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Construction and functional analysis of pathogen-inducible synthetic promoters in *Brassica napus*

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Abstract

In this study, we selected two known pathogen-inducible *cis*-acting elements, F and E17, to construct synthetic pathogen-inducible promoters for analysis in transformed canola (*Brassica napus* L.). The synthetic promoter approach was used, which involved the insertion of dimers and combining two *cis*-acting elements (E17 and F) upstream of the minimal CaMV 35S promoter. Canola plants were transformed by three constructs, pGEE, pGFF, pGFFEE containing synthetic promoters (SP), SP-EE, SP-FF and SP-FFEE, respectively. Analyses of histochemical and fluorometric GUS expression indicated that synthetic promoters responded to fungal elicitors and phytohormone treatments. The SP-FF promoter showed high responses against methyl jasmonate and *Sclerotinia sclerotiorum*, while SP-EE demonstrated inducibility only in response to salicylic acid and *Rhizoctonia solani*. The SP-EE promoter similar to SP-FFEE, did not respond to *S. sclerotiorum* and methyl jasmonate. However, SP-FFEE was highly induced by *R. solani* elicitors and showed that the level of GUS expression was greater than that by either of E17 or F elements alone. These three synthetic promoters did not activate the expression of the reporter gene in response to cold, heat, UV and wounding.

Additional key words: canola, *cis*-acting element, elicitor, fungal infection, reporter gene..

Introduction

An ideal pathogen-inducible promoter should rapidly and strongly direct expression of the specific transgene in response to a wide range of plant pathogens. Furthermore, it should express the resistant genes which are commonly used by different research groups (Rajam *et al.* 2007, Anand *et al.* 2009, Guerra-Guimaraes *et al.* 2009) temporally and locally during plant-pathogen interactions (Gurr and Rushton 2005b). Although the natural pathogen-inducible promoters have shown high potential to direct gene expression in response to pathogens, they contain a complex arrangement of *cis*-motifs and their expression patterns are not robust under various conditions (Rushton *et al.* 2002, Gurr and Rushton 2005b, Venter 2007).

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 (Rushton *et al.* 2002, Gurr and Rushton 2005b, Venter 2007). Recent reports show that individual *cis*-acting elements fused with a minimal promoter can locally direct reporter gene expression in response to pathogens (Rushton *et al.* 2002, Cazzonelli and Velten 2008, Mazarei *et al.* 2008).

Pathogen-inducible *cis*-acting elements are classified according to their reaction in response to defense components such as salicylic acid, methyl jasmonate and ethylene or signals based on the core sequences which

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Abbreviations: BAP - benzylaminopurine; BSA - bovine serum albumin; *F.g* - *Fusarium graminearum;* GUS - β-glucuronidase; MinP - minimal promoter; LB - Luria-Bertani; MJ - methyl jasmonate; MS - Murashige and Skoog; 4-MU - 4--methyl-umbelliferone; 4-MUG - 4-methyl-umbelliferone-β-D-glucuronide; PDB - potato dextrose broth; *R.s* - *Rhizoctonia solani*; *S.s* - *Sclerotinia sclerotiorum*; SA - salicylic acid; SP - synthetic promoter; X-Gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.
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they possess, such as the GCC or W boxes (Mazarei *et al*. 2008). Differences in *cis*-acting elements are often related to their background expression and signaling pathways that are involved in plant-pathogen interactions (Venter 2007). Among the defined pathogen-inducible *cis*-acting elements, only a few are not induced by wounding and mechanical damages (Gurr and Rushton 2005b). E17 as a new type of W box *cis*-acting elements was identified in the promoter of an immediate-early pathogen-responding gene (*ELI*17) belonging to parsley (Kirsch *et al.* 2001). Another *cis*-acting element (F) has been shown to respond strongly to fungal infections (Heise *et al.* 2002). Both F and E17 elements contain two functionally important W box motifs with different relative positions, whereas the F element has an additional 9 bp motif that is functionally important (Heise *et al*. 2002). Having some characteristic features such as fast responding, low background expression, local expression and non-responsiveness to wounding and mechanical damages make them suitable candidates for use in synthetic pathogen-inducible promoters. Based on the conservative nature of plant defense signaling (Singh *et al.* 2002), these elements are expected to show similar reactions in other species, but to clarify their functions in heterologous systems such as canola plants, they need to be analyzed individually in the target biological system.

