic promoters provide an efficient and flexible strategy to regulate transgene expression in a desired spatial and temporal manner at the site and time of plant-pathogen interaction and reduce the complexity of the expression pattern of natural promoters (Venter 2007; Gurr and Rushton 2005b; Rushton *et al*., 2002). Recent reports show that individual *cis*-acting elements fused with a minimal promoter can locally direct reporter gene expression in response to pathogens (Cazzonelli and Velten 2008; Mazarei *et al*., 2008; Rushton *et al*., 2002). The β-glucronidase (GUS) gene as a reporter is widely used for monitoring gene expression in plant tissues, which can be assayed qualitatively and quantitatively (Huttly 2009; Cervera 2004).

Among the defined pathogen-inducible *cis*-acting elements, only a few are not induced by wounding and mechanical damages (Gurr and Rushton 2005b). One

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of the *cis*-acting elements (F), has been shown to respond strongly to fungal infections (Heise *et al*., 2002). This element contains two functionally important W box motifs with different relative positions and an additional 9 bp motif that is functionally important (Heise *et al*., 2002). Having certain characteristic features such as fast response, low background expression, local expression and non-responsiveness to wounding and mechanical damages make it a suitable candidate for use in the construction of synthetic pathogen-inducible promoters. Based on the conservative nature of plant defense signaling (Singh *et al*., 2002), this element is expected to show similar reactions in other species, but to clarify its function in heterologous systems such as canola plants, it needs to be analyzed individually in the target biological system.

Plants defend themselves against pathogens through different signaling pathways (Zipfel 2009; De Wit 2007; Gurr and Rushton 2005b). The defense pathways were appointed by plants are very discriminative to the pathogen lifestyles (Gurr and Rushton 2005b). Salicylic acid signaling pathways are usually involved in response to biotrophic pathogens (Lu 2009), and it has been shown that jasmonic acid and ethylene-related signaling pathways control defense signaling against necrotrophic pathogens (Balbi and Devoto 2008).

In this study, we have applied the synthetic promoter approach to evaluate the function of the F element in response to *S. sclerotiorum*. Furthermore, the reaction of this element in response to salicylic acid and methyl jasmonate is investigated.

MATERIALS AND METHODS

Plant material, fungal and bacterial strains: *Brassica napus* L. (R line Hyola 308) used as a receptor, was kindly provided by the Oilseed and Development Co. Tehran, Iran *S. sclerotiorum* (2310), the causal agent of canola stem rot, was kindly provided by Dr. H. Afshari-Azad (Plant Pests and Diseases Research Institute, Agricultural Research and Education Organization, Tehran, Iran). *Escherichia coli* DH5α was used in all construction experiments and *Agrobacterium tumefaciens* LBA4404 was used for the purpose of plant transformation. Plasmids pACYC177 (Chang and Cohen 1978), pCAMBIA3301 (CAMBIA, Canberra, Australia) and pBT10 (Becker *et al*., 1992) were used in the cloning experiments and plasmid pGPTV (Sprenger-Haussels and Weisshaar 2000) was used as an expression vector.

Media and growth conditions: Bacteria were grown in Luria-Bertani (LB) medium (Bacto-tryptone 10/l, Bacto-yeast extract 5 g/l, NaCl 10 g/l) at appropriate temperatures (37ºC for *E. coli* and 28ºC for *A. tumefaciens*) with shaking (200 rpm) (Sambrook and Russell 2001). The fungal strain was grown on potato dextrose agar (PDA) medium (potatoes, infusion 200 g/l, dextrose 20 g/l and agar 15 g/l) at 22ºC for 24 h and potato dextrose broth (PDB) medium was used for liquid culturing of the fungal mycelia (Ayers *et al.,* 1976).

Constructions of synthetic promoters (SynP-FF): The *cis*-acting element F (Heise *et al*., 2002) was used for the construction of a pathogen-inducible promoter. A fragment containing individual *cis*-acting elements flanked by the *Pst*I-*Spe*I sites at the 5′ end and *Xba*I-*Bgl*I sites at the 3′ end was designated as follows: F element (Sense: 5′-GAC TAG TTT GTC AAT GTC ATT AAA TTC AAA CAT TCA ACG GTC AAT TTC TAG AGC CCT TCC-3′; Antisense: 5′-GGG CTC TAG AAA TTG ACC GTT GAA TGT TTG AAT TTA ATG ACA TTG ACA AAC TAG TCT GCA-3′).

