Extracellular Protein Elicitors from *Phytophthora*: Host-Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens

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Purified elicitor proteins (elicitins) from *Phytophthora parasitica* and *P. cryptogea* induced both localized and distal hypersensitive responses (HR) specifically in *Nicotiana* species and some radish and turnip cultivars but not in 12 other plant species. Differences between HR induction by acidic (parasiticein) and basic (cryptogein) isoforms were observed only for distal HR assays. Cryptogein consistently induced stronger distal necrosis in tobacco and radish than parasiticein. Similar results were obtained for the induction of a bean chalcone synthase promoter fused to a β-glucuronidase reporter in a transgenic tobacco line. However, in localized infiltration assays, both elicitin isoforms induced necrotic HR lesions at similar levels, suggesting that the difference between acidic and basic elicitins is related to distal HR induction and not to necrogenicity per se. Induced resistance to two *P. parasitica* isolates was observed on tobacco after pretreatment with elicitins. In radish, elicitins induced cultivar-specific HR and resistance to the bacterial pathogen, *Xanthomonas campestris* pv. *armoraciae*.

Additional keywords: black shank of tobacco, Xanthomonas leaf spots of crucifers.

Incompatible interactions between plants and pathogenic microbes often are associated with the induction in the plant of a complex set of biochemical responses believed to lead to resistance. The molecular events accompanying such incompatible interactions are presumed to occur as follows: 1) initial recognition by the plant of a pathogen molecule, 2) activation of cellular signal transduction pathway(s), and 3) induction of plant cell biochemical and physiological reactions that lead to resistance (Dixon and Lamb 1990; Ebel and Scheel 1990; Lamb *et al.* 1989). However, little is known about these events. Certain microbial and/or plant molecules, generally called elicitors, can mimic the induction of defense responses by incompatible pathogens (Anderson 1989; Darvill and Albersheim 1984; Dixon and Lamb 1990; Ebel and Scheel 1990; Lamb *et al.* 1989; Templeton and Lamb 1988). Elicitor molecules exhibit various levels of specificity. Numerous elicitors, such as 1,3-β-D-glucans, glycoproteins, chitosan, and arachidonic acid induce a defense response on both susceptible and resistant plants and are thus nonspecific (Darvill and Albersheim 1984; Dixon 1986; Lamb *et al.* 1989). Other nonspecific elicitors include molecules of plant origin, such as oligogalacturonides, which are released from the cell wall as a result of microbial degradative enzymes (Ryan 1988). Although nonspecific elicitors are probably involved in plant-pathogen signal exchange, the significance of these elicitors in resistance remains to be determined.

In contrast to nonspecific elicitors, specific elicitors induce a defense response only on plants that are resistant to the elicitor-producing pathogen. Specific elicitors are thought to mediate the action of avirulence genes (Keen 1986). For example, the *avrD* gene of *Pseudomonas syringae* pv. *tomato* confers cultivar-specific avirulence when introduced into the soybean pathogen *Pseudomonas syringae* pv. *glycinea* (Keen *et al.* 1990; Kobayashi *et al.* 1990). Bacterial strains, including *Escherichia coli*, bearing *avrD* produce a diffusible, low molecular weight molecule that elicits host-specific hypersensitivity on the resistant soybean cultivars, suggesting that *avrD* encodes an enzyme involved in the biosynthesis of the elicitor (Keen *et al.* 1990). Specific elicitors have also been identified in phytopathogenic fungi. The α race of *Colletotrichum lindemuthianum* produces an extracellular galactose- and mannose-rich glycoprotein that induces phytoalexin accumulation in an α-race resistant bean cultivar but not in a susceptible one, suggesting that this protein may directly act as an avirulence factor (Tepper and Anderson 1986; Tepper *et al.* 1989). The *Fulvia fulva* (*Cladosporium fulvum*) *avr9* gene directly encodes a processed 28-amino-acid peptide that is capable of inducing specific hypersensitivity on *Cf9*-bearing tomato cultivars (Scholtens-Toma and de Wit 1988; van Kan *et al.* 1991). Recently, it was shown that races of *F. fulva* that are virulent on *Cf9* lines do not contain sequences homologous to the cloned *avr9* gene and become avirulent after transformation with the *avr9* gene, confirming the direct role of this elicitor in host-specificity (van den Ackerveken *et al.* 1992; van Kan *et al.* 1991).

A family of small, 10-kDa extracellular elicitor proteins is produced by several species of *Phytophthora* (Ricci *et al.* 1989). These proteins, collectively termed elicitins, induce hypersensitivity on tobacco and resistance to the black shank agent *P. parasitica*. Because most virulent tobacco

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Table 1. Fungal and bacterial strains

Strains	Relevant characteristics[a]	Source or reference
Phytophthora parasitica		
P582	*Nicotiana tabacum* (Kentucky), A2, ELC−	M. Coffey[b]
P1351	*N. tabacum*, ELC−, race 1	M. Coffey[b]
P1960	*N. tabacum* (South Africa), ELC+	M. Coffey[b]
P1751	*N. tabacum* (Australia), A1, ELC+	M. Coffey[b]
6H-11A	*Hibiscus* (California), ELC+	J. McDonald[c]
P. cryptogea		
F2	*Chrysanthemum* (California), ELC+	J. McDonald[c]
Xanthomonas campestris pv. *armoraciae*		
XLS10R	Wild-type, rifampin[r]	Kamoun *et al.* 1992

[a] A1 and A2 refer to the compatibility type. ELC+ and ELC− indicate elicitin producers and nonproducers. The host species and place of origin are also indicated.
[b] University of California, Riverside.
[c] University of California, Davis.

isolates of *P. parasitica* do not produce elicitins (Ricci *et al.* 1989; Kamoun *et al.*, unpublished), these proteins have been proposed to act as avirulence factors in the *P. parasitica*–tobacco interaction. The *Phytophthora* elicitin protein family has been classified in two groups on the basis of both structural properties and biological activities (Nespoulous *et al.* 1992). The α-elicitin group comprises acidic proteins produced by *P. parasitica, P. capsici, P. cactorum,* and *P. citrophthora*, whereas β-elicitins are basic, more hydrophillic proteins produced by *P. cryptogea, P. cinnamomi,* and *P. megasperma* (Bonnet 1985; Nespoulous *et al.* 1992). Recently, an isolate of *P. drechsleri* was shown to produce both α- and β-elicitin isoforms (Huet *et al.* 1992). Using petiole dip assays on tobacco, Nespoulous *et al.* concluded that β-elicitins are more necrogenic than α-elicitins (Nespoulous *et al.* 1992).

In this paper we investigated the host-specificity of purified elicitins from *P. parasitica* Dastur and *P. cryptogea* Pethybr. & Lafferty. We also analyzed differences between the α and β isoforms of elicitins in induction of hypersensitivity, induction of the promoter of the plant gene chalcone synthase (*CHS*), and induction of resistance to *P. parasitica*. Finally, we describe the induction by elicitins of cultivar-specific resistance to the bacterial phytopathogen *Xanthomonas campestris* pv. *armoraciae* in radish.