Plant-pathogen interactions can be divided into non-host, biotrophic and necrotrophic based on the pathogens life-styles (Gurr and Rushton 2005b). Plants respond to pathogens using different signaling pathways. Salicylic acid signaling pathways are usually involved in response to biotrophic pathogens and it has been shown that jasmonic acid and ethylene-related signaling pathways control defense against necrotrophic pathogens. The signaling pathways have many cross-talks and interactions. Wounding shows considerable overlap with jasmonic acid and ethylene signaling pathways (Glazebrook 2005). The role of E17 and F elements have only been investigated in *Arabidopsis* and in interaction with a few compatible pathogens (Kirsch *et al.* 2001, Heise *et al.* 2002).

In this study, we report the function of the E17 and F elements in response to chitin, salicylic acid, methyl jasmonate and different stimuli such as cold, heat and UV. Furthermore, reaction of the elements in response to different pathogens was investigated.

Materials and methods

The rapeseed (*Brassica napus* L.) R line Hyola 308, used as receptor, was kindly provided by the *Oilseed and Development Co*., 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 experiment and plasmid pGPTV (Sprenger-Haussels and Weisshaar 2000) was used as an expression vector. Bacteria were grown in Luria-Bertani (LB) medium at temperatures of 37 °C for *E. coli* and 28 °C for *A. tumefaciens* with shaking (200 rpm).

The El7 (Kirsch *et al.* 2001) and F (Heise *et al.* 2002) *cis*-acting elements were used for construction of a pathogen-inducible promoter. Two fragments 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 were designed as follows: E17 element (sense: 5′-GACTAGTTGTCAATGGTCAACATTCAACTCT AGAGCCCTTCC C-3′; antisense: 5′-GGGCTC TAGAGTTGAATGTTGACCATTGACAACTAGTCTG CA-3′; F element (sense: 5’-GACTAGTTT GTCAATGTCATTAAATTCAAACATTCAACGGTC AATTTCTAGAGCCCTTCC-3’; antisense: 5’-GGGCTC TAGAAATTGACCGTTGAATGTTTGAATTTAATG ACATTGACAAACTAGTCTGCA-3’).

The synthetic single stranded oligonucleotides were synthesized by the *MWG Co*. (Ebersberg, Germany) and the double stranded blocks with sticky ends were generated by annealing of complementary sense and antisense strands. The annealing was achieved in reaction containing equal volume of the strands in T4 ligase buffer. It was incubated in boiling water for 5 min and then slowly cooled to room temperature. These synthetic *cis*-acting elements were introduced into *Pst*I-*Bgl*I sites (within the ampicillin resistant gene) of the pACYC177 vector.

The *Xba*I-*Asu*II fragments (~ 3.5 kb), excised from these constructs were ligated into the *Xba*I-*Cla*I (*Cla*I together with *Asu*II generates compatible ends) sites of pBT10. Both elements were dimerized by digesting the constructs with either *Spe*I or *Xba*I together with *Sac*I enzymes and ligating the 5' *Spe*I-*Sac*I 3' segment from the first reaction with the 5' *Sac*I-*Xba*I 3' segment from the second reaction to yield the synthetic promoter-EE (SP-EE) or SP-FF. The same strategy was used to make a new construct with a combination of dimerized elements in order to create SP-FFEE.

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 created promoter::GUS cassette to yield pGFF (containing SP-FF), pGEE (containing SP-EE) and pGFFEE (containing SP-FF). The pGMP vector containing the CaMV 35S minimal promoter lacking *cis*-

acting elements was constructed and used as a negative control. The accuracy of constructed promoter cassettes was confirmed by sequencing.

Seeds of *B. napus* (R line Hyola 308) were sterilized by being submerged in 70 % ethanol for 5 min and then in 0.1 % $HgCl_2$ for 8 min. They were then rinsed several times with sterilized water and plated on ½ MS (Murashige and Skoog 1962) medium and incubated under continuous irradiance of 250 μmol m^{-2} s^{-1} for 5 d. After germination, the cotyledonary petioles were cut and placed on MS solid medium with 3.5 mg dm^{-3} of benzylaminopurine (BAP) for pre-culture. After 2 d, the explants were used for transformation.

Single colonies of *A. tumefaciens* harboring the recombinant plant expression vector, pGPTV containing *cis*-acting elements were used to inoculate LB medium supplemented with 50 mg dm^{-3} of kanamycin, and allowed to grow overnight at 27 - 28 °C with constant shaking (200 rpm) to mid-log phase. The bacterial culture was then transferred to fresh medium and cultivated till an absorbance (A_{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 *et al.* (1989).