The synthetic single stranded oligonucleotides were synthesized by the MWG Co. (Germany) and the double-stranded DNA with sticky ends was generated by annealing of the complementary sense and antisense strands. The annealing was achieved in a reaction mixture containing equal volumes of the strands in T4 ligase buffer. It was incubated in boiling water for 5 min and then slowly cooled to room temperature. This synthetic *cis*-acting element was introduced into the *Pst*I-*Bgl*I sites (within the ampicillin resistant gene) of the pACYC177 vector. The *Xba*I-*Asu*II fragment (~ 3.5 kb), excised from this construct was ligated into the *Xba*I-*Cla*I (*Cla*I together with *Asu*II generates compatible ends) sites of pBT10. The element was dimerized by digesting the construct with either *Spe*I or *Xba*I and together with *Sac*I. The 5′ *Spe*I-*Sac*I 3′ segment from the first reaction was then ligated to the 5′ *Sac*I-*Xba*I 3′ segment from the second reaction, to yield the synthetic promoter SynP-FF.

The 5′ segment of the GUS encoding gene was replaced by the corresponding intron containing sequence from pCAMBIA3301, using the *Nco*I and *Asu*II enzymes. The *Spe*I-*Sac*I fragment from pGPTV vector was replaced by the corresponding fragment containing the constructed promoter::GUS cassette to yield pGFF (containing SynP-FF). The pGMP construct containing the CaMV 35S minimal promoter

lacking *cis*-acting element (Shokouhifar *et al*., 2010) was used as a negative control. The accuracy of constructed promoter cassettes was confirmed by sequencing.

Transformation and molecular analysis of transgenic canola plants: Seeds of *B. napus* (R line Hyola 308) were sterilized by being submerged in 70% (v/v) ethanol for 5 min and then in 0.1% (w/v) $HgCl_2$ for 8 min. They were then rinsed several times with sterilized water and plated on half-strength Murashige and Skoog basal medium (½MS) devoid of plant growth regulators (Murashige and Skoog 1962) and incubated in the presence of light for 5 days. After germination, the cotyledonary petioles were cut and pre-cultured on MS solid medium supplemented with 3.5 mg/l of benzylaminopurine (BAP). After 2 days, the explants were used for transformation.

Single colonies of *A. tumefaciens* harboring the recombinant plant expression vector, pGPTV containing the *cis*-acting element were used to inoculate LB medium supplemented with 50 mg/l of kanamycin, and allowed to grow overnight at 27-28ºC with constant shaking (200 rpm) to mid-logarithmic phase. The bacterial culture was then transferred to fresh medium and cultivated till an optical density (OD_{600}) of 0.4 was obtained. The bacterial cells were then collected by centrifugation and re-suspended in ½ MS medium for the subsequent inoculation step. Plant transformation was carried out as described by Moloney and colleagues (Moloney *et al*., 1989).

Leaf material from the transgenic and non transgenic canola was harvested, lyophilized and grinded into a fine powder for extraction of the genomic DNA by the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1991). The PCR procedure was used to confirm the presence of the transgene in the putative transgenic plants using different sets of primers (Table 1). The PCR reaction mixture contained 2.5 U of *Pfu* DNA polymerase (Fermentas, Canada), 1X PCR buffer, 200 µM each dNTP, 2 µM $MgSO_4$ and 0.5 µM each primer.. Reaction condition for the amplification was programmed as follows: initial denaturation at 94ºC for 4 min; 35 cycles of denaturation at 94ºC for 45 s, annealing at 60ºC for 30 s, extension at 72ºC for 60 s, and a final extension at 72ºC for 5 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel.

Table 1. The primers used in this study.

Name	Sequence
pGXX-f	5'-CTGGCTTTCTACGTGTTCCGC-3'
pGXX-r	5'-GGGTTTCTACAGGACGGACG-3'
ACC-f	5'-CTATAGCTGGGGTCAATGACAACG-3'
ACC-r	5'-GTCGACAGAAGAATGATCGCGAAC-3'

Leaf discs treatment with fungus and elicitors: Independent transgenic plants harboring the SynP-FF promoter and a wild type canola (as a control) were used for the **fungus and elicitors** treatments. Leaf discs (1cm in diameter) of different plants were harvested and placed on the MS basal medium (Murashige and Skoog 1962). The leaf discs were then infected with freshly grown mycelia of *Sclerotinia sclerotiorum*, thus acting as a necrotroph. The Plates were then incubated at 22ºC for 15 h and the discs were then analyzed using the histochemical GUS assay.

