A Promoter from Pea Gene DRR206 Is Suitable to Regulate an Elicitor-Coding Gene and Develop Disease Resistance

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ABSTRACT

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Plant nonhost disease resistance is characterized by the induction of multiple defense genes. The pea DRR206 gene is induced following inoculation with pathogens and treatment with abiotic agents, and moderately induced by wounding. A deletion series of DRR206 promoter segments was fused with the β -glucuronidase (GUS) reporter gene and transiently transferred to tobacco, potato, and pea. GUS activity revealed that two upstream regions of the DRR206 promoter were particularly important for activation in the three plant species. Putative *cis* regulatory elements within the DRR206 promoter included a wound/pathogen-

inducible box (W/P-box) and a WRKY box (W-box). Gel shift assays with nuclear extracts from treated and untreated tissue with the W/P-box revealed both similar and unique protein-DNA complexes from pea, potato, and tobacco. Tobacco was stably transformed with gene constructs of the DRR206 promoter fused with a DNase elicitor gene from *Fusarium solani* f. sp. *phaseoli*, FspHDNase. Pathogenicity tests indicated that the FspHDNase elicitor conferred resistance against *Pseudomonas syringae* pv. *tabaci* and *Alternaria alternata* in tobacco. Transgenic potatoes showed some sensitivity to the FspHDNase gene providing less protection against *Phytophthora infestans*. Thus, the elicitor-coding gene, FspHDNase, is capable of generating resistance in a heterologous plant system (tobacco) when fused with defined regions of the pea DRR206 promoter.

In the majority of plant-pathogen interactions, plants mount a rapid disease resistance response (DRR) known as nonhost resistance, a type of general resistance that is difficult to define due to its complexity, involving multiple signals and genes (22). Nonhost resistance occurs when pathogens or pathogen-derived signal molecules (elicitors) trigger the surveillance system of the plant, activating a spectrum of defense genes, thus preventing pathogen invasion. The mechanisms for inducing nonhost disease resistance are not well known (32,46). In contrast, R gene-mediated race-specific resistance is more simply defined in that it occurs in response to the recognition of a host R gene-associated product and a corresponding avirulence (Avr) gene product of the pathogen.

In plants, recognition between the host R gene product and the corresponding Avr gene product results in a heightened intensity of pathogenesis-related (PR) gene induction. Yet recent attempts to genetically engineer disease resistance by overexpressing individual PR genes have usually resulted in little or incomplete resistance (36). A more complete resistance may be possible by inducing a broader array of PR genes, as is typical of the nonhost resistance response. To facilitate this process, some type of trigger would be necessary to activate this natural defense mechanism. One such elicitor, a DNase, has been derived from the fungal pathogen, *Fusarium solani* f. sp. *phaseoli* (FspHDNase). FspHDNase is exuded by the fungus in the presence of the pea host (18) and detected in cytosolic extracts of pea following inoculation of *F. solani* on pea tissue (28). When purified FspHDNase is applied to pea endocarp tissue, the tissue becomes

immune to the true pea pathogen, *F. solani* f. sp. *pisi* (20), and the pea DRR206 gene (along with other PR genes) is induced (5). A gene coding for FspHDNase has been cloned (GenBank Accession No. AF175291) (28) and thus is available for genetic transfer to a plant of interest. Inducible expression of FspHDNase is essential because constitutive activation of FspHDNase within the plant cell may be destructive, as has been described in other gene transfers (23). The promoter driving its expression should be induced at low levels in response to wounding and environmental stresses, but activated rapidly to maximum levels when challenged by a pathogen or elicitors released from the pathogen.

The disease resistance gene DRR206 (7) from pea (*Pisum sativum* L.) encodes a protein that shares homology with a dirigent protein involved in lignin/lignan biosynthesis (9,17). Lignan has antifungal properties, while lignin is an important part of the structural protection as well as defense. DRR206 gene expression is up-regulated during nonhost pathogen interactions with *F. solani* f. sp. *phaseoli* (a bean pathogen) and fungal components such as chitosan (5,16,40). It is also activated in race-specific resistance against races of the bacterium *Pseudomonas syringae* pv. *pisi* (8) and with *F. oxysporum* (21). When the DRR206 gene is expressed constitutively in canola, increased resistance can be observed against a broad range of fungal pathogens including *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Leptosphaeria maculans* (51). In pea endocarp tissue, DRR206 is also induced by DNA-specific abiotic factors (5). Because of this multiple-factor inducibility, we further evaluated the DRR206 promoter.

As an initial step to evaluate the activity of the DRR206 promoter in a heterologous system, a construct of a 2.7-kb segment of the DRR206 promoter fused with the β -glucuronidase (GUS) reporter gene was prepared and stably transformed into tobacco (5). Some differences in GUS activity in the heterologous system of tobacco were observed relative to that of pea when the

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DRR206 promoter was activated by treatments with abiotic elicitors (5). Because nuclear proteins interacting with the DRR206 promoter are also likely to vary between plant species, differences in the gene-activating responses between pea and other plants were expected. However, when the pea DRR206 promoter::GUS reporter was heterologously transferred to tobacco, biotic elicitors, including those released by a tobacco pathogen, activated this promoter as indicated by increased GUS activity. Therefore, the pea DRR206 promoter possesses features of universal regulation that can be used to develop disease-resistant plant species other than pea.

Herein, we examined the pea DRR206 promoter to define broad regions involved in wound and pathogen inducibility and to determine if this promoter is comparably functional in other plant species. DRR206 promoter activity was characterized by promoter deletion analyses and fusions of these deletions with the GUS reporter gene. GUS activity was monitored following transient transformation via agroinfiltration. Results of these experiments indicate that two segments (2.7 and 1.9 kb) of the DRR206 promoter activated the GUS reporter in potato, tobacco, and pea. To evaluate a potential role of these promoter segments in the development of disease-resistant plants, tobacco and potato plants were transformed with constructs of the DRR206 promoter segments (2.7 and 1.9 kb) fused with the elicitor-coding gene, *FspH*DNase. Resistance generated by these transgenic lines was effective in response to certain pathogens.

