

# ***Fusarium solani* DNase Is a Signal for Increasing Expression of Nonhost Disease Resistance Response Genes, Hypersensitivity, and Pisatin Production**

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The inoculation of pea endocarp tissue with the bean pathogen *Fusarium solani* f. sp. *phaseoli* results in a non-host resistance response causing a complete cessation of fungal growth within 6 to 8 h. In addition to previously reported elicitation by chitosan, we now report that components of this response are also induced by a DNase released from this fungus. A single band of protein corresponding with DNase activity elicits phytoalexin production and the accumulation of RNA homologous with the pathogenesis-related (PR) genes DRR49, DRR206, and DRR230. Both the enzyme activity and the eliciting potential of the *Fusarium* DNase (Fsp DNase) are heat stable but susceptible to digestion by proteinase K. Fsp DNase mimics the intact fungus in inducing resistance against *F. solani* f. sp. *pisi*. Also, Fsp DNase causes similar cytologically detectable changes in pea tissue, such as increasing hypersensitive discoloration and diminishing fluorescence of Hoechst 33342-stained nuclei and fluorescein diacetate stained cells.

**Additional keywords:** fungal elicitor, hypersensitive response, plant defense.

Nonhost disease resistance is considered to be resistance expressed against plant pathogenic organisms not adapted to the host plant. Although the genetics of nonhost resistance have not been defined with the precision of those of race-specific resistance (Hadwiger and Culley 1993), the defense genes expressed in the nonhost resistance response of peas are some of the same genes ultimately activated in race-specific resistance (Daniels et al. 1986; Hadwiger et al. 1992; Wagoner et al. 1982). Some of these host genes have been cloned and characterized (Chang et al. 1992, 1993a, 1993b, 1995; Chiang and Hadwiger, 1990, 1991; Culley et al. 1995; Fristensky et al. 1985, 1988). However, much remains to be known about how the fungal genes of the pathogens and their protein products signal activation of the plant defense genes. Here we report that a single fungal protein of known function has the potential to signal this activation.

Proteins appear to employ multiple routes in eliciting defense responses. The soybean enzyme  $\beta$ -1-3-endoglucanase

can digest fungal cell walls to release phytoalexin eliciting glucans (Keen and Yoshikawa 1983). The pea enzymes  $\beta$ -glucanase and chitinase release chitosan-like oligomers from *Fusarium solani* macroconidia that elicit pisatin production in peas (Kendra et al. 1989). Certain bacterial pathogens can release proteins termed “harpins” that appear to be required for the hypersensitive response (HR) (He et al. 1993). The fungal pathogen *Rhynchosporium secalis* releases the peptide NIP1 that elicits defense reactions in barley (Hahn et al. 1993). *Erwinia carotovora* subsp. *carotovora* produces a variety of exo-enzymes, of which pectate lyase and polygalacturonase, but not cellulase, promote increased  $\beta$ -1,3-glucanase mRNA accumulation in tobacco (Palva et al. 1993). Some proteins (peptides) are specifically encoded by avirulence genes (Van den Ackerveken et al. 1992) or toxin genes (Wolpert et al. 1994) of the pathogen.

The bean pathogen *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burkholder) W. C. Snyder & H. C. Hans. produces a heat stable extracellular DNase (Fsp DNase) capable of making random single-stranded nicks in single- or double-stranded DNA (Gerhold et al. 1993). Increases in this DNase activity are induced when the fungus contacts pea endocarp tissue or when it is subjected to nutrient starvation in culture. The DNase activity from *F. solani* f. sp. *phaseoli* is released from intact fungal cells and can be detected within the nuclei of pea pod tissue 6 h after inoculation (Gerhold et al. 1993). This raises the possibility that this enzyme is one of the components responsible for the structural alterations of nuclei detectable microscopically in infected pea tissue (Hadwiger and Adams 1978) and the corresponding increase in disease resistance response (DRR) gene transcription (Chang et al. 1993a). We now report that the treatment with Fsp DNase of endocarp tissue increases production of pisatin and accumulation of RNAs homologous with three DRR genes previously shown to be elicited by the fungus or by DNA-specific compounds that affect DNA conformation (Loschke et al. 1983; Fristensky et al. 1985; Hadwiger, 1988).

## **RESULTS**

### **Fsp DNase elicits pisatin production.**

An extracellular DNase from *F. solani* f. sp. *phaseoli* (Fsp DNase) has been previously characterized and partially purified (Gerhold et al. 1993). This enzyme activity is present in

cell-free filtrates from fungal cultures that have attained maximal growth in nutrient depleted media. Pure enzyme was isolated from the culture medium following filtration to remove fungal tissue, heat treatment, ammonium sulfate fractionation, isofocusing, and sodium dodecyl sulfate (SDS) gel electrophoresis (see Materials and Methods). The enzyme eluted from an acrylamide gel strip was reconstituted following SDS removal and was shown to retain some enzyme activity (~5 enzyme units  $\mu\text{l}^{-1}$ ) (see Materials and Methods) as well as the potential to elicit pisatin production of 13  $\mu\text{g}$  per  $\text{g}^{-1}$  of tissue within 24 h after treatment of pea endocarp tissue.

**Table 1.** *Fusarium* (Fsp) DNase as an inducer of pisatin in immature pea pod endocarp tissue

Treatment <sup>a</sup>	Concentration	Pisatin, $\mu\text{g/g}$ fresh weight <sup>a</sup>	HR <sup>b</sup>
H <sub>2</sub> O		0	—
<i>Fusarium solani</i> f. sp. <i>phaseoli</i> spores	$5.5 \times 10^3 \mu\text{l}^{-1}$	$201 \pm 1$	+
Fsp DNase in H <sub>2</sub> O	64 units $\mu\text{l}^{-1}$	$97 \pm 7$	+
	32 units $\mu\text{l}^{-1}$	$54 \pm 5$	++
	16 units $\mu\text{l}^{-1}$	$22 \pm 4$	—
	8 units $\mu\text{l}^{-1}$	$25 \pm 2$	sl
	4 units $\mu\text{l}^{-1}$	$16 \pm 3$	—
	2 units $\mu\text{l}^{-1}$	$9 \pm 5$	—
	1 units $\mu\text{l}^{-1}$	$9 \pm 2$	—
Fsp DNase in 20 mM MnCl <sub>2</sub>	64 units $\mu\text{l}^{-1}$	$96 \pm 5$	+++
	32 units $\mu\text{l}^{-1}$	$33 \pm 5$	+++
	16 units $\mu\text{l}^{-1}$	$73 \pm 3$	+
	8 units $\mu\text{l}^{-1}$	$52 \pm 18$	++
	4 units $\mu\text{l}^{-1}$	$50 \pm 7$	+
	2 units $\mu\text{l}^{-1}$	$29 \pm 1$	+
	1 units $\mu\text{l}^{-1}$	$25 \pm 2$	—
<i>F. solani</i> f. sp. <i>phaseoli</i> spores in 20 mM MnCl <sub>2</sub>	$5.5 \times 10^3 \mu\text{l}^{-1}$	$176 \pm 8$	+++
MnCl <sub>2</sub> (20 mM)		$6 \pm 2$	—

<sup>a</sup> The endocarp surface of pea pods (0.3 g fresh weight) was treated with 100  $\mu\text{l}$  of the enzyme preparation (purified through isofocusing) or controls and incubated for 26 h at 22°C.

<sup>b</sup> Hypersensitive response. After 24 h: +++ = HR observed as one intensely yellow-green cell per 2 normal surface cells; ++ = one yellow-green cell per 20 normal cells; + = scattered, light yellow-green cells; — = all surface cells appear normal; sl = slight coloration.