RESULTS

Host-specificity of elicitins.

The various fungal and bacterial strains used in this study are summarized in Table 1. Elicitins were purified from the culture filtrates of *P. cryptogea* F2 and *P. parasitica* 6H-11A, P1960, and P1751 by chromatography (Materials and Methods, Fig. 1). Following infiltration into leaves of tobacco (cv. Havana), all four protein fractions containing elicitins rapidly induced water-soaking (6–10 hr), succeeded by a brown pigmented necrosis characteristic of a hypersensitive response (HR) (16–24 hr), localized to the infiltrated area. Infiltration of serially diluted solutions indicated that minimal threshold concentrations of 10–50 nM were necessary for HR induction by all four protein fractions (data not shown).

In order to investigate the host-specificity of the elicitins, 100-nM solutions of parasiticein 6H-11A (α-elicitin) and cryptogein F2 (β-elicitin) were infiltrated into fully expanded leaves of a collection of 20 plant species represented by 61 lines. All six *Nicotiana* species tested responded with a HR to both elicitins. In addition, five other *Nicotiana* species, *N. benthamiana, N. debneyi, N. glauca, N. longiflora,* and *N. plumbaginifolia* were sensitive to both elicitins when tested in a petiole dip assay (not shown). Most other plants were insensitive to elicitins and did not show any visible necrotic response, suggesting that elicitins are host-specific elicitors (Table 2). The exception was a number of crucifers, especially radish cultivars, but also including the hybrid turnip line Just Right. In contrast to the *Nicotiana* species, the radish cultivars displayed differential responses to elicitins, along with differences in the confluency and severity of the necrosis caused by 100-nM elicitin solutions. Therefore, different levels of sensitivity and/or responsiveness to elicitins occur in radish (Table 2). No difference was observed between the elicitin isoforms in the specificity and severity of HR induction on plants.

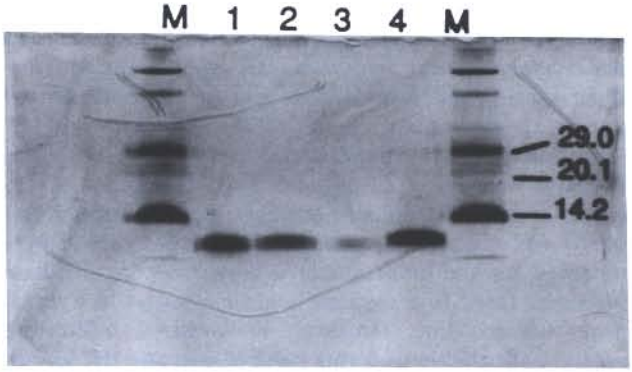

Fig. 1. Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of elicitins purified as described in Materials and Methods from *Phytophthora parasitica* 6H-11A (lane 1), P1960 (lane 2), P1751 (lane 3), and *P. cryptogea* F2 (lane 4). Each lane contains approximately 250 ng of elicitin. Sigma low molecular protein standard is shown on lanes M, along with the sizes in kilodaltons of selected markers.

Distal necrosis induction by α- and β-elicitins.

On the basis of petiole dip assays, it was proposed that β-elicitins (including cryptogein) are more necrogenic than α-elicitins (including parasiticein) (Nespoulous *et al.* 1992). However, we observed no difference between the two isoforms in HR induction following infiltration (Table 2). To further test the necrogenicity of α- and β-elicitins, we

Table 2. Species and cultivar-specificity of hypersensitive response induction by parasiticein and cryptogein

Family Species Cultivar	Source	Response to elicitins[a] Para (α)	 Cry (β)
Chenopodiaceae			
Chenopodium amaranticolor	UC Davis	− (4)	− (2)
Compositae			
Lactuca sativa		− (4)	− (2)
Vanguard 75	Richard Michelmore[b]	− (4)	− (2)
Diana	Richard Michelmore[b]	− (4)	− (2)
Dark Green Boston	Richard Michelmore[b]		
Cruciferae			
Arabidopsis thaliana		− (3)	− (3)
Colombia-0	Arabidopsis Information Service		
Brassica campestris			
Tokyo Cross	American Takii Seed Co.	− (2)	− (2)
Tokyo Turnip Hybrid	Northrup King Seed Co.	− (4)	− (2)
Turnip Just Right	American Takii Seed Co.	+ (10)	+ (5)
Takane Hybrid	Sakata Seed Co.	− (2)	− (2)
Kanamashi	Sakata Seed Co.	− (2)	− (2)
Shikimaki	Sakata Seed Co.	− (2)	− (2)
Seven Top Turnip	Northrup King Seed Co.	− (4)	− (2)
Shogoin Turnip	Northrup King Seed Co.	− (4)	− (2)
Michihili Chinese Cabbage	Northrup King Seed Co.	− (4)	− (2)
B. juncea			
Florida Broadleaf India Mustard	Northrup King Seed Co.	− (4)	− (2)
B. oleracea			
Early Jersey Wakefield Cabbage	Northrup King Seed Co.	− (4)	− (2)
Golden Acre Cabbage	Lilly Miller Seed Co.	− (4)	− (2)
Red Acre	Northrup King Seed Co.	− (4)	− (2)
Purple Kohlrabi	Northrup King Seed Co.	− (2)	− (2)
White Kohlrabi	Northrup King Seed Co.	− (2)	− (2)
Raphanus sativus			
All Season Tokinashi	Sakata Seed Co.	++ (7)	++ (5)
Daikon	Lilly Miler Seed Co.	++ (11)	++ (7)
Early Mino	Northrup King Seed Co.	++ (7)	++ (5)
Mino's Sakata Imperial	Sakata Seed Co.	+ (3)	+ (2)
Miyashige	Sakata Seed Co.	+ (4)	+ (3)
Miura	Sakata Seed Co.	+ (2)	+ (2)
Koutoaonaga	Sakata Seed Co.	+ (2)	+ (2)
Giant White Globe	Northrup King Seed Co.	++ (2)	++ (2)
Daikon Shogoin	Sakata Seed Co.	++ (5)	++ (5)
White Icicle	Northrup King Seed Co.	− (8)	− (4)
Yukikomashi	Sakata Seed Co.	− (3)	− (2)
Ideal White	Sakata Seed Co.	++ (3)	++ (3)
Red Prince	Northrup King Seed Co.	− (3)	− (3)
Red Baron	Northrup King Seed Co.	+ (10)	+ (10)
Sg5317	Sluis & Groot	++ (6)	++ (6)
Kader	Sluis & Groot	++ (2)	++ (2)
Round White	Northrup King Seed Co.	+ (4)	+ (2)
8318C	Northrup King Seed Co.	+ (3)	+ (2)
Fuego	Northrup King Seed Co.	+ (2)	+ (1)
Spanish Radish	Lilly Miller Seed Co.	− (3)	− (3)
Cucurbitaceae			
Cucumis sativus			
Armenian Cucumber	Northrup King Seed Co.	− (4)	− (2)
Salad Bar hybrid	Northrup King Seed Co.	− (4)	− (2)
Leguminosae			
Glycine max			
Williams	Richard Buzzell[c]	− (2)	− (2)
Harosoy	Richard Buzzell[c]	− (2)	− (2)
Phaseolus vulgaris			
Greencrop Bush Beans	Northrup King Seed Co.	− (4)	− (2)
Henderson Lima Beans	Northrup King Seed Co.	− (4)	− (2)

(*continued on next page*)

[a] 100 nM of parasiticein (Para) 6H-11A and cryptogein (Cry) were infiltrated in the leaves of the listed plants: — indicates no visible response, + indicates nonconfluent necrotic response, ++ indicates confluent necrotic response. The numbers in parenthesis refer to the number of independent infiltrations.
[b] University of California, Davis.
[c] Agriculture Canada.
[d] University of California, Berkeley.