MATERIALS AND METHODS

DRR206 promoter deletion constructs. Primers were designed to amplify segments of the DRR206 gene sequence of approximately 400 bp, upstream of the DRR206 start site. For certain segments, restriction enzymes were used to obtain fragments of the desired length. Using the Genetics Computer Group program (Madison, WI), primers were designed for the 2.3 kb (P206F2/P206R), 1.4 kb (P206F4/P206R), and 500 bp (P206F6/P206R) segments as follows: P206F2 (5'-ATTATACCATATTGGACCAGCC-3'), P206F4 (5'-TCCACAATTCACCCAACC-3'), P206F6 (5'-CACCTCTATCAACGTCATGGTC-3'), and P206R (5'-TTTATTCTTAGCTATTGGAAGTTG-3'). Each polymerase chain reaction (PCR) contained 0.1 µg of DC206-16A pBS plasmid/2.7-kb promoter, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 250 µM dNTPs, 2.5 mM MgCl₂, 1 µl each (1 pmol) of forward and reverse primers, and 1.5 units of *Taq* polymerase (Promega, Madison, WI) µl⁻¹. PCR amplification was performed with a thermocycler (Hybaid PCR Sprint; Midwest Scientific, Valley Park, MO) with an initial 2.5-min step at 94°C followed by 30 cycles of 15 s at 94°C, 20 s at 55°C, and 2 min at 72°C, and a final extension of 7 min at 72°C. Each fragment was cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. The 1.9-kb fragment was derived from *Pst*I/*Sca*I digestion, while the 850-bp fragment was derived from *Nsi*I/*Sca*I digestion. Each of the fragments was subcloned into pBS, sequenced, inserted into the binary vector pBI101 (Clontech Labs, Palo Alto, CA), and electroporated into *Agrobacterium tumefaciens* (strain LBA4404) with a Gene Pulser II System (Bio-Rad Laboratories, Hercules, CA).

Agroinfiltration of DRR206 promoter::GUS constructs. Wild-type tobacco (*Nicotiana tabacum* L. cv. Xanthi) was seeded and grown for 8 weeks in a greenhouse. *Solanum tuberosum* L. cv. Russet Burbank was grown from tubers and maintained in a greenhouse for 7 weeks, while *Pisum sativum* L. was grown from seed in sand for approximately 2 weeks in a greenhouse. *Agrobacterium tumefaciens* containing the above-mentioned DRR206 deletion constructs were grown for 2 days in YEP (10 g of Bacto-yeast extract, 10 g of Bacto-peptone, and 5 g of NaCl per liter) cultures to a final density adjusted to A₄₂₀ of 1.8 to 2 optical density in 5 g of Murashige and Skoog based salt mixture (MS),

1.95 g of MES, and 20 g of sucrose liter⁻¹ supplemented with 200 µM acetosyringone (Aldrich Chemical Co., Milwaukee, WI). Plants were agroinfiltrated with approximately 100 µl of the bacterial suspensions. Leaf disks were harvested from the infiltrated areas with a cork borer at 1 to 6 days postinfiltration (dpi) and immediately frozen in liquid nitrogen.

GUS assays. Leaf disks were ground in liquid nitrogen with a pestle-tipped Dremel drill immediately followed by the addition of GUS extraction buffer (50 mM NaPO₄, pH 7; 10 mM EDTA; 0.1% [vol/vol] sarcosyl; 0.1% [vol/vol] Triton X-100; and 10 mM β-mercaptoethanol) at 177 µl cm⁻² of leaf tissue. Ten microliters of plant extract was assayed for GUS activity by incubation for 15 min at 37°C with 150 µl of 1.5 mM 4-methylumbelliferyl-β-glucuronide (Sigma Chemical, St. Louis). The reaction was stopped with 0.2 M Na₂CO₃ in a final volume of 1.5 ml. Fluorescence was measured with a fluorometer (Model TKO 100; Hoefer Scientific, San Francisco) with an excitation of 365 nm and emission of 460 nm.

Statistical analysis. GUS data were subjected to analysis of variance using the least significant difference model procedure of SAS (SAS Institute, Cary, NC).

Analysis of the DRR206 promoter for *cis* regulatory elements. Analyses of the DRR206 promoter region for putative *cis*-acting elements entailed the use of several programs with default settings. The programs included BLAST (available online from the National Center for Biotechnology Information); TESS (44) (Transcription Element Search Software, available online from the Computational Biology and Informatics Laboratory, University of Pennsylvania); and TRANSFAC (Transcription Factor Database; available online from Biological Databases) containing eukaryotic *cis*- and *trans*-acting regulatory DNA elements.

Electrophoretic mobility shift assay. Pea nuclear proteins were extracted from 8-day-old seedlings that had been kept in the dark and either inoculated for 13 h with *F. solani* f. sp. *phaseoli* or noninfected. Nuclear proteins were extracted from immature pea pods that were untreated or inoculated with *F. solani* f. sp. *phaseoli* for 6 h. Tobacco leaves or potato leaflets were collected 6 h after they were wounded with a 3 MM abrasive sponge, treated with chitosan (1 mg ml⁻¹) and wounded with an abrasive sponge, or untreated. Tissue was ground using a Waring blender or mortar and pestle with liquid nitrogen. Nuclear proteins were isolated according to Jensen et al. (25) with modifications. Three milliliters of cold buffer A (10 mM NaCl, 10 mM MES-NaOH [pH 6.0], 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.6% Triton X-100 [vol/vol], 0.25 M sucrose, 20 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride) was added for each gram of fresh tissue. The crude extract was filtered through two layers of Miracloth (Calbiochem, San Diego) and centrifuged for 5 min at 4,000 × g. The pellet was suspended in 0.5 ml of buffer B/NP-40 g⁻¹ (10 mM MES-NaOH [pH 6.0], 260 mM sucrose, 10 mM NaCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1% Nonidet P-40 [vol/vol], and 14.3 mM β-mercaptoethanol) and repeated. Nuclei were pelleted by centrifugation and resuspended in buffer C (20 mM HEPES-KOH [pH 7.9], 0.2 mM EDTA, 420 mM NaCl, and 1.5 mM MgCl₂) and then were lysed by sonication for 1 min. After 30 min on ice, samples were centrifuged for 15 min at 12,000 × g, and the supernatant was quantified with a Bradford assay. Glycerol was added to the extract to 20% (vol/vol) and stored at -70°C.