Indirect treatments were carried out by released material from fungal mycelia as described by Ayers and colleagues (Ayers *et al*., 1976) with some modifications. Fourteen-days-old PDB cultures of the fungus (100 ml) were harvested using sterile miraclothes (EMD Biosciences, CA) and washed with 1000 ml of distilled water. The mycelia were then shaken for 3 h in 50 ml of distilled water at 200 rpm prior to homogenization. The debris was removed by 30 min of centrifugation at 10000 g and the upper phase was passed through 0.45 and 0.22 µm filters consecutively. The leaf discs of the canola plants were soaked in the elicitor solution and the solution infiltrated in leaf tissues under a vacuum condition (10 kPa) for 15 min. After 24 h, the discs were collected and flash frozen until required for the fluorometric GUS assay or were analyzed using the histochemical GUS assay.

Treatment of the leaf discs with salicylic acid and methyl jasmonate: Salicylic acid and methyl jasmonate, as plant defense signaling factors (Dong 1998), were used to study the responsiveness of the synthetic promoters. The leaf discs from the transgenic plants were separately immersed in 600 µg/ml of colloidal chitin, 2 mM salicylic acid or 50 µM methyl jasmonate and vacuumed for 15 min (Cao Ming *et al*., 2000). To infiltrate the solution between leaf cell spaces, the negative pressure was removed suddenly. Discs were then harvested and immediately frozen in liquid nitrogen for future GUS activity assays.

Treatment of different organs of the transgenic plants with the elicitors of *S. sclerotiorum*:

Clarification of the heterologous expression pattern of a pathogen-inducible promoter is very critical in confirming the probability of transgene expression in all of the plant tissues infected by pathogens (Gurr and Rushton 2005b). In this study, this ability was analyzed by treatment of tissues, such as flowers, leaves and stems of canola plants that can be infected by the *S. sclerotiorum* pathogen. The flowers in the middle of inflorescences of transgenic plants harboring SynP-FF and MinP were cut and soaked in the filtered crude elicitors of *S. sclerotiorum* for 24 h and then used for histochemical GUS staining. Following detachment, the untreated flowers were used as control, for the demonstration of basal GUS expression in the flowers. The leave discs from the transgenic plants were vacuumed by the elicitors for 15 min and after 24 h were subjected to histochemical GUS assay. Fresh stems from the transgenic plants were used to prepare cross sections. The sections were soaked in a solution of crude elicitors for 24 h and GUS activity was then histochemically assayed.

Histochemical and fluorometric GUS assays: Histochemical GUS assay was performed as described by Jefferson *et al*. (Jefferson *et al.,* 1987). The treated leaf discs were vacuum infiltrated for 10 min in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) staining solution 1 mg/ml of X-Gluc, 100 mM phosphate buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$ and 10 mM EDTA) and incubated at 37ºC overnight. To remove the chlorophylls and the associated green color, the discs were sequentially incubated in 50, 80 and 100% (v/v) ethanol for 1 h. The bleached leaf discs were then examined for GUS activity.

Quantitative GUS assay was carried out as described by Jefferson *et al*. (1987). 4-Methylumbelliferone (4-MU) released by the reaction was measured by reading the emission at 455 nm (excitation at 365 nm) using a fluorescence spectrophotometer micro-titer plate reader (FLUSOstar. Co., Austria). The specific GUS activity was reported in pmol of 4-MU per mg of protein per min. Total soluble protein concentration was measured according to the Bradford assay (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

RESULTS

Construction and DNA sequence of the synthetic promoter (SynP-FF): For construction of a synthetic promoter, a fragment containing the F *cis*-acting element (Heise *et al*., 2002) was generated by annealing of complementary sense and antisense strands. This synthetic *cis*-acting element was introduced into the ampicillin resistant gene of the pACYC177 vector using the insertional inactivation method. The element was then dimerized and placed upstream of the CaMV 35S minimal promoter (-46 to +8, containing the TATA box) in the pGPTV vector and designated as pGFF (Fig. 1). Also, pGMP containing the minimal promoter (without the F elements) upstream of the Gus reporter gene was used as a negative control. The construct was confirmed using PCR-based RFLP (Fig. 2) and sequencing. The 5′ segment of the GUS coding gene in pGFF was replaced by the corresponding intron-containing sequence from pCAMBIA3301. The sequence of the created promoter with a length of 151 bp (dimerized form of the F element fused to the minimal promoter) and the first part of intron containing the GUS encoding gene is shown in Figure 3. This construct was used for canola transformation.