Table 2. *Continued*

Family Species Cultivar	Source	Response to elicitins[a] Para (α)	 Cry (β)
Solanaceae			
Capsicum frutescens			
Early California Wonder (ECW)	Brian Staskawicz[d]	− (4)	− (2)
ECW10R	Brian Staskawicz[d]	− (4)	− (2)
ECW20R	Brian Staskawicz[d]	− (4)	− (2)
Datura stramonium	UC Davis	− (5)	− (2)
Lycopersicon esculentum			
Ace55 VF	Northrup King Seed Co.	− (4)	− (2)
Watters PF	Harris Moran Seed Co.	− (4)	− (2)
Nicotiana alata	John Yoder[b]	+ (4)	+ (2)
N. bonariensis	John Yoder[b]	++ (7)	+ (5)
N. glutinosa	John Yoder[b]	++ (4)	++ (2)
N. langsdorfii	John Yoder[b]	+ (5)	+ (3)
N. rustica	John Yoder[b]	+ (5)	+ (2)
N. tabacum			
Turkish	UC Davis	++ (8)	++ (3)
Havana	UC Davis	++ (4)	++ (2)
Xanthi-nc	Calgene Inc.	++ (13)	++ (13)
Solanum melongena			
Black Beauty Eggplant	Northrup King Seed Co.	− (4)	− (2)

Table 3. Induction of distal necrosis on detached leaves by parasiticein and cryptogein

Species Cultivar	Response[a] Para (α)	 Cry (β)	 ddH_2O
Nicotiana tabacum			
Xanthi-nc	− (4)	++ (4)	− (2)
Raphanus sativus			
All Season Tokinashi	+ (5)	++ (5)	− (3)
Early Mino	+ (2)	++ (2)	− (2)
Mino's Sakata Imperial	− (2)	++ (1)	− (2)
Miyashige	+ (2)	++ (2)	− (2)
Miura	+ (3)	++ (3)	− (3)
Daikon Shogoin	+ (4)	++ (4)	− (2)
White Icicle	− (2)	− (2)	− (2)
Yukikomashi	− (2)	− (2)	− (1)
Ideal White	+ (3)	++ (3)	− (2)
Red Prince	− (2)	− (2)	− (1)
Red Baron	− (3)	− (3)	− (1)
8318C	− (2)	− (2)	− (1)
Spanish Radish	− (3)	+ (2)	− (2)

[a] Detached leaves were treated by the petiole dip assay with 100 nM parasiticein (Para) 6H-11A, cryptogein (Cry) F2, or double-distilled water (ddH_2O): − indicates no visible response; + indicates single, minute necrotic spots; ++ indicates large necrotic areas resulting in collapse and drying of the leaf. The numbers in parentheses indicate the total number of leaves tested. *R. sativus* cultivars Red Baron and 8318C showed no necrotic response in this assay, even though they responded by a nonconfluent necrosis in the infiltration assay (Table 1). In Spanish Radish, one of the two leaves treated with cryptogein showed some minute necrotic lesions. All other results correlated with the infiltration assays reported in table 1.

conducted petiole dip assays on *N. tabacum* Xanthi-nc and a number of radish cultivars. In all tested cultivars, cryptogein consistently induced a much stronger distal necrosis than parasiticein (Table 3). Uptake by detached leaves of 100 nM cryptogein often resulted in a severe confluent necrosis that led to the drying and collapse of the entire leaf, whereas 100 nM parasiticein induced at most minute necrotic spots. On the other hand, infiltration of leaves with the same solutions induced necrotic lesions of similar intensity (Fig. 2, Table 3). These observations

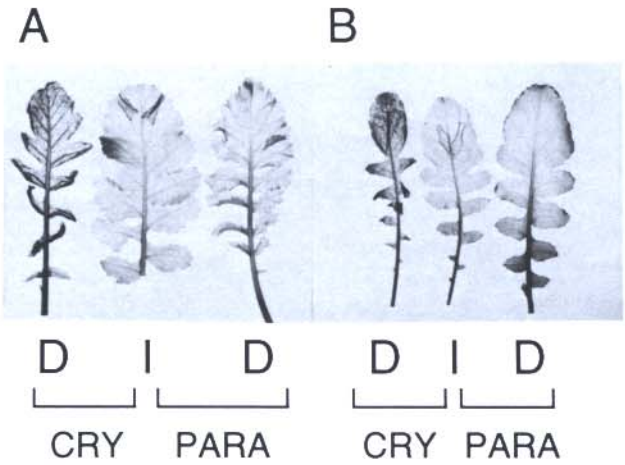

Fig. 2. Distal and local induction of hypersensitivity by α- and β-elicitins. Leaves of the radish cultivars **A,** Daikon Shogoin, and **B,** All Season Tokinashi were infiltrated (I) or petiole dip assayed (D) with 100 nM cryptogein F2 (CRY) or 100 nM parasiticein 6H-11A (PARA). Leaves were cleared of chlorophyll with 70% ethanol before photography.

suggest that the difference in HR induction between α- and β-elicitins lies in the ability to induce distal necrosis rather than in necrogenicity per se.

Temporal and spatial analysis of induction by elicitins of bean chalcone-synthase promoter in tobacco.

Using fusions to a *GUS* reporter gene, the 5′-flanking sequence of the bean *CHS8* gene was shown to be induced in transgenic tobaccos by various pathogen and stress treatments (Doerner *et al.* 1990; Stermer *et al.* 1990). To analyze the induction of the *CHS8* promoter by elicitins, leaves of a tobacco transgenic line, Xanthi-nc (*CHS8*::*GUS*), were infiltrated with a 100-nM solution of parasiticein 6H-11A or cryptogein F2, and at various times *GUS* activity was determined *in situ* by histochemical staining. As illustrated in Figure 3, 6 hr after the treatment,