Two complementary single-stranded 18-mer oligonucleotides (5'-CCTTCCACCACCTTTA-3' and 5'-GGTGTAAGGTATGGTGG-3') were synthesized (Operon Technologies, Alameda, CA) and annealed in equimolar amounts to obtain the wound/pathogen-inducible region (W/P-box) -1,209 to -1,188. This resulted in a double-stranded 22 mer with 4-bp overhangs for end labeling. The end-labeling reaction contained Klenow (3 u), approximately 1 µg of double-stranded W/P-box oligomer, 1× buffer minus dATP (-dATP) (Ambion, Austin, TX), and [³²P]dATP

(20 to 40 μCi) with a specific activity of 3,000 Ci mmol^{-1} . The reaction mix was incubated for 30 min at 37°C and separated on a Sephadex G-50 column to remove unincorporated nucleotides. For the W-box probe, complementary oligonucleotides of 18 bases in length containing a single WRKY box (W-box) (5'-TT-GACTAAACGTCATGGT-3' and 5'-TAAGACCATGACGTT-TAG-3') were synthesized (Operon Technologies) and annealed. The W-box probe was end-labeled as described previously.

The conditions of the gel shift experiments were essentially those described by Jensen et al. (25). The binding reaction mix for 30 to 40 μl contained 3.5 pmol of end-labeled DNA probe, 25 mM HEPES (pH 7.9), 10 mM CaCl_2 , 1 mM dithiothreitol (DTT), 10% (vol/vol) glycerol, 10 μg of nuclear extract, and competitors (dA/dT [1 μg], dI/dC [1 μg], or unlabeled cold probe as specified). This was incubated for 20 min at room temperature and loaded on 6% polyacrylamide gel in 1 $\times$ TGE buffer (1 liter of 10 $\times$ TGE buffer contains 30.3 g of Trizma base, 142.7 g of glycine, and 20 ml of 0.5 M EDTA). Electrophoresis was performed at 150 V for 2 h. Following electrophoresis, gels were dried under vacuum at 60°C for approximately 30 min. Gels were autoradiographed overnight at -70°C with an intensifier screen or at room temperature without an intensifier screen.

Inoculation of pea tissue with pathogens. Each of the pathogens, except *P. infestans*, were grown on potato dextrose agar (PDA) and allowed to sporulate. *Phytophthora infestans* (US#8) sporangia were maintained on infected potato leaflets. Spores were scraped from the plate or rinsed from infected potato leaflets and suspended in sterile water. Immature pea pods ranging from 1 to 2 cm in length were split and challenged on the inner endocarp surface with the following pathogens: *Colletotrichum coccodes* (6.6×10^6 spores ml^{-1}), *Verticillium dahliae* (2×10^7 spores ml^{-1}), *Phytophthora infestans* (4×10^5 spores ml^{-1}), *Rhizoctonia solani* (4×10^5 spores ml^{-1}), *Fusarium solani* f. sp. *pisi* (1.6×10^6 spores ml^{-1}), or *F. solani* f. sp. *phaseoli* (1.6×10^6 spores ml^{-1}) for 3 and 6 h.

RNA isolation and northern blotting. RNA was extracted according to Chomczynski and Sacchi (6) with modifications. About 150 mg of tissue was frozen in liquid nitrogen and ground with 1 ml of extraction buffer (0.8 M guanidine thiocyanate; 0.4 M ammonium thiocyanate; 0.1 M sodium acetate, pH 5; 5% [vol/vol] glycerol; and 38% [vol/vol] water-saturated acid phenol, pH 4.0). The mixture was incubated with 200 μl of chloroform at room temperature for 2 to 15 min and centrifuged at $10,000 \times g$ for 15 min. The supernatant was removed, and RNA was precipitated. After centrifugation at $10,000 \times g$ for 10 min, the pellet was suspended in 0.1% (vol/vol) diethyl pyrocarbonate-treated water.

Ten micrograms of total RNA was electrophoresed on a 1.2% agarose gel with 0.67 M formaldehyde. RNA was blotted in 10 $\times$ SSC (1.5 M NaCl and 150 mM trisodium citrate) onto a Hybond N nylon membrane (Amersham). Membranes were incubated at 60°C overnight in prehybridization solution (10 $\times$ Denhardt's solution [50 $\times$ Denhardt's is 1% (wt/vol) polyvinylpyrrolidone, 1% (wt/vol) bovine serum albumin fraction V, and 1% (wt/vol) Ficoll 400]; 3 $\times$ SSC; 10% [wt/vol] dextran sulfate; 7% [wt/vol] sodium dodecyl sulfate [SDS]; and 150 μg of single-stranded salmon sperm DNA ml^{-1}). A 513-bp *FsphDNase* genomic clone was labeled with a random primer labeling kit (DECAprime II; Ambion) according to the manufacturer's instructions using 50 μCi [^{32}P]dATP with specific activity of 3,000 Ci mmol^{-1} . The labeled probe was spun on a Sephadex 50 to 150 column to remove excess [^{32}P]dATP. The labeled probe was added to the prehybridization solution and incubated for 18 to 24 h at 60°C. Membranes were washed twice in 2 $\times$ SSC (for 20 $\times$, 3 M NaCl, 0.3 M trisodium citrate) containing 0.1% (wt/vol) SDS for 15 min at room temperature, and twice more for 15 min at the hybridization temperature. For northern blots involving detection of the pea DRR206 message, 5 μg of total RNA was electrophoresed,

blotted, and probed as described previously (5). Membranes were exposed overnight to X-Omat Blue film (Kodak, Rochester, NY) with an intensifier screen at -70°C.

