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Acquired resistance triggered by elicitins in tobacco and other plants

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Abstract

Elicitins are a family of proteins excreted by *Phytophthora* spp. They exhibit high sequence homology but large net charge differences. They induce necrosis in tobacco plants which then become resistant to the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. In stem-treated plants, resistance was not restricted to the site of elicitin application, but could be demonstrated by petiole inoculation at all levels on the stem. Resistance was already maximum after two days and lasted for at least two weeks. It was effective not only towards *P. p.* var. *nicotianae* infection, but also against the unrelated pathogen *Sclerotinia sclerotiorum*. In contrast to dichloroisonicotinic acid, an artificial inducer of systemic acquired resistance, which was increasingly effective with doses ranging from 0.25 to 5 μmole per plant, the basic elicitin cryptogein exhibited a threshold effect, inducing near total resistance and extensive leaf necrosis above 0.1 nmole per plant. Between 1 and 5 nmole, acidic elicitins (capsicein and parasiticein) protected tobacco plants with hardly any necrotic symptom. Elicitins exhibited similar effects in various tobacco cultivars and *Nicotiana* species, although with quantitative differences, but induced neither necrosis nor protection in other Solanaceæ (tomato, petunia and pepper). Among 24 additional species tested belonging to 18 botanical families, only some Brassicaceæ, noticeably rape, exhibited symptoms in response to elicitins, in a cultivar-specific manner. Elicitins appear to be natural specific triggers for systemic acquired resistance and provide a tool for unraveling the mechanisms leading to its establishment.

Abbreviations: AR = acquired resistance; HR = hypersensitive response; INA = 2,6-dichloroisonicotinic acid; Ppn = *Phytophthora parasitica* var. *nicotianae*; SAR = systemic acquired resistance.

Introduction

Plants can acquire resistance to pathogens as a result of a previous interaction with some pathogens. Acquired resistance (AR) appears either as an enhanced expression of inherited resistance or as a decrease in susceptibility. For instance, tobacco plants harbouring the N resistance gene which have formed local lesions in response to a primary infection by Tobacco Mosaic Virus (TMV) produce fewer and smaller lesions when they are subsequently challenged with the same virus [Ross, 1961a; Ross, 1961b]. They have also acquired partial resistance to the fungal pathogen *Phytophthora parasitica* var. *nicotianae* (Ppn), to which they are otherwise susceptible [McIntyre *et al.*, 1979]. Several features distinguish AR from inherited resistance [Kuc, 1982]. The process is initiated at the site of the inducing interaction which typically involves the formation of a restricted necrotic lesion, such as that occuring in the hypersensitive response (HR). AR does not establish immediately, but develops with time and is more or less durable. It is first observed locally, in the vicinity of the necrotic lesion, but may later extend to other parts of the plant and is then referred to as systemic AR (SAR). Finally, AR is non-specific in that it is effective on a range of unrelated pathogens.

Interest in AR has grown in the recent years as it is now perceived as a realistic alternative to classical chemicals in crop protection. Models have been proposed to describe the sequence of events involved in the establishment of (S)AR [Kloepper *et al.*, 1992; Ryals *et al.*, 1994], although the precise mechanisms are still largely unknown. During the inducing interaction a trigger is produced by the pathogen in the presence of the plant. The trigger is an event or a molecule which causes the plant to generate signal(s). These are responsible for the activation of a set of various 'SAR' genes; in addition, systemic AR implies that a signal is translocated to long distances within the plant. Many investigations have concentrated on the identification of the natural plant signals, and synthetic molecules, such as acetylsalicylic acid and 2,6-dichloroisonicotinic acid (INA), have been used to artificially induce SAR [Ward *et al.*, 1991]. In contrast very little is known on the nature of the triggers and their mode of action. It is not known, for instance, whether the same molecule which elicits the formation of the initial necrotic lesion can also trigger the various aspects of SAR. In the tobacco-TMV interactions, the viral coat protein induces a HR in tobacco plants harbouring the N' resistance gene [Culver *et al.*, 1991] and it has been suggested that the replicase protein is required for HR induction in the cultivars bearing the N gene [Padgett *et al.*, 1993]. Presently, there is no evidence that these proteins function as triggers for SAR.