**Table 2.** Effect of EDTA (in vivo) on the induction of pisatin by *Fusarium solani* f. sp. *phaseoli* (Fsp) DNase

Treatment <sup>a</sup>	Elicitor concentration	Pisatin $\mu\text{g/g}$ fresh weight
H <sub>2</sub> O	...	0
H <sub>2</sub> O + 10 mM EDTA <sup>a</sup>	...	0
<i>F. solani</i> f. sp. <i>phaseoli</i>	$3.6 \times 10^6$ spores $\text{ml}^{-1}$	$173 \pm 8^b$
<i>F. solani</i> f. sp. <i>phaseoli</i> + 10 mM EDTA	$3.6 \times 10^6$ spores $\text{ml}^{-1}$	$189 \pm 26$
Fsp DNase	32 units $\mu\text{l}^{-1}$	$49 \pm 12$
Fsp DNase + 10 mM EDTA	32 units $\mu\text{l}^{-1}$	0
Fsp DNase	16 units $\mu\text{l}^{-1}$	$28 \pm 0$
Fsp DNase + 10 mM EDTA	16 units $\mu\text{l}^{-1}$	$0 \pm 0$
Fsp DNase	8 units $\mu\text{l}^{-1}$	$43 \pm 13$
Fsp DNase + 10 mM EDTA	8 units $\mu\text{l}^{-1}$	$1 \pm 1$
Fsp DNase	4 units $\mu\text{l}^{-1}$	$25 \pm 8$
Fsp DNase + 5 mM EDTA	4 units $\mu\text{l}^{-1}$	$2 \pm 2$

<sup>a</sup> The endocarp surface of pea pod halves (0.5 g) was treated with 200  $\mu\text{l}$  of the indicated concentrations of fungal spores or Fsp DNase (from 35 to 70% ammonium sulfate fractionation after dialysis) and adjusted to the indicated molarity of EDTA immediately before use.

<sup>b</sup> Pisatin accumulation after 24 h presented as the mean and mean deviation of two samples.

sue. Table 1 relates the phytoalexin-inducing potential of the Fsp DNase activity present following purification through the isofocusing step. This enzyme (64 units  $\mu\text{l}^{-1}$ ) is capable of eliciting pisatin accumulation of 97  $\mu\text{g}$  per g of tissue after 24 h (Table 1). Within 24 h of Fsp DNase treatment, patterns of endocarp epidermal cells become yellow-green in an HR similar to those observed in the pea's response against intact *Fusarium* macroconidia. The intensity of this hypersensitive discoloration increases (Table 1) when the enzyme treatment is supplemented with MnCl<sub>2</sub>, previously shown to increase the catalytic activity of the enzyme in vitro (Gerhold et al. 1993). Although the manganese supplement produced no further increase in the maximum level of pisatin accumulation, it augmented the pisatin levels elicited by lower concentrations of the enzyme and generally enhanced the intensity of the HR.

In an alternative effort to examine the necessity for DNase catalytic activity in the elicitation of pisatin, the effect of complexing divalent cations with EDTA was evaluated (Table 2). It was previously reported that 10 mM EDTA completely inhibits the single-stranded DNA nicking action of Fsp DNase in vitro (Gerhold et al. 1993). EDTA (10 mM) applied to the endocarp surface simultaneously with eliciting concentrations of Fsp DNase also negated the pisatin-eliciting action of this enzyme. In contrast, EDTA did not inhibit the ability of *F. solani* f. sp. *phaseoli* spores to induce pisatin production. In subsequent experiments, utilizing treatments with lower spore concentrations, the level of pisatin accumulation was significantly reduced by EDTA but not eliminated, indicating the presence of other fungal elicitors not requiring metal complexes.

#### Proteinaceous nature of the DNase elicitor.

The Fsp DNase activity is stable to 100°C temperature but not to proteinase K (Gerhold et al. 1993). Therefore to determine if the pisatin-eliciting potential is stable to protease digestion, the isofocused DNase-active fractions were transiently exposed to proteinase K linked to acrylamide beads for 1 h at 65°C. The proteinase K treatment destroyed the inductive properties of the Fsp DNase active fraction, indicating the proteinaceous nature of the elicitor (Table 3). Both the enzymatic activity of Fsp DNase assayed in vitro and the po-

**Table 3.** Destruction of the phytoalexin eliciting potential of *Fusarium solani* f. sp. *phaseoli* (Fsp) DNase by proteinase K

Elicitor	Prior treatment of elicitor	Pisatin $\mu\text{g/g}$ fresh weight
H <sub>2</sub> O	None	0
<i>Fusarium solani</i> ( $4.5 \times 10^7$ spores)	None	$291 \pm 1$
H <sub>2</sub> O	Proteinase K beads only	$6 \pm 1$
	65°C, 1 h	
Fsp DNase	Digestion with Proteinase K beads 65°C, 1 h <sup>a</sup>	$4 \pm 4$
Fsp DNase only	65°C 1 h <sup>a</sup>	$43 \pm 13$
Fsp DNase only	None	$59 \pm 34$

<sup>a</sup> Fsp DNase active fractions (70 units  $\mu\text{l}^{-1}$ ) recovered from the isofocusing step of purification (see Materials and Methods) were subjected to digestion with approximately 0.5 units of proteinase K-acrylic beads (Sigma P-0803). The beads were precipitated and removed following digestion; 100  $\mu\text{l}$  of each treatment was applied to the endocarp surface of 0.25 g of pea pods for 24 h at 22°C.

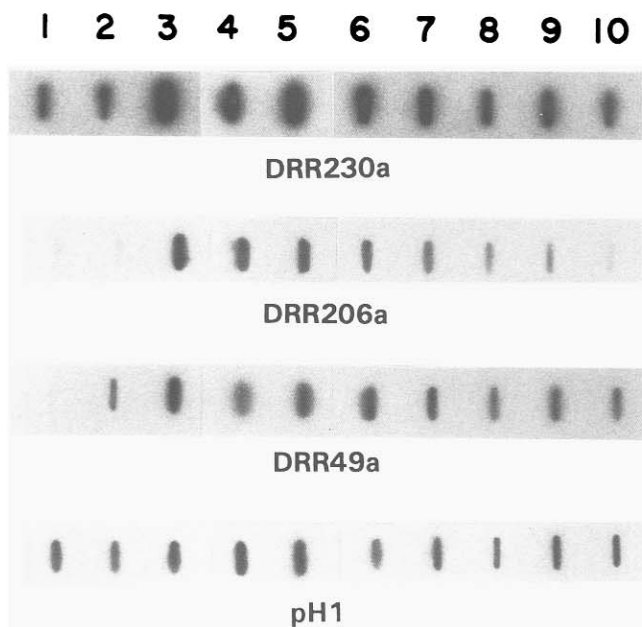
tential to stimulate pisatin accumulation are retained at this incubation temperature in the absence of proteinase K treatment. It remains to be determined unequivocally if the elicitation is dependent on the enzyme activity per se or simply its secondary protein structure.

#### Protein concentration and specific activity of the DNase elicitor.

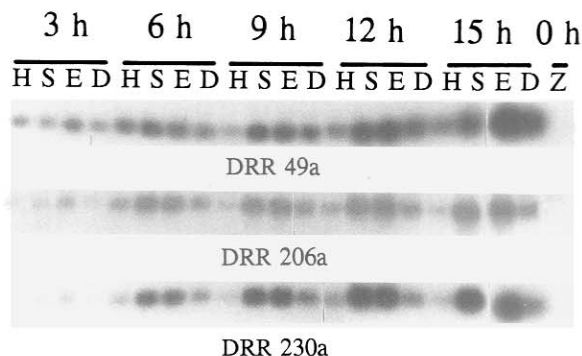
An earlier report indicated moderate pisatin production in peas can be stimulated 24 h after treatment with high concentrations (1 mg ml<sup>-1</sup>) of basic proteins, such as poly-L-lysine or cytochrome C (Hadwiger et al. 1974). Conversely, many non-basic proteins with known biological functions did not elicit pisatin accumulation. For example, a serum albumin concentration of 1 mg ml<sup>-1</sup> did not elicit pisatin accumulation. The elicitation of pisatin production by basic proteins and polymers such as chitosan may be attributable to their abundant positive charges.