Preparation of DRR206 promoter::FsphDNase constructs. The 7.1 constructs contained the 2.7-kb segment of the DRR206 promoter fused to *FsphDNase* with and without the GUS gene, respectively. The 8.1 constructs contained the 1.9-kb segment of the DRR206 promoter fused to *FsphDNase* with and without GUS, respectively. Each was ligated into the binary vector, pBI101, following removal of the GUS gene. Subsequently, the constructs were electroporated into *Agrobacterium tumefaciens* strain LBA4404 and selected on YEP plates containing kanamycin (100 μg liter^{-1}) and streptomycin (100 μg liter^{-1}). A single colony was inoculated into 2 ml of YEP supplemented with kanamycin and streptomycin (each 100 μg ml^{-1}) and grown for 1 day. The culture was transferred to 50 ml of YEP with kanamycin and streptomycin (each 100 μg ml^{-1}). Overnight bacterial suspensions were centrifuged at $1,000 \times g$ for 10 min. The bacteria were resuspended in YEP.

Stable transformation of DRR206 promoter::FsphDNase constructs. *Agrobacterium tumefaciens* containing the DRR206::FsphDNase constructs were incubated with leaf disks from *N. tabacum* cv. Xanthi in YEP medium for approximately 30 s. Leaf disks were placed onto co-cultivation media (MS salts, 30 g of kinetin liter^{-1} , 0.3 mg of benzyl adenine purine [BAP] liter^{-1} , and 1.8 g of phytigel liter^{-1}). After 3 days, the disks were transferred to the selection media (same as the co-cultivation media, except supplemented with 30 g of sucrose liter^{-1} , 50 mg of kanamycin liter^{-1} , 250 mg of carbenicillin liter^{-1} , and B5 vitamin, pH 5.7). When shoots became visible, they were transferred onto MS rooting media (MS salts, 30 g of sucrose liter^{-1} , 250 mg of carbenicillin liter^{-1} , 50 mg of kanamycin liter^{-1} , 1.8 g of phytigel liter^{-1} , B5 vitamin, pH 5.6). Only those that continued to root on kanamycin (T_0) were transported to a greenhouse and screened with *Pseudomonas syringae* pv. *tabaci* at 2×10^5 CFU ml^{-1} . T_0 plants showing delayed, increased resistance to wildfire symptoms caused by *Pseudomonas syringae* pv. *tabaci* were selfed to collect the T_1 generation. The seeds from the T_1 lines were plated on 1/2MS (30 g of sucrose and 1.8 mg of phytigel liter^{-1}) with 300 mg of kanamycin liter^{-1} . Those that grew vigorously were screened with *Pseudomonas syringae* pv. *tabaci* at three different concentrations (1×10^7 , 1×10^5 , and 1×10^3 CFU ml^{-1}). Those exhibiting resistance were selfed and the T_2 generation was collected. Seeds collected from tobacco were surface-sterilized and plated on 1/2MS containing 300 mg of kanamycin liter^{-1} .

Russet Burbank potatoes were maintained on propagation and rooting medium (MS salts, vitamin B5, MSVI vitamins [2 mg of glycine liter^{-1} , 0.5 mg of nicotinic acid liter^{-1} , 0.5 mg of pyridoxin-HCl liter^{-1} , and 0.4 mg of thiamine-HCl liter^{-1}], JHMS vitamin [0.25 mg of folic acid and 0.05 mg of d-biotin liter^{-1}], 100 mg of inositol liter^{-1} , 0.02 mg of 2-naphthalene acetic acid [NAA] liter^{-1} , 30 g of sucrose liter^{-1} , and 6 g of agar liter^{-1} , pH 5.6). Sections of internodes, petioles, and leaflets containing the major vein were cut into YEP and transformed with *Agrobacterium tumefaciens* containing each construct as described previously. The sections were placed on plates containing callus-inducing medium (MS salt, MSVI and JHMS vitamins, 100 mg of inositol liter^{-1} , 30 g of sucrose liter^{-1} , 1 mg of BAP liter^{-1} , 2 mg of NAA liter^{-1} , 50 mg of kanamycin liter^{-1} , and 6 g of agar liter^{-1} , pH 5.6). The explants were transferred onto 3C5ZR medium (MS salts [pH 5.9], vitamins [1 mg of thiamine-HCl liter^{-1} , 0.5 mg of nicotinic acid liter^{-1} , and 0.5 mg of pyridoxine-HCl liter^{-1}], 100 mg of *myo*-inositol liter^{-1} , 3 μM indole acetic acid, 5 mM *trans*-zeatin riboside, 30 g of sucrose liter^{-1} , 8 g of agar liter^{-1} , 50 mg of kanamycin liter^{-1} , and 100 mg of timentin liter^{-1}) and incubated at 25°C with a 16 h photoperiod. The regenerated shoots were excised and transferred to rooting medium (MS salt, MSVI vitamins, JHMS vitamins, 100 mg of inositol liter^{-1} , 30 g

of sucrose liter⁻¹, 0.02 mg of NAA liter⁻¹, 6 g of agar liter⁻¹, and 100 mg of kanamycin liter⁻¹, pH 5.6) to induce root formation. Regenerated shoots showing vigorous root growth in kanamycin-containing media were propagated and transplanted in a greenhouse.

DNA isolation and PCR screening of transgenics. DNA was isolated as outlined by Karcher (27). The DNA was redissolved in 100 µl of 1 mM Tris and 0.1 mM EDTA, pH 8.0. Screening of each of the transgenic lines was performed with gene-specific primers designed for FspHNDase 22KDGS3P-1 (5'-CTACCGCT-ATCATGGCCTTTGTC-3') and W822REV (5'-CAGGGTGGTG-ATGAGGTGAG-3') and amplified a fragment of 481 bp. Each PCR reaction consisted of 10 ng of genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (vol/vol) Triton X-100, 250 µM dNTPs, 2.5 mM MgCl₂, 1 µl each (1 pmol) forward and reverse primers, and 1.5 units of *Taq* polymerase (Promega). Separate reactions were performed with genomic DNA from the wild-type tobacco (– control) and *F. solani* (+ control). PCR amplification was performed with an initial 3-min step at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C, and a final extension of 7 min at 72°C.