Independent transgenic plants harboring the synthetic promoters and a wild type canola (as a control) were used for treatment. leaf discs (1 cm in diameter) of different plants were harvested and placed on the MS basal medium. The leaf discs were infected with freshly grown mycelia of *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Fusarium graminearum* as biotrophic, necrotrophic and non-host pathogens, respectively. The plates were then incubated at 22 ºC for 15 h and the discs were analyzed using the histochemical β-glucuronidase (GUS) assay.

Indirect treatments were carried out by released material from fungal mycelia as described by Ayers *et al.* (1976) with some modifications. Liquid cultures of the fungi (14-d-old) in 100 cm^3 of potato dextrose broth (PDB) medium were harvested using sterile miraclothes (*EMD Biosciences*, San Diego, CA, USA) and washed with 1 dm^3 of distilled water. Then, the mycelia were shaken for 3 h in 50 cm^3 of distilled water at 200 rpm prior to homogenization. The debris was removed by 30 min of centrifugation at 10 000 *g* and the upper phase was passed through 0.45 and 0.22 μm filters consecutively. The leaf discs were soaked in the filtered solution and vacuumed for 15 min. After 24 h, the discs were collected and flash frozen until required for the fluorometric GUS assay.

Chitin as a general plant defense elicitor (Nürnberger and Brunner 2002) together with salicylic acid and methyl jasmonate as plant defense signaling factors (Dong 1998), were used to study the responsiveness of the transgenic plants. The leaf discs were separately immersed in 600 μg cm^{-3} of colloidal chitin, 2 mM salicylic acid or 50 μM methyl jasmonate and vacuum infiltrated (10 kPa) for 15 min (Cao *et al.* 2000). To infiltrate the solution into leaf intercellulars, the negative pressure was removed suddenly. Discs were harvested and immediately frozen in liquid nitrogen for future GUS activity assays.

Response of the transgenic plants to abiotic stresses [cold (4 °C, overnight), heat (37 °C overnight) and UV (280 - 315 nm, 3 h) (Yang *et al.* 2000, Bolle 2009)] were investigated by exposing the leaf discs to these environmental factors. After the treatments, the leaf discs were harvested and flash frozen. Wounding was also carried out by cutting the leaves into discs, which after 24 h were subjected to histochemical and fluorometric GUS assays.

Histochemical GUS assay was performed as described by 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 cm^{-3} 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 37 ºC overnight. To remove the chlorophylls, 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. 4-Methylumbelliferone (4-MU) released by the reaction was determined by reading the emission at 455 nm (excitation at 365 nm) by the fluorescence spectrophotometer micro-titer plate reader (*FluoStar. Co.*, Salzburg, 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 Bradford (1976), using bovine serum albumin (BSA) as a standard.

Results

To analyze activity and inducibility of two *cis*-acting elements E17 and F in the canola plant by phytopathogenic fungi, we prepared three kinds of constructs containing these elements upstream of the CaMV 35S minimal promoter (-46 to +8, containing the TATA box). Based on the current state of knowledge, none of these elements have been functionally tested in canola plants. Our strategy to construct a pathogen-inducible promoter was to place these two *cis*-acting elements as a dimmer form of E17 or F individually or in combination (heterodimer forms of E17 and F) into the pGPTV vector (Fig. 1).