Are the nuclease units applied to pea endocarps within the same magnitude as the Fsp DNase units generated in a spore inoculum and are they biologically significant? The pisatin production-inducing Fsp DNase elicitor obtained from culture filtrates or from spore suspensions following contact with pea tissue contained concentrations of total soluble protein below 1 mg ml<sup>-1</sup>. The crude enzyme activity accumulating in the supernatant of a spore suspension (2 × 10<sup>7</sup> ml<sup>-1</sup>) 4.5 h af-

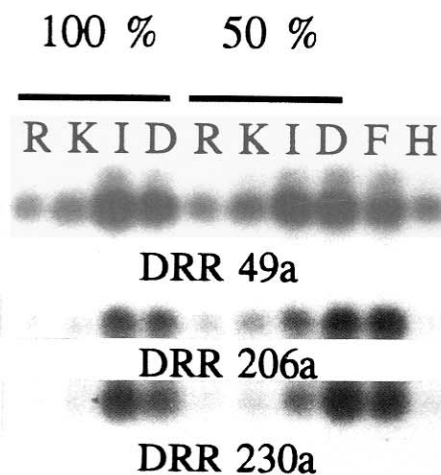
ter contact with the endocarp tissue is 8 DNase units µl<sup>-1</sup> in a solution of 0.675 µg of protein µl<sup>-1</sup> (sp. act 11.8 units µg<sup>-1</sup> protein). The projected DNA plasmid linearization capability from each spore in this suspension is 40 pg of DNA in 1 h. Each pea nucleus contains 12.6 pg of DNA. DNase from the culture filtrate purified through the isofocusing step contains 680 DNase units per µg of protein. Because this purified elicitor is low in protein and high in DNase activity, the eliciting action may result more from the high catalytic activity of DNase than a noncatalytic charge effect attributable to the protein per se.



**Fig. 1.** A slot blot of 8 µg of total RNA from 0.3 g of pea pods treated with 100 µl of H<sub>2</sub>O, *Fusarium solani* f. sp. *phaseoli* spores or dilutions of *Fusarium* (Fsp) DNase or micrococcal DNase, hybridized with probes for pea disease resistance response (DRR) genes 230, 206, and 49 (see Materials and Methods). The pH1 probe is from a bean cDNA clone often utilized for the constitutive control (Lawton and Lamb 1987). RNAs in lanes 1 and 2 were extracted from water-treated pods at 0 and 8 h, respectively. Lane 3, 8-h treatments with *F. solani* f. sp. *phaseoli* spores (3 × 10<sup>8</sup> ml<sup>-1</sup>); lane 4, Fsp DNase 100 units µl<sup>-1</sup>; lane 5, 50 units µl<sup>-1</sup>; lane 6, 25 units µl<sup>-1</sup>; lane 7, 12 units µl<sup>-1</sup>; lane 8, 6 units µl<sup>-1</sup>; lane 9, micrococcal DNase 25 units; lane 10, micrococcal DNase 2 units. Fsp DNase units are described in Materials and Methods. Micrococcal DNase units (1 unit will produce 1.0 µmole of acid soluble polynucleotide from native DNA min<sup>-1</sup>) represent total units applied per sample.



**Fig. 2.** The time course of accumulation of RNAs homologous with disease resistance response (DRR) genes 49, 206, and 230 following these treatments: Lane H, 0.3 g of pea endocarp tissue with 100 µl H<sub>2</sub>O; lane S, *Fusarium solani* f. sp. *phaseoli* spores; lanes E and D, 242 and 121 units µl<sup>-1</sup>, respectively, of isofocus-purified *Fusarium* (Fsp) DNase. Lane Z represents the RNA present in freshly harvested intact pea pods. The northern blot membrane containing 15 µg total RNA per lane was sequentially hybridized with probes from the DRR genes 49, 206, and 230 (see Materials and Methods).



**Fig. 3.** *Fusarium* (Fsp) DNase digested with proteinase K does not elicit increases in RNA homologous with probes from disease resistance response (DRR) genes 49, 206, or 230 (see Materials and Methods). Lane R, pea pod endocarps (0.3 g) treated 10 h with 100 µl of the digestion reaction buffer only; lane K, Fsp DNase digested 1 h with proteinase K at 65°C; lane I, Fsp DNase incubated 1 h at 65°C; lane D, Fsp DNase receiving no treatment; lane F, *Fusarium solani* f. sp. *phaseoli* 1.4 × 10<sup>6</sup> macroconidial ml<sup>-1</sup>; lane H, water. Designations of 100 and 50% indicate concentrations of a stock Fsp DNase that contained 282 units µl<sup>-1</sup> and 141 units µl<sup>-1</sup>, respectively.