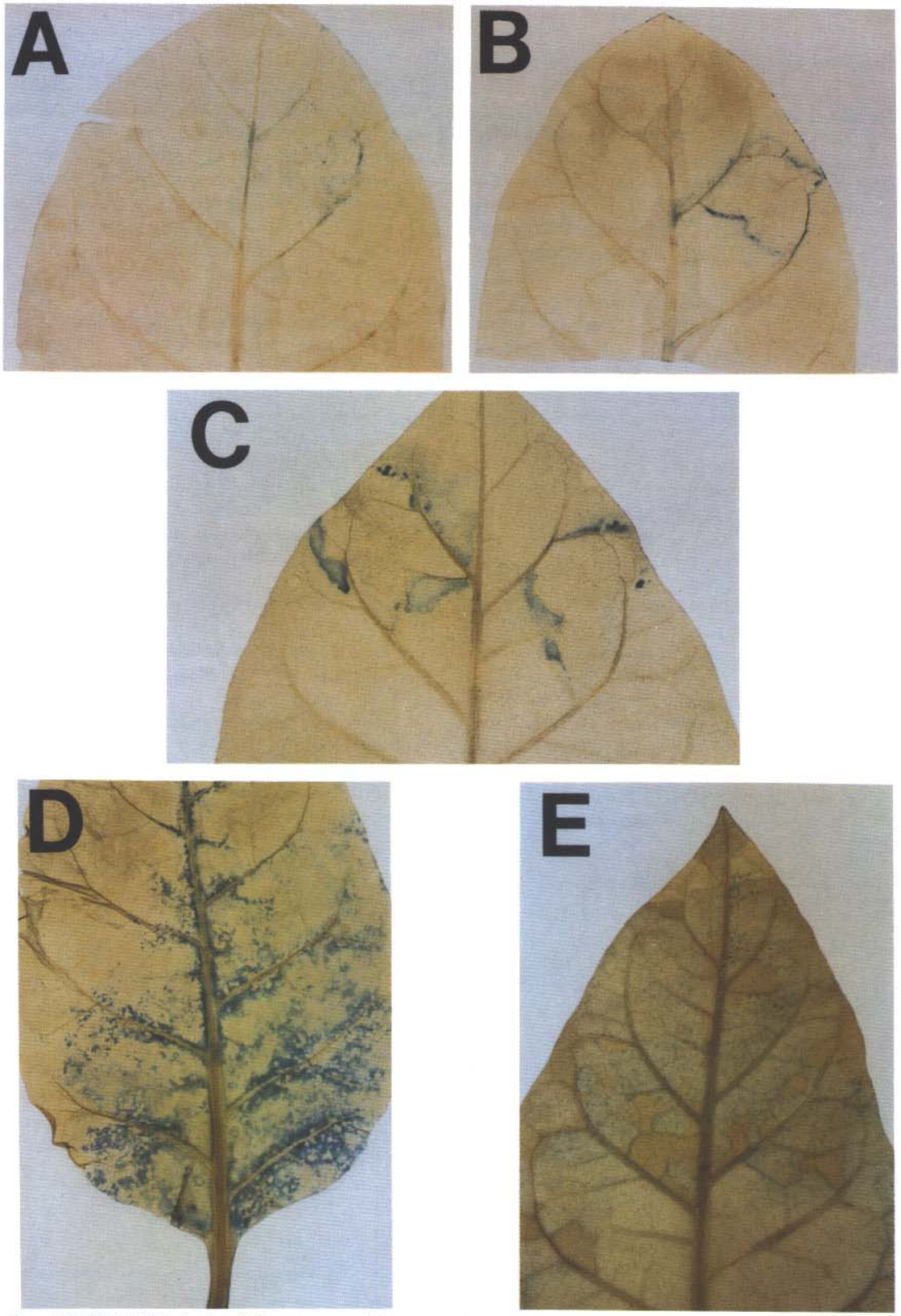

Fig. 3. Induction of *CHS8::GUS* fusion by elicitins. Leaves of the tobacco line Xanthi-nc (*CHS8::GUS*) were infiltrated with 100 nM parasiticein 6H-11A (right half of the leaves in **A, B, and C**) and 100 nM cryptogein F2 (left half of the leaf in **C**). The left half of the leaves in **A** and **B** were infiltrated with a water solution. *GUS* assays were performed 6 hr (**A**) or 24 hr (**B and C**) after infiltration. Petiole dip assays followed by *GUS* assays at 24 hr were performed on the same line with 100 nM cryptogein (**D**) and 100 nM parasiticein (**E**). Note that the elicitin infiltrated panels in **B** and **C** showed necrosis, and so did the upper right and left part of the leaf shown in **D**, but the leaf in **E** showed no necrosis. Leaves were cleared of chlorophyll with 70% ethanol before photography.

GUS activity was visible within the infiltrated area in correlation with water-soaking symptoms (Fig. 3A). At 24 hr, the entire infiltrated area became necrotic, and *GUS* activity was then localized to the immediate vicinity of the necrotic area (Fig. 3B and C). No *GUS* activity was detected within this time frame after infiltration with water (Fig. 3A and B). Also, as previously reported for necrotic symptoms, no difference in the intensity of *CHS8*::*GUS* induction between α- and β-elicitins was observed in a series of side-by-side infiltrations, confirming that the two isoforms induce a similar HR response by infiltration in leaf panels (Fig. 3C).

To determine distal induction of the *CHS8* promoter by elicitins, petiole dip assays were performed on the Xanthi-nc (*CHS8*::*GUS*) line with 100-nM solutions of elicitins. Cryptogein F2 induced distal necrosis along with large areas of *GUS* activity in nonnecrotic areas (Fig. 3D). On the other hand, 100 nM of parasiticein 6H-11A did not induce necrosis and induced only minute blue spots of *GUS* activity throughout the leaf (Fig. 3E). Again, these results confirm that cryptogein (β-elicitins) induces a stronger distal response than parasiticein (α-elicitins).

Induced resistance to the tobacco pathogen *P. parasitica* by α- and β-elicitins.

Tobacco plants pretreated with elicitins were resistant to the black shank agent, *P. parasitica* (Ricci *et al.* 1989). To determine the ability of α- and β-elicitins to induce resistance to *P. parasitica*, decapitated stems of tobacco plants (cv. Xanthi-nc) were treated with parasiticein 6H-11A (5 μg), cryptogein F2 (5 μg), or water. One day later, necrotic lesions of variable sizes appeared on several leaves of all plants treated with β-elicitin, but no necrosis was visible on α-elicitin- or water-treated plants, confirming the difference in distal HR induction between the two isoforms of elicitin. Three days after the elicitin treatment, the tobacco plants were challenged with mycelium plugs of *P. parasitica* P582 and P1351, and the length of the disease lesion was recorded at various times for 11 days. Both strains were highly virulent on the control Xanthi-nc plants. Pretreatment of Xanthi-nc tobaccos by cryptogein induced total protection against P582 and significant protection against P1351, whereas pretreatment by parasiticein induced partial protection only against P582 (Fig. 4). These results suggest that, at similar concentrations, β-elicitins induce a more effective resistance of tobacco to *P. parasitica* than α-elicitins.

Elicitins induce cultivar-specific resistance to the bacterial phytopathogen *X. c.* pv. *armoraciae*.