Transformation and confirmation of the transgenic canola plants: The pGFF was mobilized into *A. tume-*

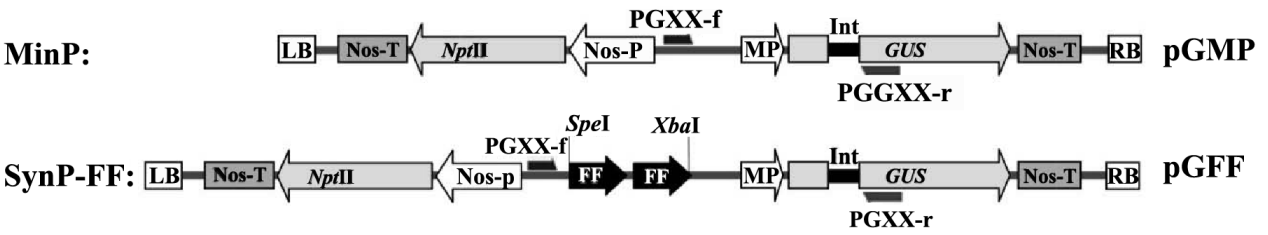

Figure 1. Schematic representation of SynP-FF and MinP synthetic promoters in the pGMP and pGFF construcs. LB: left border, NOS-ter: nopaline synthase terminator, *Npt*II: neomycin phosphotransferase, NOS-pro: nopaline synthase promoter, F: a pathogen inducible *cis*-acting element, MP: minimal promoter (the sequence -46 to +8 from the CaMV 35S promoter), GUS: β-glucuronidase gene containing an intron, NOS-ter: nopaline synthase terminator, Int: intron, RB: right border.

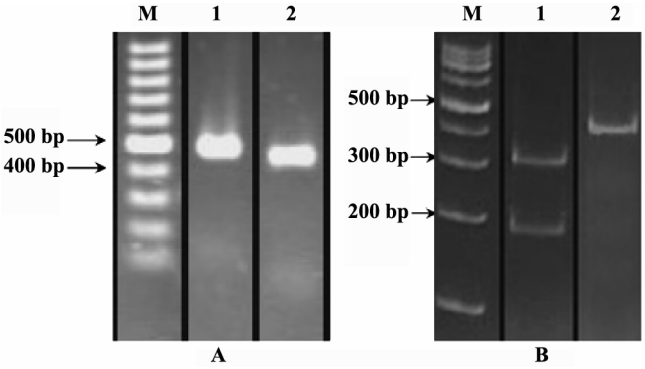

Figure 2. Confirmation of pGFF and pGMP constructs by PCR and PCR-based RFLP (PBR). A: PCR products using specific primers (pGFF-f/r) for flanking regions of synthetic promoter sequences on 1.2 % (w/v)agarose gel. Lane 1: Amplified fragment containing the SynP-FF promoter (500 bp); lane 2: Amplified fragment containing the MinP promoter (410 bp). B: Digestion pattern of PCR fragments (using PGFF-f/r primers) of synthetic promoters using *Xba*I restriction enzyme on 10% (w/v) acrylamide gel stained by ethidium bromide. Lane 1: Digestion of amplified fragment using pGFF DNA as template (expected 313 and 187 bp); lane 2: Digestion of amplified fragments using PGMP DNA as template (expected 410 bp fragment). (Due to the lack of *Xba*I sites, this fragment was not digested). The positions of PGFF-f/r primers and *Xba*I site are indicated in Figure 1.

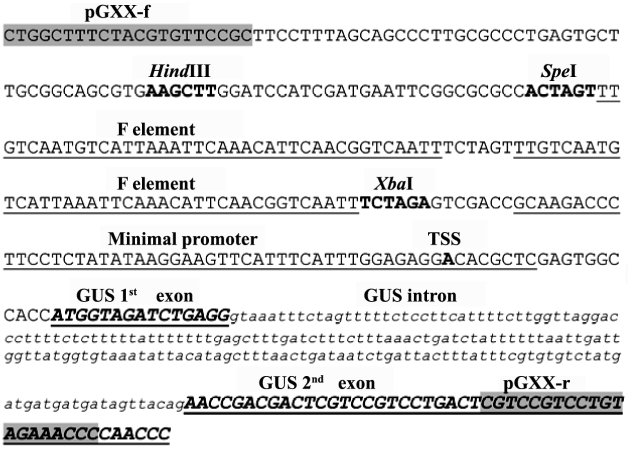

Figure 3. Nucleotide sequence of the SynP-FF promoter. The *Hin*d III, *Spe*I and *Xba*I digestion sites are in bold; F elements and the minimal promoter are underlined;, the transcription start site (TSS) is in bold; the GUS gene exons are in bold, italic and underlined; the GUS intron is lowercase. The pGXX-f/r primers attachment sites are shown as a gray background.

faciens (LBA4404) and subsequently used for transformation of 5-days old cotyledonary petioles of *B. napus*, (R line Hyola 308). The shoots were regenerated from the cotyledonary petioles, 4 to 6 weeks after inoculation. Independent transgenic canola lines were successfully rooted on kanamaycin containing selection media. The transgenic plants were hardened-off in the glasshouse (Fig. 4) and shown to contain the synthetic promoter (a fragment corresponding to 500 bp of the synthetic promoter in all of the lines tested) by PCR using PGXXf/r primers (Fig. 5). These specific primers did not amplify the corresponding fragment in the untransformed sample (Fig. 5).