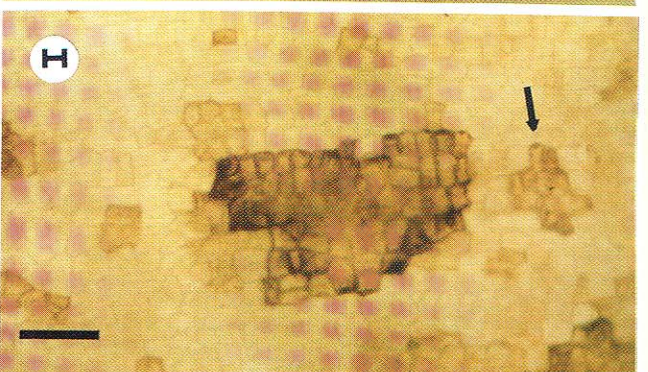
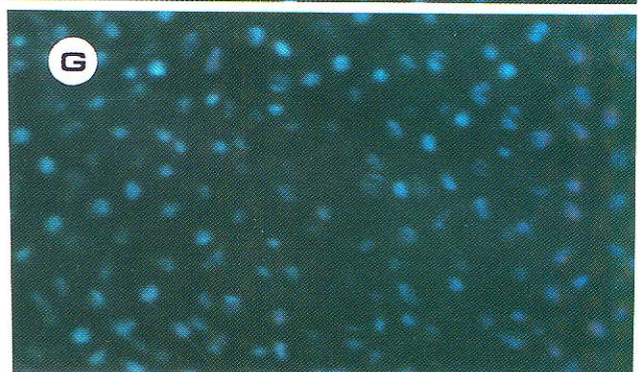
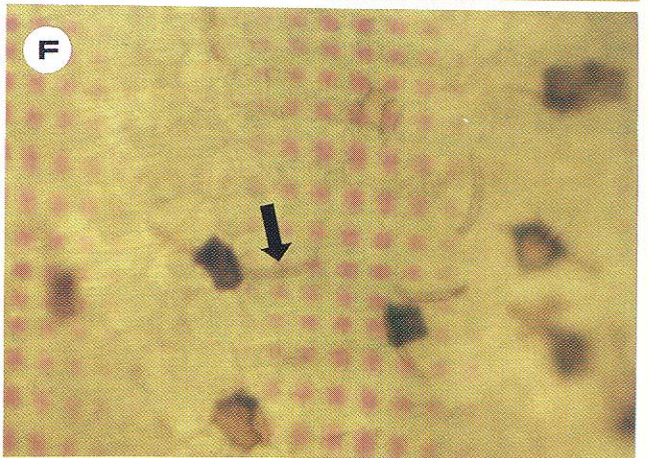
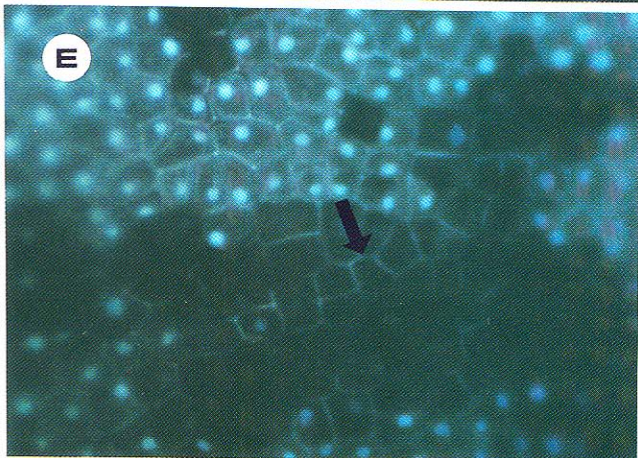
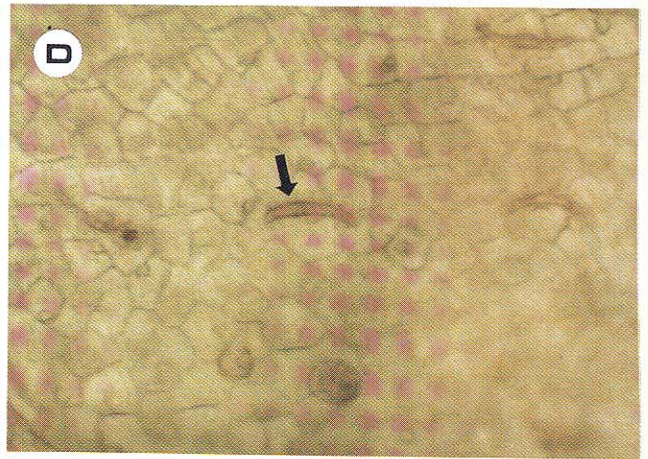
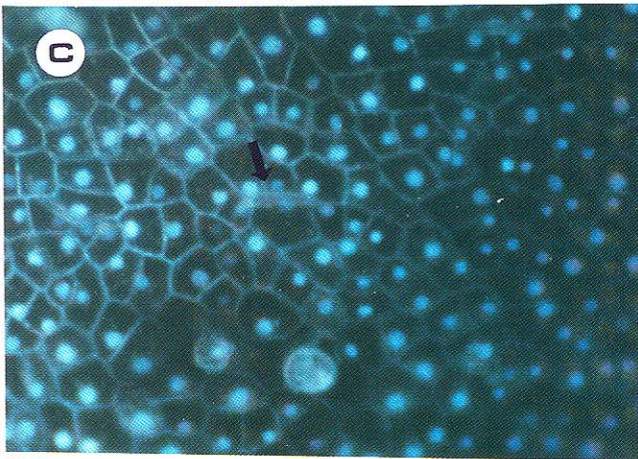
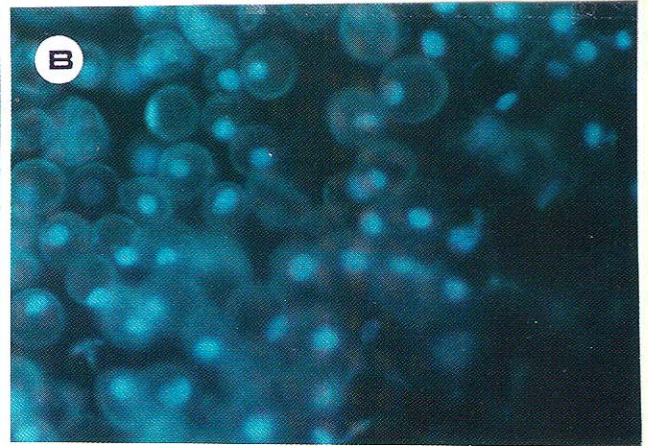
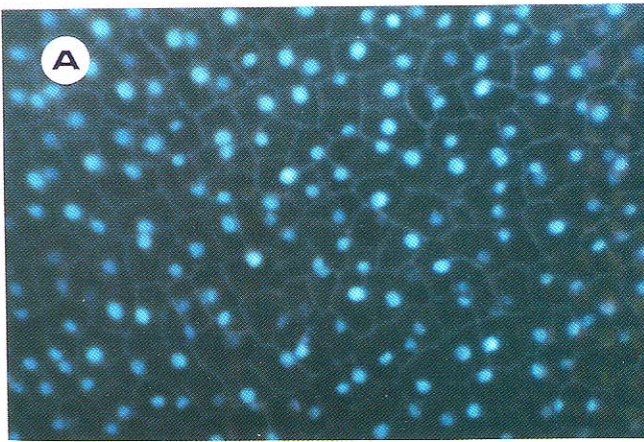




Fig. 4. Alteration of nuclear staining (Hoechst 33342) and discoloration of pea endocarp cells treated with compatible and incompatible *Fusarium solani* macroconidia ( $7 \times 10^5 \text{ ml}^{-1}$ ) or with *Fusarium* (Fsp) DNase  $\sim 25 \text{ units } \mu\text{l}^{-1}$  (12  $\mu\text{l}$  per pod half). Pea endocarp treated with  $\text{H}_2\text{O}$ : A, after 7 h; B, after 24 h. *Fusarium solani* f. sp. *phaseoli* after 21 h: C, fluorescent photo; D, corresponding light photo. *Fusarium solani* f. sp. *pisi* for 21 h, E, fluorescent photo; F, corresponding light photo. DNase: G, 7 h; H, 24 h. Matched arrows locate a nucleus in the vicinity of the spore in the fluorescent photo and the corresponding light photo respectively. Single arrow (H) identifies cells discolored in the hypersensitive response. (Bar = 50 microns.)