We showed above that elicitins induced cultivar-specific HR in radish (Table 2). To determine whether elicitins also induce an effective defense response in radish, the crucifer bacterial pathogen *X. c.* pv. *armoraciae* XLS10R (10^5–10^7 cells per milliliter) was coinfiltrated with 1 nM cryptogein or parasiticein (subnecrotic concentrations) into the leaves of five radish cultivars (Table 4). In all tested cultivars, control inoculations with XLS10R yielded necrotic lesions of variable sizes typical of the Xanthomonas leaf spot disease of crucifers. However, when XLS10R was coinoculated with parasiticein or cryptogein in the radish cultivars Early Mino and Sg5317, which respond to elicitins, the bacteria did not cause necrotic lesions, but instead only induced a localized chlorosis (Table 4, Fig. 5). On the other hand, elicitins did not interfere with the disease symptoms induced by XLS10R in the radish cultivar White Icicle, which does not respond to elicitins, suggesting that induced resistance to *X. c.* pv. *armoraciae* by elicitins is cultivar-specific (Table 4, Fig. 5). The two other cultivars, All Season Tokinashi and Red Baron,

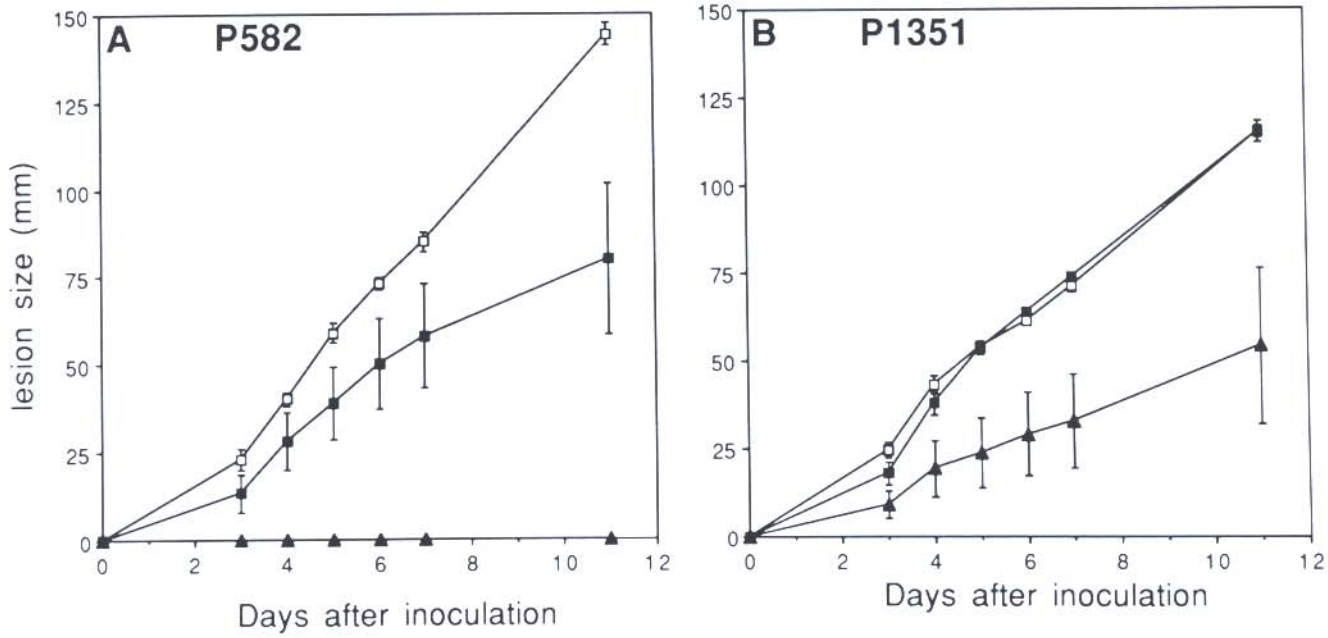

Fig. 4. Time course of the invasion by *Phytophthora parasitica* **A,** P582 and **B,** P1351 of tobacco after treatment with 5 μg of parasiticein 6H-11A (■), 5 μg of cryptogein F2 (▲), or water (□). Bars represent standard errors.

showed a necrotic response to 100 nM elicitins but did not show induced resistance with 1 nM elicitin.

To confirm the induced resistance to *X. c.* pv. *armoraciae*, *in planta* growth curves of XLS10R were determined in two radish cultivars in the presence or absence of elicitins. Coinfiltration with elicitins resulted in significant reduced growth of XLS10R in Early Mino radish correlating with the absence of necrotic lesions (Fig. 6A). On the other hand, elicitins did not affect growth of XLS10R in White Icicle radish (Fig. 6B) correlating with the presence of symptoms. To extend these observations, bacterial populations were determined in five radish cultivars on the same day and four days after inoculation. The results show that the resistance induced by parasiticein observed in both Early Mino and Sg5317 correlates with growth inhibition; however, in this experiment cryptogein did not induce statistically significant growth inhibition even though it induced resistance. Additionally, neither elicitin had a significant effect on bacterial growth in cultivars All Season Tokinashi, Red Baron, and White Icicle, which show no induced resistance (Table 4). In summary, it appears that cryptogein and parasiticein can both induce an effective defense response to *X. c.* pv. *armoraciae* that results in a reduction of leaf spot symptoms and is sometimes accompanied by a significant growth inhibition in some elicitin-responsive radish cultivars.

DISCUSSION

Host-specificity of elicitins.

The host-specificity of purified elicitin proteins from the culture filtrates of *P. parasitica* and *P. cryptogea* was examined. Solutions of 100 nM elicitin did not induce any visible response in most plant species tested including several solanaceous plants, but elicitins induced both localized and distal HR in all tested *Nicotiana* species and in some but not all *Raphanus sativus* and *Brassica campestris* cultivars. Therefore these proteins are genus-specific elicitors within the Solanaceae and cultivar-specific elicitors within the Cruciferae (Table 2). The induction by elicitors and *avr* gene products of a defense response in taxonomically unrelated plants is not a unique observation to elicitins. For example, the *Pseudomonas syringae* pv. *tomato* avirulence gene *avrRpt2* confers avirulence of *Pseudomonas syringae* in *Arabidopsis thaliana* ecotype Col-0 (Cruciferae) and in certain soybean (*Glycine max*) cultivars (Leguminosae) (Whalen *et al.* 1991). It remains to be determined whether specific recognition of pathogen signals by unrelated plants is fortuitous or results from a common biological function in the plant or pathogen.

Mechanisms of induction of defense responses are thought to be conserved among plants even though the ultimate expression of the defense response varies considerably between taxa (Dixon and Lamb 1990; Lamb *et al.* 1989; Stermer *et al.* 1990). For example, chalcone synthase is actively involved in phytoalexin biosynthesis in bean but not in tobacco. Nevertheless, when introduced into a tobacco background, the bean *CHS8* promoter is still activated by stress signals, suggesting that this gene responds to conserved regulatory systems (Doerner *et al.* 1990; Stermer *et al.* 1990). In this paper we show that even though elicitins did not induce a defense response in bean, they were able to induce the expression of a bean *CHS8* promoter in a tobacco genetic background. Therefore, these results confirm the view that specificity in the activation of plant defense responses relies essentially on the recognition of elicitors, and that the signal transduction pathways are rather conserved between unrelated plants.