Pathogenicity tests. For screening of tobacco lines, rifampicin-resistant *Pseudomonas syringae* pv. *tabaci* was grown on nutrient broth-yeast (NBY) extract (for 1 liter, 8 g of nutrient broth, 2 g of yeast extract, 2 g of K₂HPO₄ anhydrous, 0.5 g of KH₂PO₄, 15 g of Bacto-agar, 50 ml of 10% [wt/vol] glucose, and 1 ml of 1 M MgSO₄) supplemented with rifampicin (100 mg liter⁻¹). Colonies were scraped from the plates and suspended in sterile water to attain an optical density of A₄₂₀ = 0.3, the equivalent of 1 × 10⁸ CFU ml⁻¹. The bacterial suspension was further diluted to 1 × 10⁷, 1 × 10⁵, and 1 × 10³ CFU ml⁻¹ to perform screening of the T₀ and T₁ transgenic tobacco lines.

For inoculations and subsequent colony counts, concentrations of rifampicin-resistant *Pseudomonas syringae* pv. *tabaci* were adjusted to 1 × 10⁵ CFU ml⁻¹ in sterile water, and 100 µl of the suspension was infiltrated into tobacco leaves with a needleless syringe in two areas per leaf, per plant. Four plants were used in each experiment, and the experiment was repeated at least three times for each time point. Eight leaf disks (1.5 cm in diameter) were combined and ground in a mortar and pestle with 1 ml of phosphate buffer (12.5 mM, pH 7.1). A 1:10 dilution series was prepared in phosphate buffer, and 100 µl of each dilution was plated on NBY medium supplemented with rifampicin (100 mg liter⁻¹). Population counts of *Pseudomonas syringae* pv. *tabaci* were conducted at 1, 3, and 5 dpi.

Alternaria alternata (Fr.:Fr.) Keissl. *longipes* spores were harvested in sterile water and the concentration was adjusted to 1 × 10⁵ spores ml⁻¹. Fifty microliters of the spore suspension was pipetted on sterile 1 cm² Whatman filter squares and placed on immature tobacco leaves that had been misted with water. Immature leaves were placed in humid chambers for 7 to 8 days at room temperature. This was repeated with a total of six plants from each of the transgenic tobacco lines.

Fig. 1. Northern analysis of defense gene DRR206 expression in pea pod tissue following challenge with various plant pathogens: *Colletotrichum coccoodes* (6.6 × 10⁶ spores ml⁻¹), *Verticillium dahliae* (2 × 10⁷ spores ml⁻¹), *Phytophthora infestans* (4 × 10⁵ spores ml⁻¹), *Rhizoctonia solani* (4 × 10⁵ spores ml⁻¹), *Fusarium solani* f. sp. *pisi* (1.6 × 10⁶ spores ml⁻¹), and *F. solani* f. sp. *phaseoli* (1.6 × 10⁶ spores ml⁻¹) for 3 and 6 h. The blot was hybridized with a ³²P-labeled 800-bp fragment of gene DRR206. Ethidium bromide staining of rRNA is shown.

RESULTS

Previous work indicated that pea gene DRR206 can be activated by various elicitors as well as compatible and incompatible pathogens (5). To further document the range of this response, immature pea endocarp tissues were challenged with various potato pathogens (Fig. 1). Each of these pathogens induced accumulation of the DRR206 message at 6 h postinoculation (Fig. 1). As noted previously (5), a lower level of the DRR206 gene activation occurs at 6 h in pea pods, probably as a result of handling, harvesting, splitting, and water treatment (Fig. 1).

To discern the important *cis*-acting regions of the DRR206 promoter, segments of the promoter were fused upstream of the GUS reporter gene and transiently transferred to three different plants by agroinfiltration (Fig. 2) as a preliminary assay of the regions contributing to activation. The nomenclature for each of these different fragments was derived from the estimated size, namely 2.7 kb, 2.3 kb, 1.9 kb, 1.4 kb, 850 bp, and 500 bp. Overall, the transient transformation data from tobacco, potato, and pea indicated that the use of the full-length 2.7-kb and the 1.9-kb fragments resulted in a significant activation of the GUS reporter

Fig. 2. Agroinfiltration of A, potato, B, pea, and C, tobacco leaves with constructs of DRR206 promoter segments (2.7 kb, 2.3 kb, 1.9 kb, 1.4 kb, 850 bp, and 500 bp) fused with the β-glucuronidase (GUS) reporter gene. Units of fluorescence indicate reporter gene activity as observed in GUS assays. Each assay for potato and pea was performed with three leaf disks from three plants for each time point (1, 3, and 5 days postinfiltration [dpi]). For potato, the assays were replicated in triplicates, and for pea, the assays were replicated twice. Means of the three time points with the same letter are not significantly different according to Fisher's protected least significant difference (LSD) test (*P* = 0.05). For tobacco, four plants were analyzed at 6 dpi in each experiment, and data represent the combined analyses from three experiments. Means with the same letter are not significantly different according to Fisher's protected LSD (*P* = 0.05). Control = nontransformed plants; 35S = 35S::GUS construct; pBI101 = promoterless construct; and pBI121 = constitutive 35S promoter construct.

Examination of the sequence between the 1.4-kb and 850-bp segment indicated the presence of a W/P-box at -1,199, having the sequence TTACCTTCT (34,54) with the consensus sequence underlined (Fig. 3). A W-box element (TGAC-N_x-GTCA) (42) is located at -231 to -219. Other putative *cis*-acting elements of the pea DRR206 gene promoter that may be involved in defense are symbolized and listed in Figure 3.