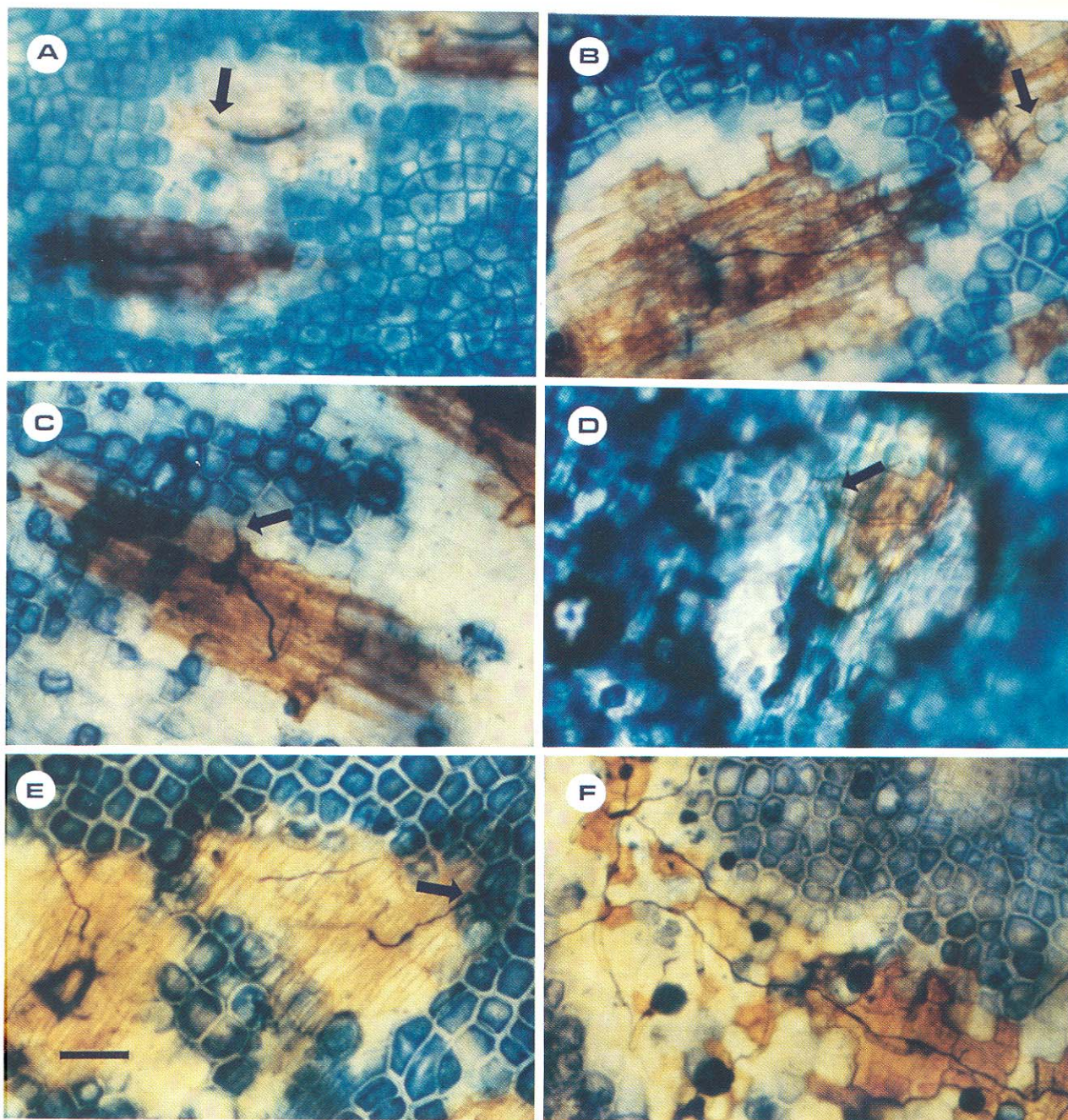


Fig. 5. Effect of *Fusarium* (Fsp) DNase on the resistance of pea endocarp tissue to *Fusarium solani* f. sp. *pisi*. A, Growth of *F. solani* f. sp. *phaseoli* (incompatible); B, *F. solani* f. sp. *pisi* (compatible) 44 or 72 h (F) following water treatment (25  $\mu\text{l}$ ) and simultaneous inoculation of each pod half with 5  $\mu\text{l}$  of a  $5 \times 10^3$  spore  $\text{ml}^{-1}$  suspension in  $\text{H}_2\text{O}$ . Growth of *F. solani* f. sp. *pisi* 44 h following inoculation in 25  $\mu\text{l}$  solutions of Fsp DNase per pod half containing 8 units  $\mu\text{l}^{-1}$  (C), 2 units  $\mu\text{l}^{-1}$  (D), or 1 unit  $\mu\text{l}^{-1}$  (E) simultaneously inoculated with 5  $\mu\text{l}$  of the *F. solani* f. sp. *pisi* inoculum. Small lesion size and sound condition of closely peripheral cells (A and D) are associated with suppression of fungal growth. Also, the hypersensitive discoloration of tissue is not always associated with suppressed fungal growth (A). (Bar = 50 microns.)



### Fsp DNase elicits accumulation of DRR RNA.

The RNA homologous with the DRR genes DRR230, DRR206, and DRR49 has been shown to increase in the non-host resistance response of peas against *F. solani* f. sp. *phaseoli* (Fristensky et al. 1985). Three separate pure preparations of Fsp DNase, renatured from a gel slice following SDS electrophoresis (see Materials and Methods) elicited the accumulation of these RNAs in treated pods (data not shown). Undenatured Fsp DNase purified through the isofocusing step elicited increases in these DRR RNAs within 8 h following application of 25 to 100 units  $\mu\text{l}^{-1}$  of Fsp DNase to pea tissue (Fig. 1). A commercial preparation of micrococcal DNase marginally increases the accumulation of RNA homologous with DRR230 and DRR206.

Within 3 h of the Fsp DNase application RNA homologous with DRR genes 49, 206, and 230 begins accumulating (Fig. 2). The times required for these increases are comparable to those required for their induction by the *F. solani* f. sp. *phaseoli* macroconidial treatment of pod tissue. Proteinase K treatment of Fsp DNase (Fig. 3) effectively reduced its DRR gene eliciting potential, again indicating the proteinaceous nature of the elicitor.

### Effect of Fsp DNase on nuclear staining.

*Fusarium solani* spores readily released DNase following contact with pea tissue (Gerhold et al. 1993), thus the effects of DNase and spore inoculum on pea tissue stained with the nuclear stain Hoechst 33342 are compared in Figure 4. In the course of the water treatment period the endocarp surface cells progress from cells with intensely staining intact nuclei to cells with actively extended endocarp hairs possessing somewhat larger intact nuclei (Fig. 4A, B). The cells in the vicinity of the incompatible spore retain an intact appearance except for those in cells immediately in contact with an individual spore (Fig. 4C, D). Multiple incompatible spores at a single location successfully reduce nuclear staining in cells

they contact. In contrast, a larger area of epidermal endocarp cells with unstained nuclei occur adjacent to the compatible fungal spores from *F. solani* f. sp. *pisi* (Fig. 4E, F). Uniform DNase applications reduce fluorescence in large regions and the entire surface loses fluorescence within 20 h (Fig. 4G). The nuclei with detectable fluorescence are distorted in shape. A hypersensitive discoloration of some cells occurs within 24 h following Fsp DNase application (Fig. 4H).

These results suggest that the pea nuclear DNA in pea cells becomes changed as a receptor of the stain, possibly by cellular exclusion of the stain, by production of fluorescence quenching compounds, by interference with the DNA specific action of the dye, or by the alteration of pea DNA. The nuclear distortions of pea nuclei caused by inoculation with *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* have previously been shown to influence the migration of isolated pea nuclei in a sucrose gradient (Hadwiger and Adams 1978). The nuclei from  $\text{H}_2\text{O}$ -treated tissue migrate with the greatest density, nuclei were less dense from *F. solani* f. sp. *phaseoli*-treated tissue and least dense from *F. solani* f. sp. *pisi*-treated tissue. The catalytic action of Fsp DNase, some of which reportedly localizes in the pea nuclei (Gerhold et al. 1993), may be associated with such nuclear alterations.

### The effect of Fsp DNase on the viability of pea endocarp tissue.

Fluorescein diacetate treatments were utilized to estimate the viability of treated tissue (not shown). In the incompatible response following inoculation of endocarp cells with *F. solani* f. sp. *phaseoli* the fluorescence of cells immediately in contact with spore is progressively reduced to nonfluorescence during the period from 6 through 13 h, a period in which water-treated tissue remained brightly fluorescent. Fsp DNase treatments of pods with 16 through 64 units  $\mu\text{l}^{-1}$  eliminated fluorescence starting at 6 and progressing through 22 h. Treatments with 1 to 4 units  $\mu\text{l}^{-1}$  partially reduced fluorescence at 6 h with a progression to normal fluorescence at 13 h. Surface cell viability at 24 h for control and resistant tissue was also indicated by a lawn of epidermal "hairs" that developed uniformly on water-treated tissue and from epidermal cells peripheral to lesions with suppressed fungal growth. These hairs did not develop until after 48 h following treatments with high concentrations of Fsp DNase.