Elicitins were proposed to act as avirulence factors in the *Nicotiana–P. parasitica* interaction. Most *P. parasitica* isolates virulent on tobacco do not produce elicitins and thus may evade tobacco defense responses (Ricci *et al.* 1989; Kamoun *et al.*, unpublished). Similarly, the recognition of elicitins by cruciferous plants could play a role in the interaction of crucifers with *Phytophthora*. Strains of *P. megasperma* and *P. cryptogea* pathogenic on *Brassica oleracea* (cabbage and cauliflower) and *B. napus* have been reported (Downes and Loughnane 1969; Farr *et al.* 1989; Grand 1985; Hamm and Koepsell 1984; Kontaxis and Rubatzky 1983; Robertson and Ogilvie 1953; Tompkins *et al.* 1936); however, no *Phytophthora* disease of radish or turnip has been described (Farr *et al.* 1989). Additionally, several *Phytophthora* isolates from *B. oleracea* and *B. napus* were found to produce elicitin (Kamoun *et al.*, unpublished). Whether elicitin recognition by radish and other crucifer cultivars confers resistance to *Phyto-*

Table 4. *In planta* growth and disease induction of *Xanthomonas campestris* pv. *armoraciae* in the presence of elicitins in various radish cultivars

Cultivar	Elicitin response[a]	Leaf spots[b]	Bacterial population growth[c] (ratios of cfu/cm² leaf tissue)		
			H_2O	Para (α) 1 nM	Cry (β) 1 nM
Early Mino radish	+	−	25.7 a	3.0 b	4.9 ab
Sg5317	+	−	24.3 a	7.1 b	14.2 ab
All Season Tokinashi	+	+	23.3 a	21.6 a	9.7 a
Red Baron	+	+	32.4 a	51.9 a	25.5 a
White Icicle	−	+	22.8 a	41.0 a	42.9 a

[a] + Indicates hypersensitive response induction after infiltration with 100 nM elicitin solutions; − indicates no response.
[b] + Indicates necrotic leaf spot lesions induced by *X. c.* pv. *armoraciae* in the presence of 1 nM elicitin solutions 5–7 days after infiltration; − indicates inhibition of leaf spot lesions by elicitins.
[c] Para refers to parasiticein 6H-11A and Cry to cryptogein F2. Numbers represent the ratios of geometric averages between day 4 and day 0. Numbers in a row followed by the same letters did not significantly differ ($P > 0.05$) under Student's unpaired t test, which was performed independently for each cultivar, using logarithm transforms of the population data.

phthora pathogens awaits further investigation and should help determine the role of elicitins in the interaction between *Phytophthora* and crucifers.

α- and β-Elicitins vary in distal HR induction.

The *Phytophthora* elicitin protein family has been classified into two groups based on both structural properties and biological activities (Nespoulous *et al.* 1992). Using detached-leaf assays of tobacco, Nespoulous *et al.* concluded that β-elicitins are more necrogenic than α-elicitins (Nespoulous *et al.* 1992). In this paper, we confirmed the observation that β-elicitins (i.e., cryptogein) induce larger necrotic areas than α-elicitins (i.e., parasiticein) in distal HR assays (detached tobacco or radish leaves or decapitated tobacco stems). However, both isoforms induced HR at identical levels when directly infiltrated into leaf mesophyll (Table 3, Fig. 2). The patterns of induction by elicitins of the *CHS8*::*GUS* fusion in tobacco also pointed to differential distal inductions between parasiticein and cryptogein, whereas both proteins induced similar levels of *GUS* activity through leaf infiltrations (Fig. 3). In induced resistance assays against *P. parasitica*, a pathogen that spreads extensively from the site of infection, cryptogein (β) induced better protection than parasiticein (α) in a tobacco stem decapitation assay (Fig. 4). However, both cryptogein and parasiticein induced similar resistance to *X. c.* pv. *armoraciae*, a pathogen with limited spread, when infiltrated in radish leaves with the bacteria (Table 4). Together, these observations suggest that the difference in biological activity noted between the two elicitin isoforms may reside in a greater ability of β-elicitins to induce distal HR rather than in a difference in the interaction between elicitins and target leaf cells.

Recently, Zanetti *et al.* (1992) analyzed the systemic movement and translocation of radiolabeled elicitins in tobacco. They demonstrated that both cryptogein (β) and capsicein (α) are equally able to migrate in the tobacco vascular system without undergoing any detectable molecular alteration. Thus, it appears that the difference in induction of distal necrosis by α- and β-elicitins does not reflect differences in *in planta* vascular movement. The necrosis-inducing ability of the elicitin isoforms could then depend on the capacity of these proteins to translocate after migration from the vascular to the mesophyll tissue or to penetrate plant cell walls. Such properties could depend on the overall structure or other features of these proteins (such as the charge) rather than a single mutated amino-acid site (Nespoulous *et al.* 1992; Ricci *et al.* 1989). Further analysis of the properties of additional isoforms should help identify the structures involved in high distal necrogenicity.