Effect of *S. sclerotiorum* on induction of the SynP-FF synthetic promoter: To study the activity of the synthetic promoter in response to phytopathogenic fungi, the gene expression pattern of transgenic and wild type plants were investigated using the histochemical assay. In addition, the effect of *S. sclerotiorum* elicitors on GUS expression was investigated using the fluorometric GUS assay. *S. sclerotiorum*, the causal agent of stem root, is a necrotrophic and destructive pathogen of *B. napus*. To reveal the promoter response to this fungus during direct interaction, leaf discs of transgenic plants harboring synthetic promoters were infected with freshly grown mycelia of *S. Sclerotiorum* and after 12 h, GUS activities were determined using the histochemical assay. The results indi-

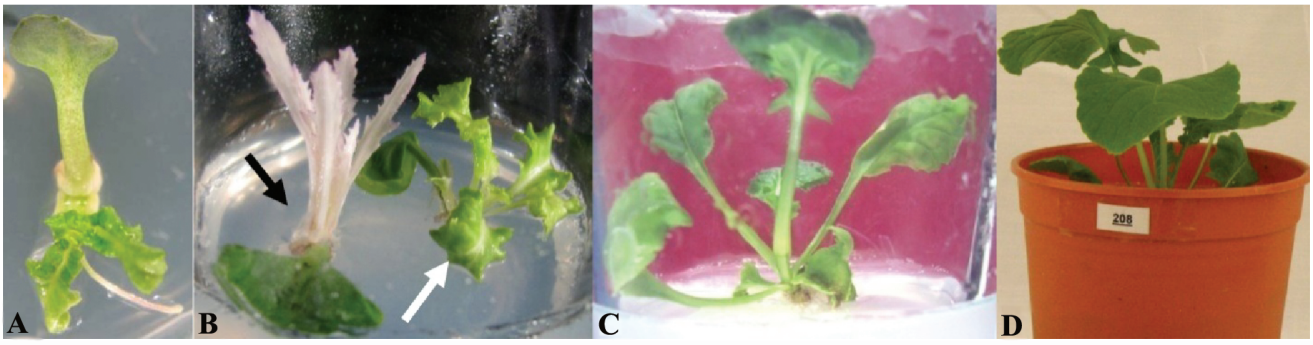

Figure 4. The transformation process and developmental stage of transgenic canola. A: Regeneration of new shoots from cotyledonary petioles in shoot induction medium; B: transgenic (white arrow) and non-transgenic (black arrow) shoots in medium containing 15 mg/l of kanamycin; C: shoots in elongation and root-induction medium, D: transgenic canola plants hardened-off under greenhouse condition.

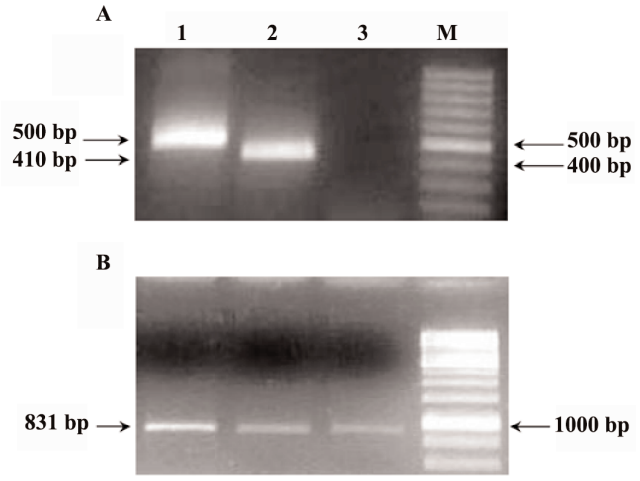

Figure 5. PCR analysis of kanamycin resistant transgenic canola. A: PCR products using specific primers (pGXX-f/r) for flanking regions of synthetic promoter sequences. Samples were electrophoresed on 1.2 % (w/v) agarose and stained with ethidium bromide. M: 100 bp DNA ladder (Fermentas, Canada). B: the amplified seqment of acetyl-CoA carboxylase gene (ACC) (831 bp) as a DNA quality control using ACC-f/r primers. Samples were electrophoresed on 1% (w/v) agarose and stained with ethidium bromide. Lanes 1 and 2: transgenic plants harboring SynP-FF (500 bp) and MinP (410 bp) promoter cassettes, respectively; lane 3: non-transgenic plant, M: 1 Kb DNA ladder, (Fermentas, Canada).