### Effect of Fsp DNase on pea disease resistance.

Pea endocarp tissue challenged with the bean pathogen *F. solani* f. sp. *phaseoli* completely suppresses growth of these spores shortly following germination (Fig. 5A). Fsp DNase purified from the culture filtrate through the isofocus step (see Materials and Methods) and applied to pea tissue simultaneously with *F. solani* f. sp. *pisi*, a pathogen of peas influences the rate of hyphal extension (Fig. 5C, D, and E). Surprisingly, resistance was elicited most efficiently when the pea tissue was treated with 1 or 2 units  $\mu\text{l}^{-1}$  of Fsp DNase (Table 4). High levels of Fsp DNase (32 units  $\mu\text{l}^{-1}$ ) resulted in deterioration of both plant and fungal cell structure making low-power microscope photography difficult. This high DNase level totally inhibited growth of both *F. solani* f. sp. *phaseoli* and *f. solani* f. sp. *pisi* in Vogel's media at 18 h (data not shown). After 7 days pea tissues treated with these high levels supported prolific growth of the pathogen spores surviving the treatment.

**Table 4.** Effect of *Fusarium* (Fsp) DNase on suppression of *Fusarium solani* f. sp. *pisi* growth following inoculation of  $5 \times 10^{-3}$  spores  $\text{ml}^{-1}$  onto pea endocarp tissue

Treatment	Observation <sup>a</sup>		Resistance <sup>b</sup>
	1	2	
<i>F. solani</i> f. sp. <i>phaseoli</i> in $\text{H}_2\text{O}$	<1.0	<1.0	++++
<i>F. solani</i> f. sp. <i>pisi</i> in $\text{H}_2\text{O}$	4.11	3.55	0
<i>F. solani</i> f. sp. <i>pisi</i> in 32 U/ $\mu\text{l}$ DNase <sup>c</sup>	det. <sup>d</sup>	det.	0
<i>F. solani</i> f. sp. <i>pisi</i> in 16 U/ $\mu\text{l}$ DNase	1.01	det.	0
<i>F. solani</i> f. sp. <i>pisi</i> in 8 U/ $\mu\text{l}$ DNase	1.70	3.40	0
<i>F. solani</i> f. sp. <i>pisi</i> in 4 U/ $\mu\text{l}$ DNase	1.62	2.81	0
<i>F. solani</i> f. sp. <i>pisi</i> in 2 U/ $\mu\text{l}$ DNase	1.46	1.57	+
<i>F. solani</i> f. sp. <i>pisi</i> in 1 U/ $\mu\text{l}$ DNase	2.46	1.46	++
<i>F. solani</i> f. sp. <i>pisi</i> in 0.5 U/ $\mu\text{l}$ DNase	4.07	2.40	++
<i>F. solani</i> f. sp. <i>pisi</i> in 0.25 U/ $\mu\text{l}$ DNase	...	2.09	0

<sup>a</sup> Each observation value is an average of 50 to 100 observed spore germ tube extensions calculated as the fold extension beyond the 45 micron length of macroconidia after 48 h.

<sup>b</sup> Observation of resistance at 7 days, a continuation of the same group of pod halves as observation 2: 0 = no resistance; + = degree of preservation of sound pod condition and fungal growth suppression, arbitrarily compared with the incompatible interaction with the *F. solani* f. sp. *phaseoli*-treated pods.

<sup>c</sup> U = enzyme units.

<sup>d</sup> The condition of host surface tissue and fungal mycelia was sufficiently deteriorated to prevent measurements.

Thus, Fsp DNase applied to tissue at low concentrations (1 to 2 units  $\mu\text{l}^{-1}$ ) suppressed the compatible pea pathogen *F. solani* f. sp. *pisi*, possibly because of the plant defense response elicited. The resistance generated was not as complete or as long-term as the natural resistance response elicited in pea tissue against *F. solani* f. sp. *phaseoli*. Potentially, the Fsp DNase elicitation contributes along with other elicitors, such as chitosan, to the signaling of the total defense response.

#### Genomic DNA following Fsp DNase treatment *in vitro* and *in vivo*.

Genomic pea DNA is readily digested *in vitro* with Fsp DNase (Fig. 6, lane 3). DNA extracted from endocarp tissue 4 h following Fsp DNase treatment (100  $\mu\text{l}$  of 16 units  $\mu\text{l}^{-1}$  per 0.25 g of pods) was not visibly altered (Fig. 6, lanes 6 and 10), indicating that the enzyme was unable to digest pea DNA *in vivo*, that the levels of digestion were below the limits of detection, or that the repair processes within the pea cells efficiently repaired DNA nicked *in vivo*. An attempt to digest the strand opposite from DNA nicks, possibly generated *in vivo* by the Fsp DNase, by treating the genomic DNA *in vitro* with S1 nuclease did not result in a visibly altered gel migration pattern (Fig. 6, lane 12).

#### DISCUSSION

Fsp DNase is released in the culture medium of *F. solani* f. sp. *phaseoli* under conditions of nutrient depletion or contact with plant tissue (Gerhold et al. 1993). It has been shown that Fsp DNase activity is transferred to the nuclei of pea tissue within 6 h, establishing the necessary logistics for a signaling function (Gerhold et al. 1993). The Fsp DNase purified from the *F. solani* f. sp. *phaseoli* culture media is capable of enhancing the accumulation of both the phytoalexin pisatin and RNAs homologous with the DRR genes 49, 206, and 230, suggesting that a single enzyme can mimic the induction of a response similar to that induced by *F. solani* f. sp. *phaseoli* macroconidia. Protease K-digested DNase is incapable of eliciting the defense responses. However, the elicitation capacity and the enzyme activity are retained following heat treatment. These results suggest that the elicitor is protein and that the eliciting potential is heat stable, implying that the enzyme protein's catalytic activity is associated with the eliciting potential. Additionally, the chelation of divalent cations negates both the catalytic activity of the Fsp DNase and its elicitation of pisatin accumulation, whereas the addition of  $\text{Mn}^{+2}$  increases both of these properties. The Fsp DNase functions as a nicking enzyme leaving a 5' phosphate group and a 3' hydroxyl group on the nicked DNA strand (Gerhold et al. 1993) and may utilize this attribute for elicitation. Such an effect may be difficult to detect *in vivo* because of the size of the genome, the transient DNase action, and the low percentage of cells effected. Thus far the only detectable changes in the DNA of the host cell are in the reduced fluorescence of pea Hoechst 33342 stained nuclei and the nuclear distortion that also occurs following infection by *F. solani* f. sp. *phaseoli* macroconidia or treatment with Fsp DNase. The specific cellular mode of eliciting action by Fsp DNase remains to be determined.

Because of the variety of DNA-specific and other compounds capable of eliciting plant defense responses, their modes of action are difficult to assimilate within a single hy-

pothesis. Interestingly, the elicitor-active-sized chitosan oligomers such as those released from *F. solani* spore (Kendra et al. 1989) have in common with Fsp DNase the ability to cause single-strand nicks in plasmid DNA *in vitro* in the presence of copper ions (Kashige et al. 1994). The potential nicking action on DNA by chitosan octamers and Fsp DNase *in vivo* could be capable of releasing helical tensions in genomic DNA (Hadwiger et al. 1989). Such DNA damage would be followed by DNA repair but possibly not until some transcription positive changes have occurred. The interrelationships of DNA damage, repair, replication, and transcription are only now becoming fully recognized (Culotta and Koshland 1994).

#### MATERIALS AND METHODS

##### Plant and fungal source.

*Fusarium solani* f. sp. *pisi* strain PA (ATCC No. 38136) pathogenic on peas and *F. solani* f. sp. *phaseoli* strain W-8 (ATCC No. 38135) pathogenic on beans were maintained on pea shoot supplemented potato dextrose agar (PDA; Difco, Detroit, MI). Pods were harvested from *Pisum sativum* cv. Alcan plants grown in sand in a greenhouse.

