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BRIEF COMMUNICATION

Transcriptional properties of eight synthetic pathogen-inducible promoters in transgenic *Arabidopsis thaliana*

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Abstract

Synthetic pathogen-inducible promoters (SPIP) hold a great promise to meet the demands for a desired temporal and spatial regulation of transgenes. Four pathogen-inducible *cis*-elements (F-box, S-box, Gst1-box, and W-box) and the minimal cauliflower mosaic virus 35S (CaMV 35S) promoter (-46 to +8 TATA box) were used to design SPIP. Eight SPIP were synthesized and named FSGW, FSWG, GWFS, GWSF, SFGW, SFWG, WGFS, and WGSF according to the order of *cis*-element dimers. They were used to replace the CaMV 35S promoter in the plasmid pBI121 to control expression of the *β-glucuronidase* (*gus*) gene. The transcriptional properties of each SPIP were evaluated in homozygous T_3 lines of transgenic *Arabidopsis thaliana* by histochemical staining *gus* expression and real time quantitative PCR. FSGW and FSWG had a very low basal level and a poor inducibility. The other six SPIP showed different levels of background and inducibility. Using *Ralstonia solanacearum*, the spores of *Phytophthora capsici*, and salicylic acid as inducing factors, GWSF showed the advantages of a low basal expression, rapid response, and efficient transcriptional activity in the rosette leaves of five-week-old plants. The results indicate that the permutation and combination of the *cis*-elements had important effects on transcriptional activities of SPIP. Synthetic pathogen-inducible promoters like GWSF are valuable because it can potentially be further improved to apply to plant genetic engineering for disease resistance.

Additional key words: β-glucuronidase, pathogen-inducible *cis*-element, *Phytophthora capsici*, *Ralstonia solanacearum*, salicylic acid.

Precise control of transgene expression is pivotal to plant genetic engineering for disease resistance. Pathogen-inducible promoters are important tools to achieve a desired temporal and spatial regulation of transgenes. Ideal pathogen-inducible promoters must meet the demands of a low background expression, a wide range of inducing pathogens, rapid responses, and efficient transcriptional activities (Gurr and Rushton 2005). However, few native promoters show all the advantages. Synthetic pathogen-inducible promoters (SPIP) may prove valuable in engineering of plants with an increased pathogen resistance (Niemeyer *et al.* 2014).

There are many factors that affect transcriptional activities of SPIP such as motif functionality, copy number, combinatorial relationships, space to TATA-box and location relative to their regulatory targets, and so on (Zou *et al.* 2011). Studies of a stringent transcriptional regulation of pathogen-inducible genes have identified many inducible promoters and *cis*-elements (Roberts *et al.* 2013, Lehmeyer *et al.* 2016). These *cis*-elements, which are binding sites for transcription factors, are conserved among plant species enabling them to be used efficiently in synthetic inducible promoters in heterologous expression systems (Venter 2007).

Four *cis*-elements with a pathogen-inducible expression function including an F-box (Heise *et al.*

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Abbreviations: CS - connection sequence; GUS - β-glucuronidase; MS - Murashige and Skoog; qPCR - quantitative PCR; SPIP - synthetic pathogen-inducible promoters.
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2002, 5'-TTGTCAATGTCATTAAATTCAAACATT CAACGGTCAATT-3'), S-box (Kirsch *et al.* 2000, 5'-CAGCCACCAAAGAGGACCCAGAAT-3'), Gst1-box (Malnoy *et al.* 2006, 5'-TTCTAGCCACCA GATTTGACCAAAC-3'), and W-box (Wang *et al.* 1998, 5'-TTATTCAGCCATCAAAAGTTGACCAATAAT-3') were used to design SPIP. The dimers were assembled with two of the same *cis*-elements interspaced with a 6 bp DNA insert sequence (ACTAGA). Then, four different dimers were connected with a 10 bp DNA connection sequence (CS, GAAGATAATC, Cazzonelli and Velten 2008) to form a heterologous octamer. The total of eight SPIP, with a *Hind* III site at the 5' end and a *Bam H* I site at the 3' end, were assembled by linking one of the octamers with the minimal 35S promoter (Benfey *et al.* 1990) by three CS repeats. The eight constructed candidate promoters were named FSGW, FSWG, GWFS, GWSF, SFGW, SFWG, WGFS, and WGSF, respectively, according to the order of dimers (Fig. 1 shows GWSF as an example), and synthesized and cloned into pUC19 by *Sangon Biotech* (Shanghai China). The eight SPIP were then used to replace the CaMV 35S promoter in the binary vector pBI121, which controls transcription of the *gus* gene (Jefferson *et al.* 1987). The inserted SPIP, *NPT II*, and *gus* of the recombinant pBI121 plasmids were verified by restriction analysis, PCR, and partial sequencing. The primers for PCR and DNA sequencing were as follows: FSGW and FSWG, forward/reverse (5'-3'), GAAGATAATCCAGCCACCAAAG/GGAAGG GTCTTGCGGATTAT; SFGW and SFWG, forward/ reverse (5'-3'), CAAGCTTCAGCCACCAAAG/AGA TTATCTTCAATTGACCGTTG; GWFS, GWSF, WGFS and WGSF, forward/reverse (5'-3'), TTGAAGATA ATCCAGCCACCA/AGCGTGTCCTCTCCAAATGA; *NPT II* gene forward/reverse (5'-3'), GAGGCTATT CGGCTATGACTG/ATCGGGAGCGGCGATACCGTA; *gus* gene forward/reverse (5'-3'), ACACCGATACCA TCAGCG/TCACCGAAGTTCATGCCAGT. The recombinant plasmids and original vector plasmid pBI121 were transformed into *Agrobacterium tumefaciens* strain GV3101 as described Chen *et al.* (1994).