cated that SynP-FF exhibited an area of blue color in the leaf discs, while there was no GUS expression in the untreated samples and in the transgenic plants harboring the MinP promoter (Fig. 6). In the indirect interaction, the leaf discs of transgenic plants were treated by elicitors of *S. sclerotiorum* and the promoter activity was quantified using the fluorometric GUS assay. The promoter exhibited an induction pattern in response to the elicitor (Fig. 7). GUS activity of transgenic plants harboring SynP-FF was induced approximately 6-fold higher than that of non-induced plants representing the control.

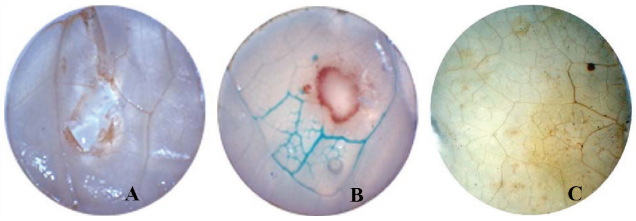

Figure 6. Histochemical analysis of GUS expression in transgenic canola plants harboring the synthetic promoter infected by *S. sclerotiorum*. A and B: infected leaf discs of transgenic canola plants harboring MinP and SynP-FF, respectively,;c: uninfected leaf disc of transgenic canola plant harboring SynP-FF as control.

Characterization of the SynP-FF promoter activity in response to salicylic acid and methyl jasmonate: This study examined the effects of chemical components, such as salicylic acid and methyl jasmonate (as plant defense signaling factors) on expression of the gene under the control of the synthetic SynP-FF promoter. To characterize the expression pattern of the SynP-FF promoter in response to SA, the leaf discs of the transgenic lines were exposed to 2 mM salicylic acid and GUS activity was estimated after 24 h. The

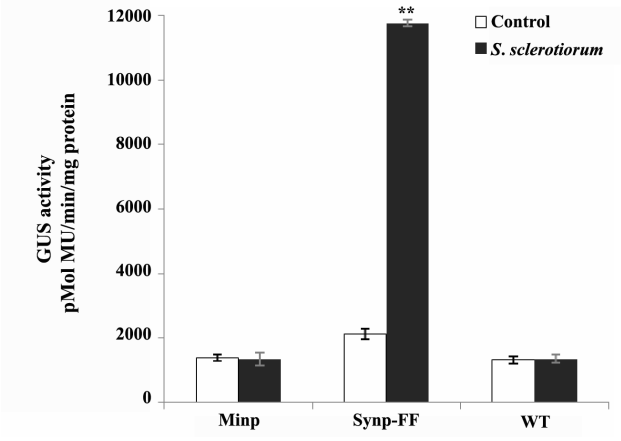

Figure 7. Analysis of the GUS activity of the SynP-FF promoter in response to *S. sclerotiorum* in canola plants. The leaf discs were exposed to crude *S. sclerotiorum* elicitors for 24 h. Each value represents the mean (± standard error) of three independent experiments. Control: leaf discs before treatment with *S. sclerotiorum*. ** value significantly different at $P < 0.01$.

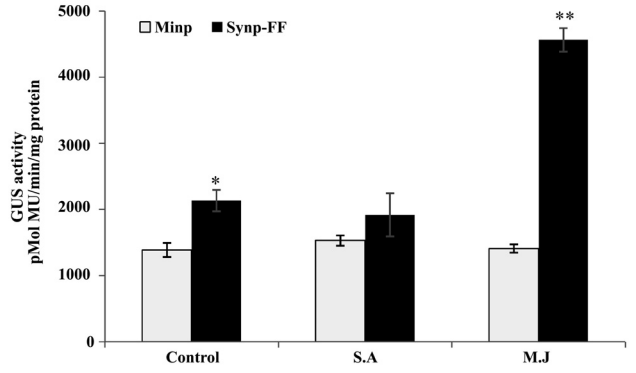

Figure 8. Analysis of GUS activity of the pathogen-inducible promoter in canola plants exposed to salicylic acid (S.A) and methyl jasmonate (M.J). The leaf discs were exposed to S.A (2 mM) and M.J (50 μM) for 24 h. Each value represents the mean (± standard error) of three independent experiments. Control: leaf discs before treatment. * and ** values significantly different at $P < 0.05$ and 0.01 respectively.