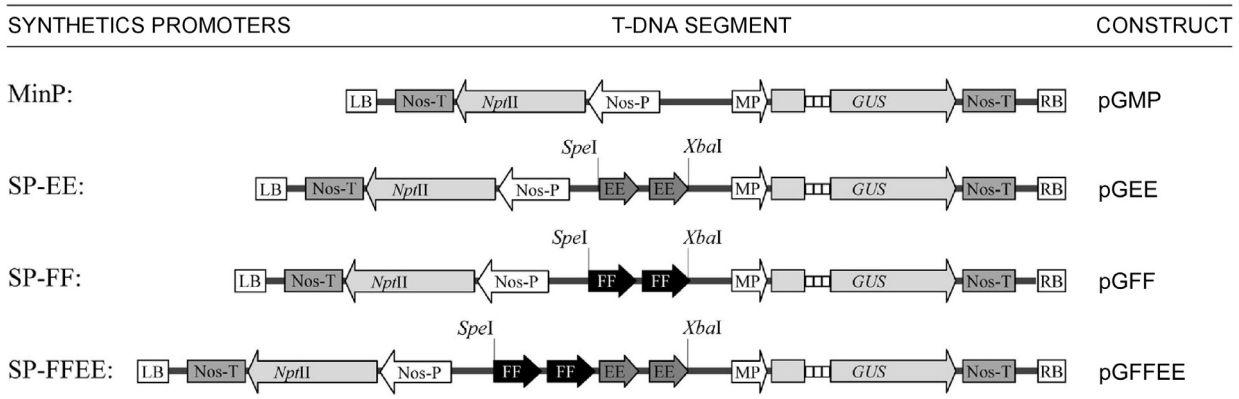

Fig. 1. Schematic diagrams of T-DNA region of constructed synthetic promoters. pGEE, pGFF, and pGFFEE contain EE, FF, and FFEE elements upstream of CaMV 35S minimal promoters, respectively. LB - left border, Nos-T - nopaline synthase terminator, *Npt*II - neomycin phosphotransferase II, Nos-P - nopaline synthase promoter, EE - a pathogen inducible *cis*-acting element from *Petroselinum crispum* (Kirsch *et al*. 2001), FF - a pathogen inducible *cis*-acting element from *Arabidopsis thaliana* (Heise *et al*. 2002), MP - sequence of -46 to +8 from CaMV 35S promoter as minimal promoter, GUS - β-glucuronidase containing intron, RB - right border. pGMP contains CaMV 35S minimal promoter used as negative control.

Table 1. Analysis of GUS activity [pmol(MU) mg^{-1}(protein) min-1] in the transgenic canola plants harboring different synthetic pathogen-inducible promoters exposed to various treatments. Means ± SE of three independent experiments. *, ** - values significantly different at $P < 0.05$ and 0.01 respectively. Control: leaf discs before treatment.

Treatments	MinP	SP-FF	SP-FFEE	SP-EE	Wt
Control	1529 ± 313	2006 ± 383	2567 ± 383	1490 ± 383	1467 ± 313
Chitin	1520 ± 296	6970 ± 296 **	3973 ± 296 *	2160 ± 241 *	1597 ± 241
Methyl jasmonate	1371 ± 176	4524 ± 176 **	1998 ± 176 *	1404 ± 107	1405 ± 107
Salicylic acid	1497 ± 232	1927 ± 232	3194 ± 232 *	3247 ± 189 **	1350 ± 328
S. sclerotiorum	1352 ± 383	12000 ± 500 **	3046 ± 313	2661 ± 383 *	1272 ± 313
R. solani	1647 ± 239	5210 ± 239 **	12160 ± 138 **	4675 ± 138 **	1187 ± 239
F. graminearum	1423 ± 371	8292 ± 371 **	10690 ± 303 **	5256 ± 371 **	1203 ± 300

To investigate the inducibility of the synthetic promoters, four transformation vectors, pGMP (containing the minimal promoter), pGEE and pGFF (containing the minimal promoter fused to the dimerized forms of the E17 and F elements, respectively), pGFFEE (containing the minimal promoter and hetero-dimerized forms of F and E17 elements) were introduced into canola plants and analyzed for GUS induction.

We examined the effects of chemical components such as chitin (as a general plant defense system elicitor) and salicylic acid (SA) and methyl jasmonate (MJ) (as plant defense signaling factors) on expression of the GUS gene under the control of the synthetic promoters. The results indicated that the promoters containing FF, EE and FFEE elements were significantly induced by chitin when compared with those of untreated plants (Table 1). The GUS activity in the SP-FF promoter was higher than SP-EE while SP-FFEE containing a combination of both elements showed an intermediate level of activity. There was no significant difference in the basal expression levels of the SP-EE, SP-FF and minimal promoter (MinP) from the wild type (Wt) plant, but SP-FFEE showed a higher background expression level. The inducibility of SP-FF was approximately 3.5 fold while in SP-FFEE and SP-EE was 1.6 and 1.4 fold, respectively (Table 1). The GUS activity of transgenic plants harboring the MinP promoter was not induced after treatment with chitin and the GUS expression was not significantly different from those of WT plants.

To characterize the expression pattern of the synthetic promoters in response to SA, the transgenic lines were exposed to 2 mM SA and GUS activity was estimated after 24 h. Transgenic plants with SP-EE and SP-FFEE promoters were sensitive to SA (Table 1). The SP-EE transgenic plants increased GUS activity with more than 2 fold in response to SA. However, the SP-FF plants did not respond to SA and the GUS activity in the treated and untreated samples was not significantly different.