##### Fsp DNase purification.

One liter of crude enzyme was prepared by growing *F. solani* f. sp. *phaseoli* strain (W-8) macroconidia on a rotary

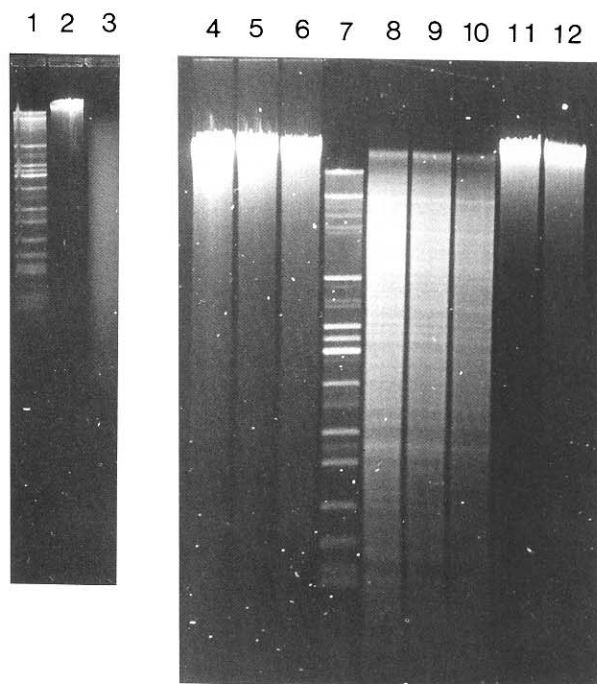


Fig. 6. Effect of *Fusarium* (Fsp) DNase on pea genomic DNA. Lambda DNA digested with *Hind*III, *Eco*RI, and *Bam*HI (marker lanes 1 and 7). Pea genomic DNA (5  $\mu\text{g}$ ) from nontreated pods nondigested (lane 2) and pea genomic DNA digested *in vitro* 1 h with 16 units of Fsp DNase (lane 3). Pea genomic DNA (5  $\mu\text{g}$ ) extracted from 0.5 g of pods: directly after harvest (lane 4), 4 h after pods were split and treated with  $\text{H}_2\text{O}$  (lane 5) or 4 h after treatment with 200  $\mu\text{l}$  of Fsp DNase (16 units  $\mu\text{l}^{-1}$ ) for 4 h (lane 6). Aliquots (5  $\mu\text{g}$ ) of the same DNA lots separated in lanes 4, 5, and 6 were digested overnight with 40 units *Bam*HI and separated in lanes 8, 9, and 10 respectively. Aliquots (5  $\mu\text{g}$ ) of the same genomic DNA lots shown in lanes 4 and 6 after digestion with 400 units S1 nuclease for 1 h are separated in lanes 11 and 12, respectively.

shaker for 8 days at 22°C in liquid minimal Vogel's media (Vogel 1956) supplemented with 0.2% casein hydrolysate, 1% glycerol, and 1% sucrose. The culture medium was separated from the mycelial growth by filtration through nylon fabric (0.2 × 0.2 mm pore size) followed by a brief heat treatment (to 100°C) and centrifugation 10 min at 12,000 × g. The supernatant containing the enzyme was fractionated with ammonium sulfate and the enzymatically active 35 to 70% pellet was retained (Gerhold et. al. 1993). The ammonium sulfate was removed with dialysis against buffer D (1 mM KPO<sub>4</sub> buffer pH 7.4, containing 1 mg phenylmethylsulfonylfluoride liter<sup>-1</sup>). The enzyme preparation was fractionated by isofocusing in a Rotofor cell (BioRad, Hercules, CA) within an ampholyte pH range of 4.0 to 6.5 (Sigma, St. Louis, MO). The activity peaked at pH 4.25. The active fractions were dialyzed against buffer D and reduced in volume with spin filters (Amicon, Inc., Beverly, ME) that retained molecules in excess of 10,000 MW and Fsp DNase. The DNase was further purified to a single band of activity utilizing discontinuous SDS electrophoresis in 10% acrylamide gel (Laemmli 1970). Gel strips 25 mm long and 4 mm wide were placed in dialysis tubing with 100 µl of H<sub>2</sub>O and were subsequently eluted and renatured by dialysis against buffer D. The DNase activity assay was recovered as a single band from an individual gel slice. Three such preparations of Fsp DNase, regarded as pure enzyme, were renatured in this manner and utilized as elicitor. The isofocus purified Fsp DNase fraction was utilized in most elicitor assays because it had not been subjected to denaturation and provided adequate catalytic units for large-scale experiments. Six additional bands lightly stained with brilliant blue were detected in the isofocused fraction when total Fsp DNase from a 2-liter culture filtrate preparation through isofocusing was electrophoresed.

#### Enzyme assay.

One unit of Fsp DNase enzyme activity was designated as that able to convert 50% of 0.2 µg of supercoiled circular DNA of plasmid pDC49e-40 (pea DRR49e promoter-GUS construct in pUC 18) to the linear form in 1 h at 37°C. pBR322 was also utilized as alternate substrate. The reaction mixture (6 µl volume) typically contained 0.2 µg of supercoiled plasmid DNA in 5 µl of reaction buffer (50 mM MES pH 6.0, 10 mM MnCl<sub>2</sub>) and 1 µl of the enzyme preparation.

#### Proteinase K digestion of DNase.

Fsp DNase active fractions 121 units µl<sup>-1</sup> in 100 µl of H<sub>2</sub>O from the isofocusing stage of purification (see above) were incubated 1 h at 65°C in PK buffer (50 mM Tris, pH 7.5, 5 mM CaCl<sub>2</sub>) with or without 0.1 g (0.5 units) of proteinase K-acrylic beads. The proteinase K beads (Sigma) were removed by centrifugation and the supernatant dialyzed against 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5. The digested enzyme and controls were assayed for DNase activity as described above and assessed as elicitors.

#### DNase elicitation of defense responses.

Immature pea pods 2 cm in length were freshly harvested from greenhouse-grown plants and broken free from the calyx end. The pod halves were separated and treatments of 100 µl applied to the exposed endocarp tissue. Initially, a treatment containing electrophoretically pure Fsp DNase (see Materials

and Methods) was utilized to determine that the single protein band comigrating with the DNase activity was able to elicit pisatin production. Experiments requiring greater quantities of enzyme utilized the Fsp DNase enzyme from the isofocusing purification step (see above). One hundred microliters of the enzyme preparations were typically applied to 0.25 g of immature pod for each experimental sample. Samples were stored at -70°C following the incubation period.

#### Pisatin production.

After 24 h of incubation at 22°C following elicitor applications to the endocarp surface, the pods were immersed in 5-ml hexanes for 18 h. The hexanes were evaporated in a dark hood, the pisatin-containing residue was dissolved in 1 ml of 95% EtOH, and the pisatin quantitated by OD<sub>309</sub> absorbance (Cruickshank and Perrin 1961).

#### DRR gene RNA accumulation.

The RNA accumulating after elicitor treatment was extracted as described previously (Fristensky et al. 1985). Fifteen micrograms of total pea RNA was applied to each well of a 1% agarose formaldehyde gel and electrophoretically separated. Slot and Northern blotting utilized the following probes: DRR49a, *EcoRI* fragment containing 740 bp of the coding region of pI49 (Fristensky et al. 1988); DRR206a, 480 bp *EcoRI/HindIII* fragment containing the entire coding region of DRR206 (Fristensky et al. 1988); DRR230a, *BamHI/HindIII* fragment with the entire 480-bp coding region of DRR230 (Chiang and Hadwiger 1990); and pH1, a bean cDNA (Lawton and Lamb 1987) utilized as a constitutive control.

#### Cytological observations.

Pea pods treated with *Fusarium solani* macroconidia or preparations of DNase were placed endocarp surface down in solutions (50 µg ml<sup>-1</sup>) of the DNA-specific Hoechst 33342 stain for 10 min. The intact endocarp surface was viewed directly and photographed in a fluorescence microscope without fixation, sectioning, or compression by a cover slip. Endocarp surface cell viability was estimated utilizing fluorescein diacetate (FDA). Fifteen microliters of FDA stock solution (1 mg of acetone per ml) was added to a 500-µl drop of H<sub>2</sub>O. The pod endocarp surface was inverted into this drop for 10 min and was subsequently viewed directly in a fluorescent microscope.