Transformation of *Arabidopsis thaliana* (Col-0) plants was performed *via A. tumefaciens* strain GV3101 by the floral dip method (Zhang *et al.* 2006). Ten to twenty independent T_0 plants were selected for each SPIP:*gus* reporter transgene and CaMV 35S:*gus* by sowing seeds on a half strenght Murashige and Skoog (MS) medium with 50 μg cm^{-3} kanamycin. The four-week-old putative transgenic plants were confirmed by PCR of SPIP, *NPT II*, and *gus* as mentioned above. To determine the number of T-DNA loci, more than 5,000 T_1 progenies of each independent T_0 line were screened for kanamycin resistance. The corresponding T_0 plants with an approximately 3:1 segregation ratio of green seedlings to white seedlings in the T_1 progeny were taken as single-loci transgenic plants. Five T_1 progenies of putative single-copy T_0 lines were planted to harvest T_2 seeds (FSGW: lines 1, 2, 3, 5, 6; FSWG: lines 1, 2, 4, 7, 9; GWFS: lines 1, 2, 3, 4, 5; GWSF: lines 1, 2, 5, 6, 7; SFGW: lines 1, 3, 5, 7, 9; SFWG: lines 4, 5, 8, 9, 12; WGFS: lines 3, 5, 7, 8, 9; WGSF: lines 3, 5, 8, 9, 11; CaMV 35S: lines 1, 3, 5, 6, 7). The T_2 progeny was confirmed by kanamycin resistance and PCR as mentioned above. At last, homozygous lines were identified when the rate of green seedlings of each T_3 lines, selected by kanamycin, were higher than 99 %.

A. thaliana T_3 homozygous transgenic lines were grown in a growth cabinet at an irradiance of 300 μmol(photon) m^{-2} s^{-1} during a 12-h photoperiod and day/night temperatures of 26/20 °C, and relative humidities of 70/80 %. The one-, two- and three-week-old plants were histochemically stained for GUS activity as described Jefferson *et al.* (1987) to detect a basal expression of SPIP. The rosette leaves of five-week-old plants were inoculated with 70 mm^3 of a 1×10^7 CFU cm^{-3} liquid suspension of *Ralstonia solanacearum* or spores of *Phytophthora capsici* at each inoculation spot, six spots on every piece of leaf, by using a 1 cm^3 needleless syringe to rub adaxial surfaces gently (Yang *et al.* 2000). Leaves were also inoculated with 25 μmol dm^{-3} salicylic acid by spraying. Three to 6 hours later, the leaves from 5 independent lines were GUS-stained at 37 °C for 15 h to evaluate the basal level and inducibility of each SPIP.

Five-week-old *A. thaliana* T_3 lines were inoculated with the method described above. Then, five pieces of leaves from five individual lines (30 to 40 mg) were ground in liquid nitrogen at 0, 6, 12, and 24 h after inoculation for total RNA isolation by using a *UNIQ-10 Column Trizol* total RNA isolation kit (*Sangon Biotech*). The quality and concentration of the total RNA were determined with a *NanoDrop 2000C* spectrophotometer. Reverse transcription was performed according to the manufacturer's protocol (a *PrimeScript* RT reagent kit with gDNA eraser, *TaKaRa*, Dalian, China) and the cDNA was used for qPCR. The cDNA from five individual lines of the transgenic CaMV 35S lines was mixed to use as a reference to evaluate transcriptional activities of SPIP. The plant materials of each treatment were in triplicates.