The transgenic plants were treated with 50 μM MJ and the results indicated that the GUS activity in SP-FF plants was induced by MJ treatment more than 2 fold when compared to the untreated plants (Table 1). The

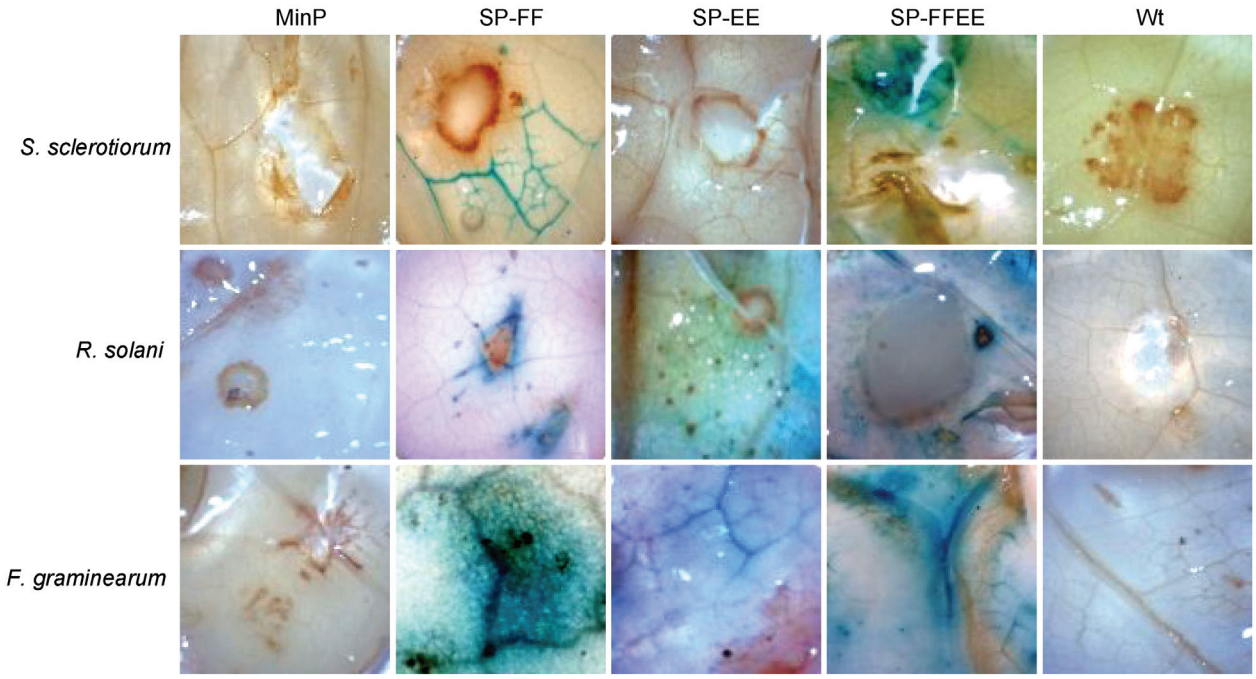

Fig. 2. Histochemical GUS analysis of transgenic canola plants harboring synthetic promoters fused to GUS gene 12 h after infection by *S. sclerotiorum*, *R. solani* and *F. graminearum*.

SP-EE plants did not respond to MJ and the interaction of EE and FF *cis*-acting elements and their effects on GUS expression in SP-FFEE unexpectedly did not lead to induction (Table 1). However, no obvious differences in GUS activities were observed in WT and MinP.

In addition, the effect of fungal elicitors of *S. sclerotiorum*, *R. solani* and *F. graminearum* on GUS expression was investigated. *S. sclerotiorum* is a necrotrophic pathogen and causes stem rot in *B. napus*. The transgenic plants exhibited different expression patterns in response to the elicitors (Table 1). GUS activity of transgenic plants harboring SP-FF was about 6-fold higher than that of non-induced plants, while SP-EE induced approximately 1.5-fold. In contrast, the FFEE plants had not significantly increased inducibility. In the direct interaction, leaf discs of transgenic plants harboring synthetic promoters were infected with fresh grown mycelia of *S. sclerotiorum* and after 12 h the GUS activities was determined. The results indicated that there was no GUS expression in the transgenic plants harboring MinP and Wt plants, while SP-FF and SP-FFEE plants exhibited areas of blue colour on the leaf discs (Fig. 2).