The progression of *F. solani* spore germination and growth was followed by cotton blue-lactic acid staining and viewed under low-power magnification. The distance the hyphae progressed was evaluated in multiples of the *F. solani* f. sp. *pisi* spore lengths (45 microns). At least 50 individual observations were incorporated into the average growth for each treatment point.

#### Assay of Fsp DNase effect on *F. solani* growth in vitro.

DNase activity 64 units µl<sup>-1</sup> purified through isofocusing was applied to three wells of a micro-dilution plate and each continuously diluted by half to cover the range 32 units µl<sup>-1</sup> to 0.03 units µl<sup>-1</sup>. *Fusarium solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli* macroconidia (~50 spores 25 µl<sup>-1</sup>) in Vogel's medium (Vogel 1956) were applied to those and control wells and growth at 25°C observed microscopically and visually through 48 h.



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## LITERATURE CITED

- Chang, M.-M., Chiang, C. C., Martin, M. W., and Hadwiger, L. A. 1993a. Expression of a pea disease resistance response gene in the potato cultivar Shepody. *Am. Pot. J.* 70:635-647.
- Chang, M.-M., Culley, D. E., and Hadwiger, L. A. 1993b. Nucleotide sequence of a pea (*Pisum sativum* L.)  $\beta$ -1,3-glucanase gene. *Plant Physiol.* 101:1121-1122.
- Chang, M.-M., Hadwiger, L. A., and Horovitz, D. 1992. Molecular characterization of a pea  $\beta$ -1,3-glucanase induced by *Fusarium solani* and chitosan challenge. *Plant Mol. Biol.* 20:609-618.
- Chang, M.-M., Horovitz, D., Culley, D., and Hadwiger, L. A. 1995. Molecular cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. *Plant Mol. Biol.* 28:105-111.
- Chiang, C. C., and Hadwiger, L. A. 1990. Cloning and characterization of a disease resistance response gene in pea induced by *Fusarium solani*. *Mol. Plant-Microbe Interact.* 3:78-85.
- Chiang, C. C., and Hadwiger, L. A. 1991. The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. *Mol. Plant-Microbe Interact.* 4:324-331.
- Cruickshank, I. A. M., and Perrin, D. R. 1961. Studies on phytoalexins III. The isolation, assay and general properties of a phytoalexin from *Pisum sativum* L. *Aust. J. Biol. Sci.* 14:336-348.
- Culley, D. E., Horovitz, D., and Hadwiger, L. A. 1995. Molecular characterization of disease resistance response gene DRR206-d from *Pisum sativum* (L.). *Plant Physiol.* 107:301-302.
- Culotta, E., and Koshland, D. E. 1994. DNA repair works its way to the top. *Science* 266:1926-29.
- Daniels, C. H., Fristensky, B. W., Wagoner, W., and Hadwiger, L. A. 1986. Pea genes associated with non-host disease resistance to *Fusarium* are also active in race-specific disease resistance to *Pseudomonas syringae*. *Plant Mol. Biol.* 8:309-316.
- Fristensky, B., Horovitz, D., and Hadwiger, L. A. 1988. cDNA sequences for pea disease resistance response genes. *Plant Mol. Biol.* 11:713-715.
- Fristensky, B., Riggelman, R. C., Wagoner, W., and Hadwiger, L. A. 1985. Gene expression in susceptible and disease resistant interactions of peas induced with *Fusarium solani* pathogens and chitosan. *Physiol. Plant Pathol.* 27:15-28.
- Gerhold, D. L., Pettinger, A. L., and Hadwiger, L. A. 1993. Characterization of a plant-stimulated nuclease from *Fusarium solani*. *Physiol. Mol. Plant Pathol.* 43:33-46.
- Hadwiger, L. A. 1988. Possible role of nuclear structure in disease resistance of plants. *Phytopathology* 78:1009-1014.
- Hadwiger, L. A., and Adams, M. J. 1978. Nuclear changes associated with the host-parasite interaction between *Fusarium solani* and peas. *Physiol. Plant Pathol.* 12:63-72.
- Hadwiger, L. A., Chiang, C. C., and Horovitz, D. 1992. Expression of disease resistance response genes in near-isogenic pea cultivars following challenge by *Fusarium oxysporum* Race 1. *Physiol. Mol. Plant Pathol.* 40:259-270.
- Hadwiger, L. A., Chiang, C., Victory, S., and Horovitz, D. 1989. The molecular biology of chitosan in plant/pathogens interaction and its application in agriculture. Pages 119-138 in: *Chitin and Chitosan, Sources, Chemistry, Biochemistry, Physical Properties and Applications*. G. Skjak-Braek, T. Anthonsen, and P. Sanford, eds. Elsevier, London.
- Hadwiger, L. A., and Culley, D. E. 1993. Nonhost resistance genes and race-specific resistance. *Trends Microbiol.* 1:136-141.
- Hadwiger, L. A., Jafri, A., Von Broembsen, S., and Eddy, R. 1974. Mode of pisatin induction. Increased template activity and dye-binding capacity of chromatin isolated from polypeptide-treated pea pods. *Plant Physiol.* 53:52-63.
- Hahn, M., Jüngling, S., and Knogge, W. 1993. Cultivar-specific elicitation of barley defense reactions by the phytotoxic peptide NIP 1 from *Rhynchosporium secalis*. *Mol. Plant-Microbe Interact.* 6:745-754.
- He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* Harpin Pss: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.
- Kashige, N., Yamaguchi, T., Ohtakara, T., Mitsutomi, M., Brimacombe, J. S., Miake, F., and Watanabe, K. 1994. Structure-activity relationships in the induction of single-strand breakage in plasmid pBR322 DNA by amino sugars and derivatives. *Carbohydr. Res.* 257:285-291.
- Keen, N. T., and Yoshikawa, M. 1983.  $\beta$ -1, 3-glucanase from soybean releases elicitor active carbohydrate from fungus cell walls. *Plant Physiol.* 71:460-465.
- Kendra, D. F., Christian, D. A., and Hadwiger, L. A. 1989. Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/ $\beta$ -glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. *Physiol. Mol. Plant Pathol.* 35:215-230.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680-682.
- Lawton, M. A., and Lamb, C. J. 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. *Mol. Cell. Biol.* 7:335-341.
- Loschke, D. C., Hadwiger, L. A., and Wagoner, W. 1983. Comparison of mRNA populations coding for phenylalanine ammonia lyase and other peptides from pea tissue treated with biotic and abiotic phytoalexin inducers. *Physiol. Plant Pathol.* 23:163-173.
- Palva, T. K., Holmstrom, K.-O., Heino, P., and Palva, E. T. 1993. Induction of plant defense response by exoenzymes of *Erwinia caratovora* subsp. *caratovora*. *Mol. Plant-Microbe Interact.* 6:190-196.
- Van den Ackerveken, G. F. J. M., Van Kan, J. A. L., and DeWit, P. J. G. M. 1992. Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *Plant J.* 2:359-366.
- Vogel, H. J. 1956. A convenient growth medium for *Neurospora*. *Microb. Genet. Bull.* 13:43.
- Wagoner, W., Loschke, D. C., and Hadwiger, L. A. 1982. Two-dimensional electrophoretic analysis of in vitro and in vivo protein synthesis in peas inoculated with compatible and incompatible *Fusarium solani*. *Physiol. Plant Pathol.* 20:99-107.
- Wolpert, T. J., Navarra, D. A., Moore, D. L., and Macko, V. 1994. Identification of the 100-kD victorin binding protein from oats. *Plant Cell* 6:1145-1155.