Transcription of *gus* gene was verified by qPCR using an *iCycler4* (*Bio-Rad*, Hercules, CA, USA) and *SYBR Premix Ex* Taq™ II (*Tli RNaseH Plus*, *TaKaRa*). Relative expression levels were calculated using endogenous *EF1-α* (AT5G60390) as a normalization control. Primers used were: *gus* gene forward/reverse (5'-3'), CTGATA GCGCGTGACAAAAA/GGCACAGCACATCAAAGA GA; *EF1-α* gene forward/reverse (5'-3'), TGA GCACGCTCTTCTTGCTTTCA/GGTGGTGGCATCCA TCTTGTTACA. Amplification efficiencies were calculated with the *Opticon Monitor* analysis software *v. 3.1.32*. Thermal-cycling conditions were as follows: an initial denaturation step at 95 °C for 15 s, 40 cycles at 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. The specificity of primer pairs was verified by melting curve

analysis from 70 to 90 °C at the end of qPCR. Analysis of the relative levels of *gus* gene expression was performed from three independent experiments by using the method of Pfaffl *et al.* (2002) with the transcription level of CaMV 35S-*gus* normalized to 1.

A low background expression is critical for SPIP in a desired regulation of transgenes without pathogen invasion. Eight SPIP showed different basal levels and variation trends of transcription at one, two, and three week stages of the transgenic *A. thaliana* T_3 lines (Fig. 1 Suppl.). GWSF showed a high basal level at the first week but decreased gradually since the second week (Fig. 2*A*,*B*,*C*).

A wide range of inducing pathogens and rapid responses are two other essential criteria for ideal pathogen-inducible promoters. FSGW and FSWG had very low basal levels, but neither of them showed an inducible response to salicylic acid. The results of histochemical staining for GUS activity in the beginning illustrate that GWSF had a lower basal level than the other five SPIP (Fig. 2*G*, Fig 2 Suppl.). When induced by salicylic acid, *R. solanacearum*, or spores of *P. capsici*,

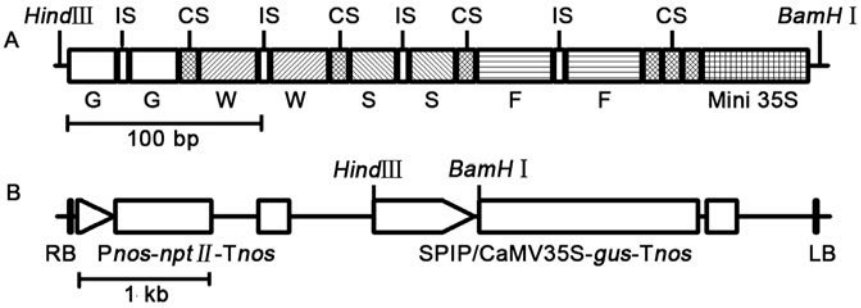

Fig. 1. A schematic diagram of synthetic pathogen-inducible promoters (SPIP) of GWSF (*A*) and recombinant pBI121 harboring SPIP (*B*). G - Gst1-box; W - W-box; S - S-box; F - F-box; IS - 6 bp DNA insert sequence; CS - 10 bp DNA connection sequence; Mini 35S - minimal CaMV 35S promoter; Pnos - nopaline synthase promoter; *NPT II* - neomycin phosphotransferase II gene; *gus* - β-glucuronidase gene; Tnos - nopaline synthase terminator. Scales are marked (100 bp at *A* and 1 kb at *B*).