R. solani, as a biotrophic pathogen, was exploited to clarify the expression patterns of the promoters in response to biotrophic interaction in canola (Table 1). The SP-FFEE plants showed a higher increase in GUS expression after treatment and its inducibility was close to 5-fold. The SP-FF and SP-EE plants also exhibited significant increases when compared to the untreated samples. The inducibility of these two transgenics was different due to their different basal expression levels (Table 1). The GUS expression in the transgenic plants harboring SP-FF, SP-EE and SP-FFEE was higher as compared to those containing MinP and also Wt plants (Fig. 2).

To investigate the response and inducibility of the promoters to non-host pathogens, the transgenic plants were treated by fungal elicitors and infected directly by *F. graminearum*. GUS activity in SP-EE, SP-FF and SP-FFEE transgenic plants was increased in response to fungal elicitors of *F. graminearum* (Table 1). The inducibility was higher than 4-fold in SP-FF and SP-FFEE and approximately 3.5-fold in SP-EE (Fig. 2).

These synthetic promoters did not activate the expression of the reporter gene in response to cold, heat and UV (data not shown). Moreover, we observed no detectable induction of GUS activity by cutting of leaf discs representing wounding stress.

Discussion

To define exactly how individual *cis*-acting elements contribute to pathogen-inducibility of a promoter, the introduction of *cis*-acting elements upstream of minimal promoters generates reconstructed promoters for functional testing. This “gain of function approach” allows us to study the correlation of specific *cis*-acting

elements, their copy number and combinations 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). The synthetic promoter approach was used in this study by dimerizing and combining two *cis*-acting elements (E17 and F) upstream of the CaMV 35S minimal promoter to produce phytopathogenic-inducible promoters. Their transcription patterns were then analyzed by *in vivo* expression of the intron containing *GUS* reporter gene. These two elements used in this research, were selected for their ability to be induced by fungal pathogen elicitors but not by the wounding (Heise *et al*. 2002, Kirsch *et al*. 2001).

In this study, the influence of the dimerized forms of the E17 (EE), and F elements (FF) and a combination of E17 and F elements (FFEE) on the expression pattern of the minimal CaMV 35S promoter was characterized in stably transformed canola plants. These expression patterns were compared quantitatively in response to chitin, plant defense signaling factors and crude elicitors of *S. sclerotiorum*, *R. solani* and *F. graminearum* pathogens.

The transgenics containing dimer forms of E17 responded to SA and *R. solani* as a biotrophic pathogen but not to MJ and *S. sclerotiorum* as a necrotrophic pathogen. These results suggest that the E17 element is induced through the SA-signaling pathway. By contrast, higher expression and inducibility by MJ and *S. sclerotiorum* elicitors occurred in the transgenic canola plants harboring F element. This is where, SA did not induce and *R. solani* had lower inducibility when compared with MJ and *S. sclerotiorum* treatments. Based on these data, F element is considered to be involved in the MJ-defense signaling pathways. As described earlier, the MJ pathways are responsible for resistance against necrotrophic pathogens (Pozo *et al.* 2004, Glazebrook 2005).

Based on the modular characteristic of some pathogen-inducible promoters, which contain a set of combined *cis*-acting elements (Singh 1998, Venter and Botha 2004, Roychoudhury and Sengupta 2009), we investigated the activity of E17 and F *cis*-acting elements in combination, on the inducibility of SP-FFEE. This transgenic plant showed unpredictable behaviour in response to some of the treatments. Especially, the SP-EE plants similar to SP-FFEE, did not respond to *S. sclerotiorum* and MJ, suggesting that the E17 element has a dominant effect and inhibits F element inducibility. Furthermore, this promoter was highly induced by *R. solani* elicitors and the GUS expression was greater than in transgenics harbouring either E17 or F elements alone (Table 1). Moreover, the inducibility of these synthetic promoters was histochemically confirmed by direct infection of fungal pathogens. Stably transformed canola plant lines with all three promoter::GUS constructs exhibited β-glucuronidase activity (Fig. 2) suggesting that despite the repeated structure of the synthetic promoter sequences, they do not appear to be subject to transgene silencing.

In conclusion, the dimerized forms of the E17 element (EE) alone or in combination with FF elements have high potential for use as biotrophic sensitive elements within the structure of pathogen-inducible promoters in canola, but are not functional against necrotrophic pathogens. While the dimerized form of the F element has higher potential to be used as a MJ sensitive element in the construction of the necrotrophic pathogen-inducible promoters. The synthetic promoters are considered as useful tools to control more specifically the expression of resistant genes in transgenic plants. This technology has the potential to play an important role in plant biotechnology applications in the future.

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