The binding affinity of pea seedling nuclear extracts to the 22-bp oligonucleotide probe (W/P-box) in comparison with extracts from potato leaves or pea pod tissue was sufficiently intense to obscure individual complexes. Therefore, a nonspecific competitor, poly[dA/dT], was added at 1 μ g to the pea pod and seedling nuclear extract (Fig. 5, lanes 2 to 4) to more clearly resolve these bands. Tobacco nuclear proteins also had a relatively high affinity and therefore 0.5 μ g of poly[dA/dT] was added to increase the resolution of the complex (Fig. 5, lanes 8 to 10). When the migration patterns of the complexes between the W/P-box and the nuclear extracts from the three different plant species were compared, different patterns of protein–DNA complexes were observed (Fig. 5). For example, in potato, the same numbers of protein–DNA complexes were observed between wounded and untreated tissues. However, the intensity of several complexes increased when the leaves were wounded and treated with chitosan (Fig. 5, lanes 6 and 7) relative to the untreated control (Fig. 5, lane 5). That pattern was different than the binding pattern observed between the W/P-box and tobacco nuclear extracts (Fig. 5, lanes 8 to 10). In this instance, the low mobility complexes (upper bands) were undetectable while the faster migrating complex (lower band) increased in intensity when the leaves were wounded and chitosan was applied (Fig. 5, lanes 9 and 10). In contrast, gel shift assays with pea pod extracts (Fig. 5, lanes 2 and 3) revealed

Fig. 3. Schematic representations of the putative *cis*-acting elements present in the pea DRR206 promoter sequence and the lengths of the various promoter segments used in this study. Elements are shown as stated, two hashed arrows (two perfect repeats). ¹Palm et al. (34) and Wu et al. (53); ²Goldsbrough et al. (19); ³Rushon and Somssich (42); ⁴Puzio et al. (37) and Escobar et al. (12); ⁵Baker et al. (1); ⁶Buttner and Singh (3); ⁷DeGreve et al. (10); and ⁸Foster et al. (15) and Singh et al. (45).

that the lower mobility DNA–protein complex exhibited substantially reduced binding affinity when challenged with *F. solani* f. sp. *phaseoli*. A similar slow migrating complex was abolished when tobacco was wounded or treated with chitosan.

To examine the role of the pea DRR206 promoter in the generation of disease resistance in a heterologous plant system, tobacco and potato were stably transformed with gene fusions of the DRR206 promoter (2.7- and 1.9-kb segments) and a cDNA encoding the FspHDNase elicitor. PCR screenings were conducted and plants lacking the 481-bp PCR product were terminated. When the DRR206 promoter::FspHDNase gene fusion was stably expressed in tobacco and challenged with *Pseudomonas syringae* pv. *tabaci*, decreased numbers of colony forming units were observed following dilution plating at 3 dpi (Fig. 6A) relative to the control plant, pBI. Disease spread was readily evident on tobacco leaves not containing the DRR206 promoter::FspHDNase construct when inoculated at 2×10^5 and 2×10^7 CFU ml⁻¹ of *Pseudomonas syringae* pv. *tabaci* (Fig. 6B). In contrast, the water-soaked lesions and disease spread were not readily apparent in two T₁ lines (8.1-4b and 7.1-6) of transgenic tobacco containing the DRR206 promoter::FspHDNase construct (Fig. 6B). Subsequently, the T₂ lines were screened and tested for resistance against *Alternaria alternata*. These experiments revealed that some of the transformants decreased the size of the necrotic lesions (Fig. 7) and the radius of the toxin diffusion around these lesions. The DRR206 promoter::FspHDNase construct was also stably transferred to Russet Burbank potatoes (data not shown). Of the 20 transformation lines, lines 16A and 34B were the most resistant against *Phytophthora infestans* strain US#8. These and several other lines were subsequently propa-

gated by cuttings, and tubers were repeatedly tested for resistance against *P. infestans* and *C. coccodes*. Northern analysis of the expression of FspHDNase in two of these potato lines (7.1-16A and 7.1-34B) indicated that the expression of FspHDNase was similar between the two lines when inoculated with *Phytophthora infestans* and chitosan treatment (Fig. 8). Wounding induced the expression of FspHDNase at a higher level in potato line 16A than in potato line 34B (Fig. 8). The inoculated potato tissue appeared sensitive to the enhanced presence of FspHDNase product, and the resistance to *Phytophthora infestans* generated in the engineered plants requires further promoter modification. Most of the non-challenged transformants showed no detectable morphological differences from the wild type when stably transformed with the DRR206::FspHDNase construct.

These same transgenic lines were also challenged with *C. coccodes* either in the intercellular spaces of the leaf or as a soil inoculum. *C. coccodes* is known to produce copious units of DNase activity on its own (52). As a consequence, the leaf infusions rapidly formed lesions that restricted further lateral growth that was bordered by regions of dead chlorotic cells. The chlorosis continued in the transgenic tissue to engulf most of the leaf, making this transgenic construct of doubtful use for resistance to *C. coccodes* in potato. Similarly, the potatoes grown in *C. coccodes*-inoculated soil also developed symptoms comparable to the controls, except that the control plants remained smaller.

DISCUSSION

A major objective of this study was to identify segments of the pea DRR206 promoter that may be responsive to various stimuli, including plant pathogens or elicitors released from pathogens. Such an inducible promoter may ultimately prove to be an in-

Fig. 4. Electrophoretic mobility shift assay of pea seedling nuclear protein with ³²P-labeled oligonucleotides containing a single copy of either the WRKY box (W-box) or the wound/pathogen-inducible box (W/P-box). Ten micrograms of the protein was incubated with W-box probe (lanes 2 to 5) or W/P-box probe (lanes 7 to 10) along with competitor poly[dA/dT] oligonucleotides (1 µg each lane). Competition with unlabeled cold probe was performed at the indicated concentrations in micrograms. Lanes 1 and 6 contain labeled probe without additional proteins.