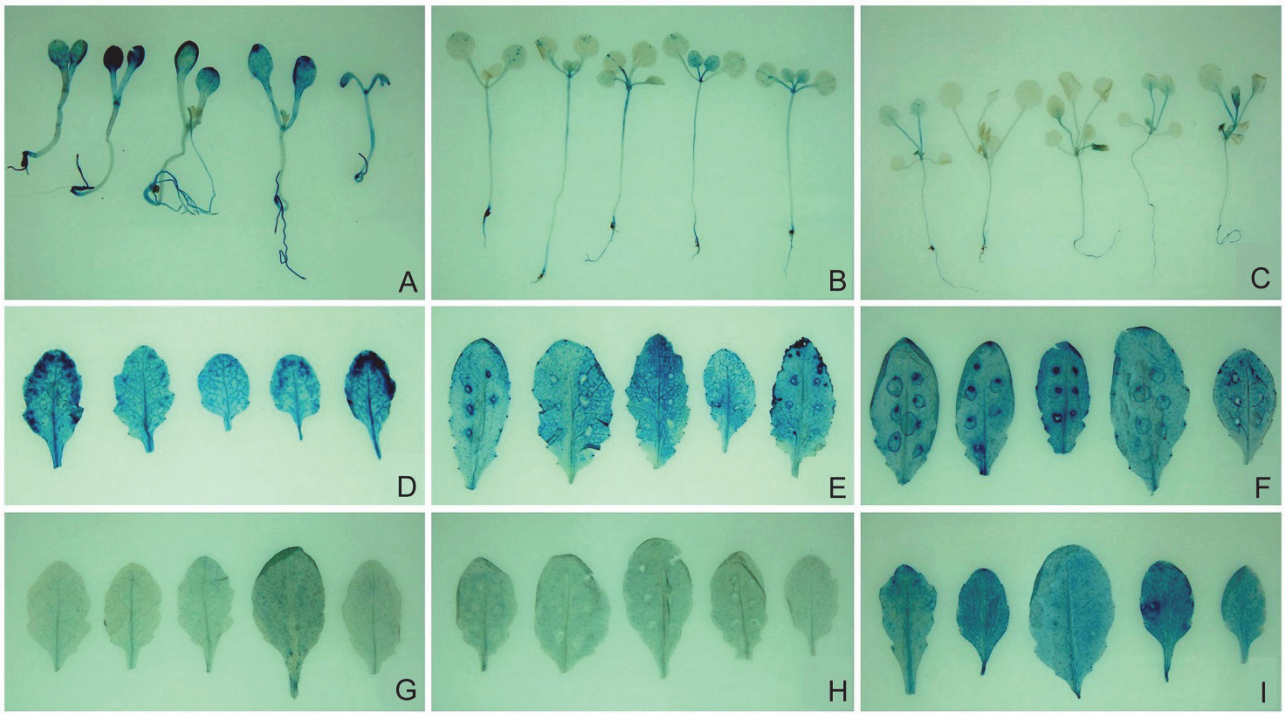

Fig. 2. Histochemical staining GWSF-*gus* transgenic *Arabidopsis thaliana* T_3 lines (lines 1, 2, 5, 6, 7 from left to right). *A*, *B*, *C* - basal GUS activities of GWSF in one-, two- and three-week-old transgenic *A. thaliana* T_3 lines. *D*, *E*, *F* - GUS staining GWSF at five-week-old transgenic *A. thaliana* T_3 lines within 6 h after being treated with salicylic acid, *Ralstonia solanacearum*, or spores of *Phytophthora capsici*, respectively. *G* - basal GUS activities of GWSF at five-week-old transgenic *A. thaliana* T_3 lines. *H* - GUS staining five-week-old non-transgenic *A. thaliana*. *I* - GUS staining five-week-old CaMV 35S-*gus* transgenic *A. thaliana* T_3 lines (lines 1, 3, 5, 6, 7 from left to right).

GWSF showed rapid responses and high expression levels within six hours (Fig. 2*D*,*E*,*F*).

An efficient transcriptional activity upon induction is an important criterion for ideal SPIP in order to enhance disease resistance. The transcriptional activities of SPIP were evaluated by using the Pfaffl method with the transcription level of *gus* under the control of CaMV 35S normalized to 1. GWSF had the lowest basal level, which was 0.151 of that of the CaMV 35S promoter (Fig. 3). After induction by *R. solanacearum*, GWFS, GWSF, WGFS, and WGSF showed efficient transcription activities. Taken GWSF as an example, transcriptional activities were 0.81, 2.58, and 1.44 times higher than that of the CaMV 35S promoter after 6, 12, and 24 h, respectively. When induced with spores of *P. capsici*, GWFS, SFWG, and WGSF showed high transcription activities after 24 h and they were 2.90, 6.54, and 2.41 times higher than that of the CaMV 35S promoter, respectively. GWSF showed more rapid responses, and the transcription activity after 6 h was 1.44 times higher than that of the CaMV 35S promoter. When treated with 25 µmol dm^{-3} salicylic acid, GWSF showed a rapid response and had the highest transcription activity after 6 h, 5.35 times higher than that of the CaMV 35S promoter (Fig. 3).