The relatively poor ability of parasiticein to protect to-

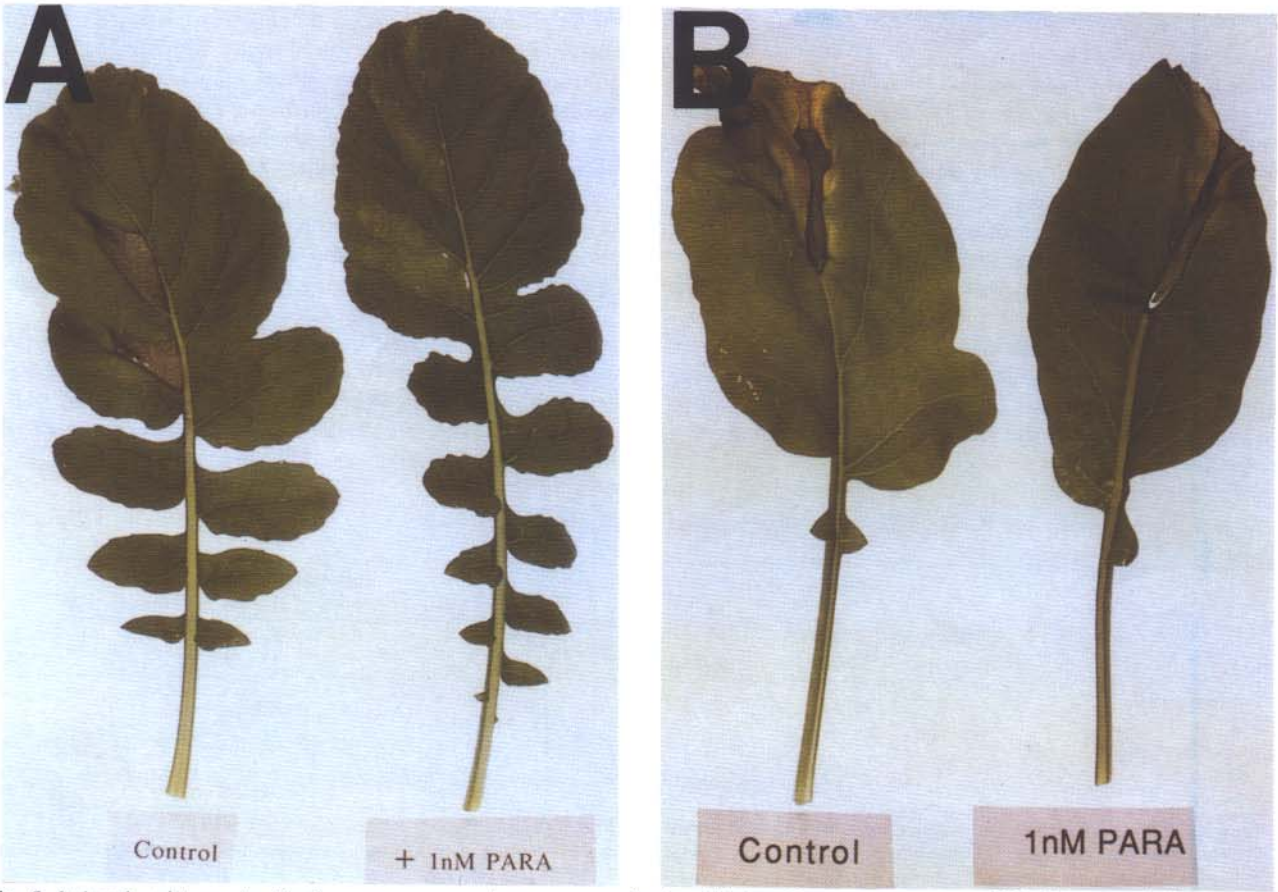

Fig. 5. Induced resistance to *Xanthomonas campestris* pv. *armoraciae* by elicitins. *X. c.* pv. *armoraciae* XLS10R was coinfiltrated alone (left leaves) or with 1 nM parasiticein (right leaves) in the radish cultivars **A,** Early Mino and **B,** White Icicle. Photographs were taken 7 days after inoculation.

bacco against *P. parasitica* in the induced resistance assays raises a question as to the actual role of elicitins as avirulence factors in the *P. parasitica*-tobacco interaction. An explanation may be that *P. parasitica* simply outgrows the local protection provided by exogenous application of 5 μg of parasiticein; continuous production of parasiticein by a pathogen might result in continuous protection against the pathogen. This could be tested most conclusively by forcing the expression of a cloned elicitin gene in a transgenic *P. parasitica* tobacco pathogen.

Elicitins induce cultivar-specific resistance to a bacterial phytopathogen.

X. c. pv. *armoraciae* causes the Xanthomonas leaf spot disease of crucifers (McCulloch 1929; Moffett *et al.* 1976; White 1930). The interaction of *X. c.* pv. *armoraciae* with cruciferous hosts is highly tissue-specific. Even though this bacterium multiplies and causes disease lesions in the mesophyll tissue of crucifers, it is unable to invade the same host plants through the vascular system and induces a tissue-specific HR in vascular tissue (Kamoun *et al.* 1992). We have shown that coinfiltration into the mesophyll tissue of subnecrotic concentrations of elicitins along with *X. c.* pv. *armoraciae* XLS10R results in the suppression of disease lesion formation in certain radish cultivars. This induced resistance is sometimes associated with a significant inhibition of the growth of the bacterium (Figs. 5 and 6, Table 4). Therefore the plant response to elicitin, a fungal elicitor, can provide protection against a bacterial pathogen.

Acquired resistance of radish to Xanthomonas leaf spot disease was found to be cultivar-specific. In *R. sativus* cvs. Early Mino and Sg5317, elicitins induced a necrotic HR at 25–100 nM and induced resistance to *X. c.* pv. *armoraciae* XLS10R at 1 nM, suggesting that, at subnecrotic levels, elicitins can induce effective antibacterial defense response(s) in radish. Of the other three radish cultivars tested, cv. White Icicle did not respond to elicitins at all concentrations tested, whereas cvs. All Season Tokinashi and Red Baron produced an HR at 100 nM elicitin but were not protected against *X. c.* pv. *armoraciae* by 1 nM elicitin (Table 4). This discrepancy could be explained by a lesser sensitivity to 1 nM elicitin or by a generally poor antibacterial defense in these two cultivars. In conclusion, it is unclear at this stage whether the biological basis of the cultivar-specificity observed for acquired resistance by elicitins overall reflects the specificity observed in HR induction in radish.

In tobacco, subnecrotic levels of cryptogein are known to induce various physiological defense responses including sesquiterpenoid phytoalexin and ethylene production (Milat *et al.* 1991). Additionally, we observed that subnecrotic levels of elicitins do induce the bean *CHS8* promoter (Fig. 3E, unpublished data). Therefore, the induction of antibacterial defenses in radish by subnecrotic concentrations of parasiticein and cryptogein was not totally unexpected. Since radish has the ability to become resistant to *X. campestris*, it should be possible to identify the pertinent defense mechanisms that result in induced resistance and eventually to alter the expression of these defense responses to yield resistant engineered plants (Kamoun and Kado, in press). Elicitin-based, induced resistance constitutes an ideal model system for such an endeavor because of the abundant biochemical and biological information amassed for these specific fungal elictors.

MATERIALS AND METHODS

Fungal and bacterial strains.

The various fungal and bacterial strains used in this study are summarized in Table 1.