Fig. 5. Electrophoretic mobility shift assay comparing binding affinities of nuclear proteins from pea, tobacco, and potato to a ³²P-labeled wound/pathogen-inducible box (W/P-box) oligonucleotide derived from the pea DRR206 promoter. Nuclear proteins were derived from untreated (U) pea pods or pea pods treated (T) with *Fusarium solani* f. sp. *phaseoli* for 6 h (lanes 2 and 3); pea seedlings (lane 4); potato leaves untreated (U), wounded (W), or treated with chitosan (C) at 1 mg ml⁻¹ (lanes 5 to 7); and tobacco leaves untreated (U), wounded (W), or treated with chitosan (C) at 1 mg ml⁻¹ (lanes 8 to 10). Lane 1 contains the ³²P-labeled W/P-box probe only. The binding reaction loaded on lane 4 contained 1 µg of poly[dA/dT] oligonucleotides and lanes 8 to 10 contained 0.5 µg of poly[dA/dT] oligonucleotides due to higher affinity of nonspecific binding.

valuable tool in developing disease resistance in other plants. Figure 2 indicates that full activity of the DRR206 promoter is dependent on regions of the promoter spanning from 2.7 to 2.3 kb and 1.9 to 1.4 kb. The pea DRR206 gene, inherently within pea endocarp tissue, is inducible from essentially an inactive state in nontreated tissue, to a low level by wounding, and to a highly active state when induced by fungal challenge, fungal elicitors, and abiotic elicitors (5). Wound inducibility of the DRR206 promoter has a positive role in engineering resistance. Wounding of plant tissues provides a means of entry for pathogens into the plant. Therefore, the DRR206 promoter in combination with the appropriate elicitor should provide additional protection prior to the challenge presented by the pathogen. The DRR206 promoter was further investigated to understand features of the promoter that are associated with up-regulation or down-regulation so that the appropriate promoter segments could be utilized to trigger a broad nonhost resistance response when fused upstream of a gene encoding the FspHDNase elicitor.

Additional characterization of the DRR206 promoter revealed that the 2.7- and 1.9-kb segments of the pea DRR206 promoter were significantly activated across three different plant species using the transient transformation method of agroinfiltration, a technique for nonstable transformation of plant cells. Agroinfiltration bypasses positional effects of gene expression that arise when the transgene is incorporated into regions of the chromatin that are not actively transcribed or are subject to regional influences (26,41). Yang et al. (54) demonstrated the powerful use of

agroinfiltration in tobacco leaves by identifying the *cis*-regulatory regions of promoters that are responsive to salicylic acid signaling. But, agroinfiltration can confound evaluations of elicitor treatments because the transfer relies on the presence of substantial amounts of bacterial cells, which possess elicitors of their own. Thus, the process of agroinfiltration, and the wounding that accompanies this process, activates the DRR206 promoter and the activity is maintained for many days following infiltration. Consequently, agroinfiltration was confined to analyzing potential response elements of the DRR206 promoter.

Transient transformation also required optimization for each plant species. Agroinfiltration of tobacco resulted in optimal GUS activity throughout 6 dpi, yet the optimal activity was observed at 3 dpi for potato and 5 dpi for pea. Reproducibility of the agroinfiltration experiments was dependent on the use of optimal tissue under uniform conditions, as observed by Yang et al. (54), who also found that consistent GUS activity requires the use of leaves that are not yet fully expanded. Also, *Agrobacterium tumefaciens* requires a wound-released phenolic signal molecule from the plant to trigger the *vir* genes necessary for T-DNA transfer. Hence, supplementation of acetosyringone as a signal is an integral part of agroinfiltration.

Analysis of GUS reporter activity in the various plants revealed that the DRR206 gene promoter was not similarly responsive between different plant species, even within the same family (i.e., Solanaceae). Different patterns of protein–DNA complexes observed with different plant nuclear extracts in the electrophoretic

Fig. 6. Resistance of T₂ transgenic tobacco lines stably transformed with a DRR206 promoter::FspHDNase construct. **A**, Leaves of four tobacco plants from each transgenic line were inoculated with approximately 100 μ l of a bacterial suspension of *Pseudomonas syringae* pv. *tabaci* (10⁵ CFU mL⁻¹). Two areas were inoculated on each leaf for a total of eight areas on each tobacco plant line. Leaf disks were harvested at 1 or 3 days postinfiltration (dpi), combined (eight disks), and ground in a mortar and pestle with phosphate buffer and plated in replicate for counting. Tobacco lines included pBI (35S::GUS), 7.1 (2.7-kb DRR206 promoter::FspHDNase), and 8.1 (1.9-kb DRR206 promoter::FspHDNase). Letter designations (b to d) represent plants within each transformation event. Error bars indicate standard deviation from the mean. **B**, Pathogenicity symptoms of tobacco T₁ lines 8.1-4b and 7.1-6 transformed with constructs of 1.9-kb DRR206 promoter::FspHDNase and 2.7-kb DRR206 promoter::FspHDNase, respectively. The pBI line contains 35S::GUS. The symptoms were observed at 7 dpi following inoculation of the leaves with 2 $\times$ 10³, 2 $\times$ 10⁵, and 2 $\times$ 10⁷ CFU mL⁻¹ of *Pseudomonas syringae* pv. *tabaci*.

mobility shift assays with the W/P-box may be indicative of this result. That is, various components of the transcriptional machinery that drive PR gene expression are likely to be different between plant genera (39). Electrophoretic mobility shift assays with the W/P-box and nuclear extracts revealed binding by multiple factors in pea, potato, and tobacco. In contrast, only a single complex was observed between pea nuclear extracts from seedlings and the W-box. Mobility shift assays with the W/P-box probe resulted in similar-sized protein–DNA complexes in potato, tobacco, and pea, with potato possessing an additional complex. Pea nuclear extract from the seedling stage had an ≈ 50 -fold affinity for the pea-based probe relative to tobacco, potato, and even pea pod nuclear proteins binding to the same region. Thus, the W/P-box may be an important element recognized during development, recruiting factors in developing seedlings. Additionally, similar to the observations of Van der Hoorn et al. (50), only a moderate level of GUS expression associated with agroinfiltration was observed in *Pisum sativum* in comparison to the level observed in solanaceous plants. One plausible explanation is that pea plants have smaller leaves with less intercellular space accessible to bacteria. Furthermore, potato, a solanaceous plant, had diminished reporter gene activity when compared with *N. tabacum* cv. Xanthi, another solanaceous plant.