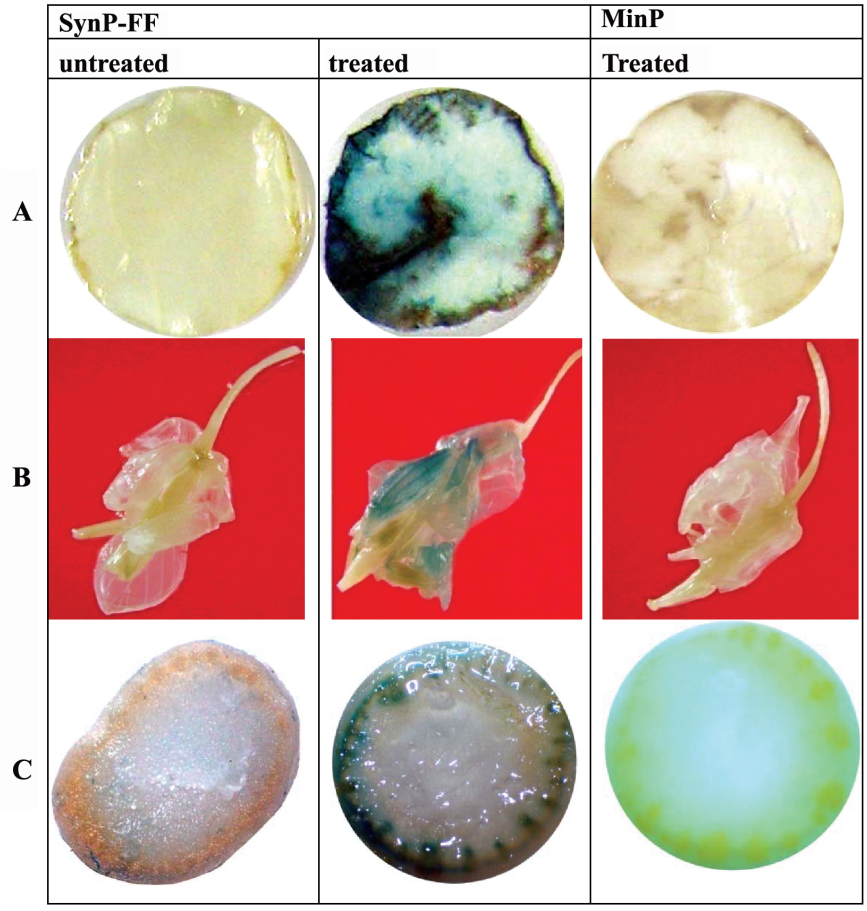

Figure 9. Histochemical GUS expression pattern of transgenic plants harboring SynP-FF or MinP promoters 24 h after infection by the crude elicitor of *S. sclerotiorum* A: leaf discs; B: flower and C: cross section of stem.

quantitative GUS assay data show that the SynP-FF promoter did not respond to salicylic acid and the level of GUS activity in the treated and untreated samples was not significantly different.

To reveal the reaction of the SynP-FF promoter to MJ, the leaf discs of the transgenic plants were treated with 50 µM methyl jasmonate and GUS activity was quantified using the fluorometric assay. The results indicated that the SynP-FF promoter was induced by methyl jasmonate treatment by more than 2-fold when compared to the untreated plants as control (Fig. 8). The results of expression patterns in the leaf discs of transgenic canola harboring MinP demonstrated that the GUS expression level was not significantly different between treated (with salicylic acid or MJ) and untreated samples.

Expression pattern of SynP-FF in different organs of transgenic plants in response to the elicitors of *S. sclerotiorum*: To study the activity of the synthetic promoter (SynP-FF) in response to the elicitors of *S. sclerotiorum*, the gene expression pattern of different organs from the transgenic plants (harboring SynP-FF or MinP) were investigated using the histochemical GUS assay. The results indicated that SynP-FF exhibited an area of blue color in the leaf, flower and stem tissues, while there was no GUS expression in the untreated samples (Fig. 9). Also, the results of the expression pattern in the leaf flower and stem tissues of the transgenic canola plants harboring MinP showed no detectable GUS activity (Fig. 9).