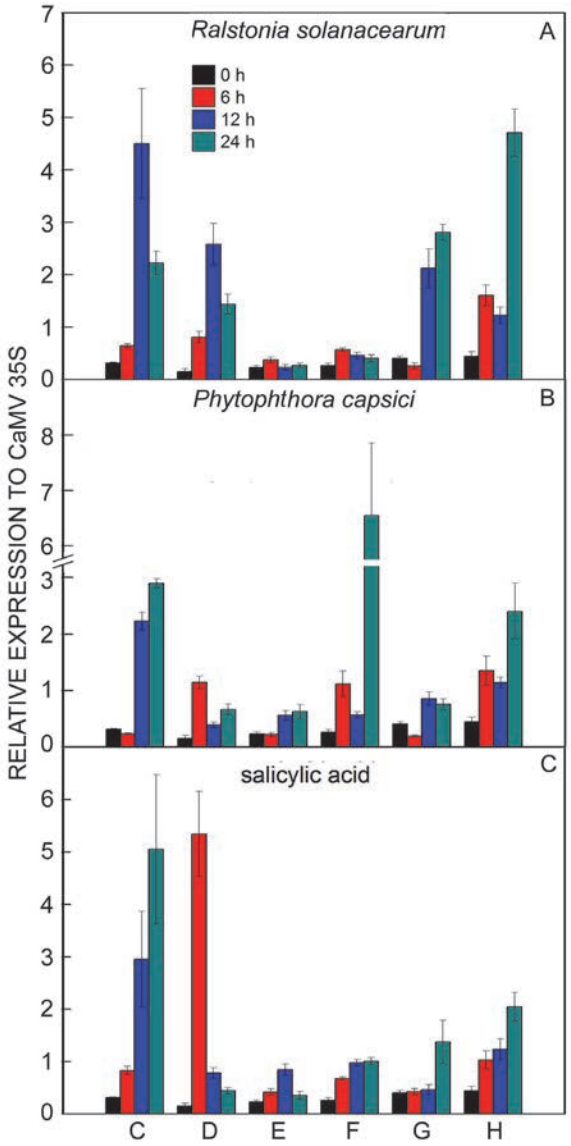

Fig. 3. Transcriptional activities of six synthetic pathogen-inducible promoters (SPIP) in five-week-old transgenic *Arabidopsis thaliana* T_3 lines after being inoculated with *Ralstonia solanacearum* (*A*), *Phytophthora capsici* (*B*), or treated with salicylic acid (*C*). Letters C, D, E, F, G, H mean SPIP GWFS, GWSF, SFGW, SFWG, WGFS, and WGSF, respectively. Analysis of relative *gus* gene expression was performed from three independent experiments by using the Pfaffl method with the transcription level of CaMV 35S-*gus* normalized to 1. The experiments were performed at least three times with similar results. Values are the means ± SDs.

There are many factors that have effects on transcriptional properties of SPIP such as *cis*-element function, *cis*-element copy number, space to TATA-box and location relative to their regulatory targets, and so on (Shokouhifar *et al.* 2011). In the present study, F-box, S-box, Gst1-box, W-box, and the minimal 35S promoter were used to design eight SPIP. These eight SPIP were named FSGW, FSWG, GWFS, GWSF, SFGW, SFWG, WGFS, and WGSF according to the order of DNA oligo-dimers. Except the order of dimers, all other factors were exactly the same in the constructs of the eight SPIP (Fig. 1). However, the basal levels of the eight SPIP showed marked differences and obvious variation trends of transcription at different development stages. As a surprise, FSGW and FSWG had very low basal levels of transcription and almost no inducibility (Fig. 1 Suppl., Fig. 2 Suppl.). When induced by *R. solanacearum*, spores of *P. capsici*, and salicylic acid, the responses of the other six SPIP were markedly different. In other words, each of the SPIP had different response to the same inductor. When treated with different inductors, each of the SPIP showed different transcription activity at different time points upon induction (Fig. 3). GWSF had a relatively low basal level, and the transcriptional level arrived to peak within 12 h (Fig. 3).

Compared with GWSF, GWFS showed a higher basal transcription activity although there was only one position transposition between the S-box dimer and F-box dimer. Compared with GWSF, FSWG had the opposite orientation of dimer order. But the orientation change led to the loss of inducibility. The above results imply that the permutation and combination of motifs had an important influence on transcriptional properties of SPIP. In general, GWSF had the advantages of a low background expression, rapid response, efficient transcriptional activity, and wide range of inducible factors. GWSF is valuable because it could potentially be further improved to apply to plant genetic engineering for disease resistance.

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