Eukaryotic gene regulation involves the concerted efforts of both *cis*- and *trans*-acting elements. The DRR206 promoter contains one site that bears identity with a W-box binding site for WRKY transcription factors (13,14). These factors, having the conserved WRKYGQK amino acid motif, are unique to plants and are defined by their ability to recognize and bind to W-boxes (T)TGAC(C), TGAC-Nx-GTCA (42). Several recent studies have underscored the importance of the W-boxes in host–pathogen interactions (13,55). For example, the St-WRKY1 gene encoding a WRKY-like transcription factor, is induced when potato is challenged with *Erwinia carotovora* subsp. *atroseptica* (11). The W-box also occurs in many defense gene promoters including the class I basic chitinase promoter of potato and tobacco (2,17). Promoter deletion analyses in this study indicated that the single W-box in the pea DRR206 promoter did not significantly affect the activation of the reporter gene. Maleck et al. (33) screened a DNA microarray with 10,000 *Arabidopsis* expressed sequence tags (representing 25 to 30% of the genome) with different pathogens. Of the induced genes, the promoters from 25 genes contained an average of four copies of the W-box (TTGAC) clustered within a kilobase. Promoters of genes not coordinately regulated with PR-1 had less than two copies of the W-box. Since the pea DRR206 promoter contains only one W-box, the relevance of this

site in DRR206 defense gene induction may be brought into question. Lebel et al. (31) used linker-scanning constructs to demonstrate that the W-box (TTGACC) and the PR-box (GCCGCC) of the *Arabidopsis* PR-1 gene were not necessary for PR-1 induction. Instead, the TCA-element (ACGTCA) and the NF- κ B (GGACTTTTC) binding site were essential for induction. However, the results of the mobility shift assays in this study with the W/P-box indicate that this element is bound by response-related *trans*-acting factors, particularly in pea and tobacco. The E-box/nematode-responsive element, CANNTG, present in multiple copies within the functional sequence of the pea DRR206 promoter, is an evolutionarily conserved recognition site for nuclear factors (47) and may contribute to inducibility (37).

Some yeast and plant promoters, including the pea DRR206 promoter (Fig. 3), contain long stretches of AT-rich DNA, which typically have been associated with the positive regulation of transcription (4,43,48,49). Similarly, long stretches of AT-rich DNA sequence in the pea DRR206 promoter were previously implicated in gene activation. Photoactivated psoralens, which preferentially bind AT-rich DNA sequences, proximal to gene DRR206 induce its expression in pea tissue (35). Alternately, these sequences appear to be associated with the HMG-I/Y family of transcription factors that bind to AT-rich duplex DNA (24) and alter the structure as well as the thermal stability of the region (38). Recently, a pathogen-elicited suppression in the transcription and subsequent synthesis of the pea HMG-I/Y protein has been associated with an enhanced expression of PR genes (29). The purified pea HMG-I/Y protein efficiently binds to the AT-rich sequences of the DRR206 promoter (referred to in Fig. 3). Plant

Fig. 8. Northern analysis of FspHDNase expression in two resistant potato lines (7.1-16A and 7.1-34B) transgenic with the 2.7-kb DRR206 promoter::FspHDNase construct. Potato leaves were untreated (U), wounded (W), treated with 10 mg of chitosan (C) ml^{-1} , or inoculated with 1×10^4 spores ml^{-1} of *Phytophthora infestans* (P). The blot was probed with a 513-bp ^{32}P -labeled FspHDNase cDNA. Ethidium bromide staining of rRNA is shown.

Fig. 7. The relative resistance of tobacco containing the DRR206::FspHDNase gene construct. Immature leaves were inoculated with 50 μl of 1.2×10^5 spores ml^{-1} of *Alternaria alternata* and incubated at 22°C for 7 days. Designations: pBI121 = 35S::Gus; 7.1 = 2.7-kb DRR206 promoter::FspHDNase; 8.1-4b = 1.9-kb DRR206 promoter::FspHDNase. Lesion size (mm^2) was taken as the average of a total of six plants per plant line, with two areas inoculated per leaf. Error bars indicate the standard deviation from the mean.

HMG-I/Y proteins have been implicated in the regulation of developmental, defense-related, and light-regulated gene expression (30).

Stable transfer of the chimeric gene fusion of the DRR206 promoter::FspHDNase elicitor to tobacco resulted in significant fungal and bacterial resistance, although the resistance observed was not complete immunity. When challenged with *Pseudomonas syringae* pv. *tabaci*, there was a significant decrease in colony forming units in the transgenic tobacco lines 8.1-4b (P206-1.9kb::FspHDNase) and 7.1-6 (P206-2.7kb::FspHDNase) lines. Likewise, these same lines, challenged with a fungal pathogen *Alternaria alternata*, decreased lesion size and diminished spread of toxin. Thus, when fused with the FspHDNase gene and driving its expression, these two segments are effective in triggering a nonhost resistance response. The FspHDNase elicitor is presumed to act by causing single-strand nicks in host DNA although FspHDNase may be recognized by cellular factors, which in turn activate defense gene expression (28). Increasing evidence indicates the existence of different pathways for wound/pathogen-induced signaling in different plant species. In spite of the differences, it appears there may be some similarities in signaling for wounding, nonhost resistance, and specific R gene-mediated resistance since each of these can induce PR gene expression.

In summary, these findings provide insight into the regulatory regions of the pea DRR206 promoter and demonstrate the suitability of this promoter, in combination with a gene coding a PR gene eliciting nonhost resistance in plants. Results of this study indicate that the FspHDNase elicitor was capable of inducing resistance, heterologously, in tobacco. However, the pathological assays of transgenic potato containing the DRR206::FspHDNase construct appeared to be sensitive to the probable increases in FspHDNase and did not exhibit useful resistance to *P. infestans* or *C. coccodes*. Because of this sensitivity, a less responsive promoter or a down graded DRR206 promoter construct may prove to be more suitable. DRR206 promoter sequences were identified that may subsequently assist in the purification of certain *trans*-acting factors associated with the promoter elements. For example, the sequence from the W/P-box can now be utilized in affinity purification of putative *trans*-acting factors.

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