DISCUSSION

The "gain of function approach" allows studying the correlation of specific *cis*-acting elements and their copy number with changes in expression patterns of the generated synthetic promoters. This approach has been adopted by other research groups to analyze the

influence of various *cis*-acting elements on promoter function (Gurr and Rushton, 2005b; Rushton *et al*., 2002). To define exactly how individual *cis*-acting elements contribute to pathogen-inducibility of a promoter, introduction of a *cis*-acting element upstream of minimal promoters generates a reconstructed promoter for functional testing. The synthetic promoter approach was used in this study by dimerizing a *cis*-acting element (F) upstream of the CaMV 35S minimal promoter to produce a phytopathogenic-inducible promoter, resulting in the pGFF construct. Based on the modular characteristic of some pathogen-inducible promoters, which contain a set of combined *cis*-acting elements (Roychoudhury and Sengupta, 2009; Venter and Botha, 2004; Singh 1998), this study investigated the activity of the F *cis*-acting element in the dimerized form.

The F element used in this research, was selected for its ability to be induced by fungal pathogen elicitors (PEP25) (Nurnberg *et al*., 1994) but not by the wound stress (Heise *et al*., 2002; Kirsch *et al*., 2001). These two criteria are desirable phenomena for breeding of disease resistant plants (Venter and Botha, 2010; Gurr and Rushton, 2005b). In this research, the influence of the dimerized form of the F element (FF) on the expression pattern of the minimal CaMV 35S promoter was characterized in stably transformed canola plants.

To analyze the influence of the F element, the pGMP construct containing only the CaMV 35S minimal promoter fused to the GUS reporter gene (Shokouhifar *et al*., 2010) was used as a negative control. The expression patterns were analyzed by *in vivo* expression of the intron containing GUS reporter gene. Comparison of expression patterns of the SynP-FF and MinP promoters before and after treatment clearly revealed the roles of the F *cis*-acting element in response to elicitors and inducers. Also, wild type plants were treated along with the transgenic plants to confirm the expression levels directed by the minimal promoter. Furthermore, the wild type plants exhibited the fluorescent signals that can be attributed to the componenets of the plant cells.

The expression patterns were compared quantitatively in response to plant defense signaling factors and crude elicitors of *S. sclerotiorum* pathogen. The transgenic canola plants harboring the F element were induced by methyl jasmonate and *S. sclerotiorum* elicitors. This is where; salicylic acid did not induce when compared with methyl jasmonate treatment. Based on these data, the F element is considered to be involved in the MJ-mediated defense signaling pathways. It has been reported that the methyl jasmonate pathways are responsible for resistance against necrotrophic pathogens (Glazebrook 2005; Pozo *et al*., 2004), which is in agreement with the findings of this research.

The F element has three copies of a core sequence (GTCA), which are separated by a 19-nucleotide spacer (Heise *et al*., 2002). This core sequence belonging to the W-box *cis*-acting element family is a target site for WRKY transcription factors (Gurr and Rushton 2005b; Heise *et al*., 2002). The authors have previously investigated another *cis*-acting element belonging to this family (E17) that contains only two GTCA core sequences. It has been shown that the synthetic promoter containing the E17 *cis*-acting element is induced by salicylic acid (Shokouhifar *et al*., 2010). It seems that the spacer sequence of the F element is responsible for induction by methyl jasmonate treatment.

To show the expression pattern of the SynP-FF promoter in different organs of canola plants, it was shown that the SynP-FF prompter is active in the canola tissues that could be attacked by *S. sclerotiorum*. This ability of a synthetic promoter is very important in the protection different plant tissues, and is thus one of the advantages of synthetic promoters (Gurr and Rushton 2005b).

The histochemical assay demonstrated that the expression of the repoter gene by the SynP-FF promoter occurs locally around the infected area. The same results have been reported in interactions between *Alternaria brassicola* and transgenic *Arabidopsis* plants harboring a native promoter containing the F element in fusion with the reporter gene (Heise *et al*., 2002). The absence of GUS activity in the untreated transgenic plants harboring SynP-FF indicated that the basal expression of the promoter is very low. In this interaction the transgenic plants harboring MinP did not show detectable GUS activity. These results indicate that the sequence of the F element is responsible for the detected GUS activities.

Analysis of the GUS expression observed in leaf, flower and stem tissues suggest that the SynP-FF is active in a range of different tissues that could be attacked by the pathogen. This finding is consistent with the report of the expression of a reporter gene under the control of the synthetic promoter in *Nicotinia tabacum* and *Arabidopsis taliana* (Cazzonelli and Velten 2008).

The promoter::GUS construct exhibiting β-glucuronidase activity in stably transformed canola plant

lines suggests that despite the repeated structure of the synthetic promoter sequences, they do not appear to be subject to transgene silencing. In conclusion, the dimerized form of the F element has the potential to be used as a MJ-sensitive element in the construction of the necrotrophic pathogen-inducible promoters.

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