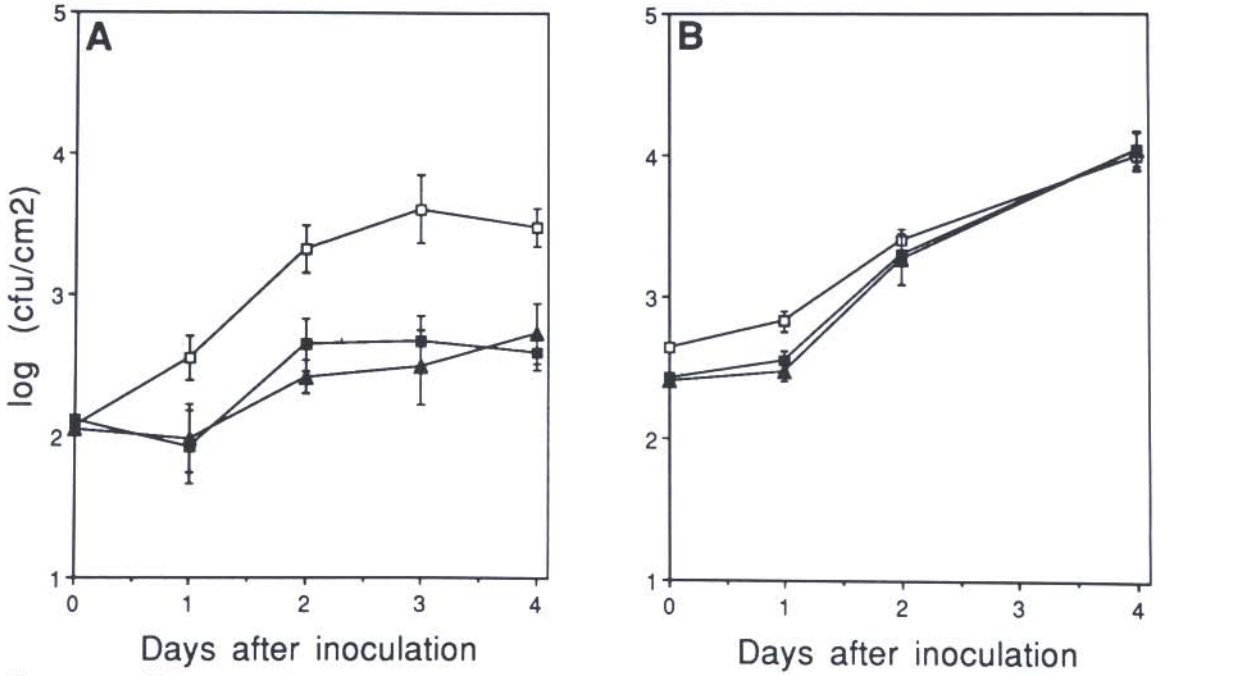

Fig. 6. Time course of the growth of *Xanthomonas campestris* pv. *armoraciae* in **A,** Early Mino radish and **B,** White Icicle radish, in the presence of 1 nM parasiticein 6H-11A (■), 1 nM cryptogein F2 (▲), or water (□). Bars represent standard errors.

Culture conditions.

Phytophthora strains were routinely grown in cleared or uncleared 20% vegetable juice (V8) medium supplemented with 1.5% agar at 24° C (Ribeiro 1978).

X. c. pv. *armoraciae* was grown in medium 523 broth or 523 1.5% agar plates at 28° C (Kado *et al.* 1972). Rifampin (50 μg/ml) was supplemented when necessary.

Purification of elicitins.

For elicitin production, *P. cryptogea* and *P. parasitica* were grown for 2–3 wk at 25° C in still culture in medium containing, per liter, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1 g of asparagine, 1 mg of thiamine, 0.5 g of yeast extract, and 25 g of glucose (modified from Bonnet *et al.* 1985). The culture medium was harvested by filtration through Miracloth (Calbiochem) and then through a 0.22-μm filter. Proteins were precipitated by addition of 70.7 g of $(NH_4)_2SO_4$ per 100 ml of medium at 0° C and then resuspended in 0.01 volume of 10 mM Tris·HCl (pH 7.4) with 1 mM EDTA. Elicitins were purified from the crude proteins by chromatography on Sephacryl HR100 (Pharmacia) in 75 mM NaCl with 10 mM Tris·HCl (pH 7.4). Parasiticeins were further purified by diluting the pooled Sephacryl fractions to 10 mM NaCl with 10 mM Tris·HCl (pH 7.4) and loading them onto a Fast-flow Sepharose Q (Pharmacia) column equilibrated with 10 mM Tris·HCl (pH 7.4). The column was then eluted with a linear gradient of 0–300 mM NaCl in 10 mM Tris·HCl (pH 7.4). Cryptogeins were further purified by diluting the pooled Sephacryl fractions with two volumes of 10 mM NaOAc (pH 5) and loading them on a Fast-flow Sepharose S (Pharmacia) column equilibrated with 10 mM NaOAc (pH 5) and 25 mM NaCl. The column was then eluted with a linear gradient of 25 mM to 1 M NaCl in 10 mM NaOAc (pH 5). The elicitins were identified throughout the purification by Coomassie blue R250 staining of 20% polyacrylamide/sodium dodecyl sulfate gels. Elicitin concentrations were determined from the absorbance at 205 nm. Purity of the elicitins (from other proteins of different mobility) was greater than 99% as determined by silver-staining of overloaded polyacrylamide/sodium dodecyl sulfate gels (Fig. 1). All experiments described in this paper were carried out using elicitins purified by both Sephacryl HR100 and ion-exchange chromatography.

Hypersensitivity assays.

Induction of hypersensitivity by elicitins was determined by infiltration of sterile distilled water solutions of elicitin (1–100 nM) into attached leaves as described by Klement *et al.* (1964). Hypersensitive response (HR) was scored when a brown necrosis occurred in the infiltrated area 16-48 hr after inoculation.

To test for induction of distal HR, petiole dip assays were conducted on freshly cut tobacco and radish leaves. Leaf petioles were dipped into a 0.5-ml Eppendorf tube containing 500 μl of a 100-nM elicitin solution or water. The solution was usually taken up after about 2 hr. Then, the leaves were transferred to small beakers containing sterile water and kept at 24° C for the duration of the assay. Necrotic lesions were visible after 24 hr and ranged from minute necrotic spots to large, confluent necrotic areas. For each experimental set, leaves of approximately the same size were selected.

To better visualize hypersensitive necrosis of radish, leaves were thoroughly washed for several days in 70% ethanol at room temperature to remove chlorophyll. In crucifers, necrotic areas correlate with the accumulation of an ethanol-insoluble brown to black material, whereas no similar material was observed in HR lesions of tobacco.

Histochemical *GUS* assays.

Histochemical localization of β-glucuronidase (*GUS*) activity in leaves of a trangenic tobacco line (cv. Xanthi containing a bean *CHS8*::*GUS* fusion [Stermer *et al.* 1990]) was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc; Biosynth International, Skokie, IL) as a chromogenic substrate (Jefferson *et al.* 1986; Jefferson *et al.* 1987). Staining and destaining procedures were described previously (Kamoun *et al.* 1992). Stained leaves were photographed with Kodak Ektachrome 100 film at magnifications of from 1:1 to 1:4.

Fungal pathogenicity assays.

Virulence of *P. parasitica* on tobacco was determined using a stem assay. The stems of fully bolted Xanthi-nc tobacco plants (50–60 days old) were decapitated near the terminal bud. Elicitin solutions (5 μg in 30 μl) or the same volume of sterile distilled water were applied to the decapitated stem and allowed to be absorbed. Three days after the treatment, the dry surface of the decapitated area was cut (usually a few millimeters), and an infested agar plug taken from a V8 agar plate inoculated with the appropriate strain (P582 or P1351) was immediately placed on the decapitated surface. Disease lesions first appeared after 2–4 days and were measured at various time intervals. Virulence was measured as the linear rate of progression of the lesion. For each combination, a minimum of five plants was used. At the end of the assay (day 11), the stems of all plants were sliced longitudinally to examine internal lesions. No major discrepancies between the lengths of internal and external lesions were observed on tobacco plants inoculated with *P. parasitica* strains P582 and P1351.

Bacterial pathogenicity and *in planta* growth.

Pathogenicity of *X. c.* pv. *armoraciae* was determined by leaf infiltration of a bacterial solution (10^5 to 10^7 cells per milliliter) in sterile distilled H_2O into the mesophyll of radish leaves as described for the HR assays. *In planta* bacterial growth in radish mesophyll was determined as previously described (Kamoun *et al.* 1992).

Statistics.

Bacterial numbers were calculated as geometric averages from three or four independent measurements (time 0), and three to eight independent measurements (all other time points). Student's *t* test (Little and Hills 1978) statistical analyses were performed on logarithm transforms using StatView software (Brain Power, Inc., Carlsbad, CA).

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