For several years, we have been studying the interaction of tobacco with *Phytophthora* spp [Ricci *et al.*, 1993]. Tobacco is not a host-plant for these fungal pathogens, with the exception of Ppn, the black shank-causing fungus. For instance, tobacco plants cv. Xanthi nc. develop a rapid HR when inoculated with *P. cryptogea*. A challenge inoculation of such plants demonstrated the acquisition of a partial resistance to Ppn, to which they are genetically fully susceptible [Bonnet *et al.*, 1986]. A ca. 10 kD holoprotein, cryptogein, excreted by *P. cryptogea* grown *in vitro* [Billard *et al.*, 1988], was immunologically detected in inoculated tobacco plants during the development of the HR [Devergne *et al.*, 1992]. The pure protein induces necrotic symptoms and associated activation of defense responses in tobacco plants [Bonnet, 1988; Milat *et al.*, 1991]. Added to a tobacco cell suspension culture, it causes some rapid physiological modifications (H^+/K^+ exchange, oxidative burst) which are typical of hypersensitively reacting cells [Blein *et al.*, 1991; Viard *et al.*, 1994]. Altogether, these results suggest that cryptogein is a major trigger of the HR in the tobacco-*P. cryptogea* incompatible interaction.

Several proteins of 98 aminoacid residues with high sequence homology to cryptogein have been isolated from the culture filtrate of other *Phytophthora* species, including capsicein from *P. capsici* [Ricci *et al.*, 1989] and parasiticein from *P. parasitica* [Ricci *et al.*, 1992]; they have been given the generic name 'elicitins' [Nespoulos *et al.*, 1992]. We have previously reported that when tobacco plants are stem-treated with low amounts (0.1–10 nmole) of pure elicitins they acquire a high level of resistance to a local challenge performed 2 days later with a virulent isolate of Ppn [Ricci *et al.*, 1989, 1992]. Elicitin-induced resistance therefore provides a model system for analyzing AR development in tobacco. In this paper we demonstrate that elicitin-induced resistance extends to different parts of the plant distant from the site of application of the protein and has several features of AR, such as durability and lack of specificity. These results indicate that the interaction of a single fungal protein with plant cells, presumably by binding to specific sites [Blein *et al.*, 1991], can initiate the whole set of events necessary for the establishment of acquired resistance.

Materials and methods

Plant material

Four cultivars of tobacco (*Nicotiana tabacum*) and six other species of *Nicotiana* (Table 6) were obtained from the seed collection of the Institut du Tabac de Bergerac (ITB, France). Seeds of pepper (cultivars YoloWonder and Phyo 636) and of tomato (cv. Marmande) were a gift from A. Palloix (INRA, Montfavet, France); 30 lines of rape (*Brassica napus*) were kindly provided by M. Renard (INRA, Rennes, France); commercial seeds of *Petunia hybrida* were used. These plants were sown and transplanted into peat soil (Kanifrance, pH 5.6–6.0) and grown under controlled conditions (24 ± 2 °C, 16 h light, 100 $\mu Em^{-2}\ s^{-1}$).Three accessions of *Arabidopsis thaliana* (Columbia, Wassilieska, Landsberg) and five mutants of Landsberg (chl1, 5-2, aba, Abi1 and Abi2) were generously given by C. Soave (Universita di Milano, Italy); the seeds were cold-treated for one week in the dark, then germinated and grown in soil at 17 °C under light (300 $\mu Em^{-2}\ s^{-1}$) for 15 days. Other plants were collected in the Villa Thuret Botanical Garden (INRA, Antibes, France) and tested for elicitin effects on detached leaves.

Fungal material

Phytophthora cultures were from the INRA Antibes culture collection. The elicitins cryptogein, cinnamomin, capsicein and parasiticein were prepared from *P. cryptogea* (isolate 52 from gerbera), *P. cinnamomi* (isolate 127 from azalea), *P. capsici* (isolate 147 from sweet pepper) and *P. parasitica* (isolate 26 from carnation) respectively. The following isolates were used for inoculations: *P. parasitica* var. *nicotianae* from tobacco Ppn 183 (USA, race 0), Ppn 308 (Cuba, race 0), Ppn 310 (Australia, race 0), Ppn 181 (USA, race 1), *P. parasitica* 237 (from petunia) and 179 (from tomato), and *P. capsici* 147 (from pepper). Cultures of *Sclerotinia sclerotiorum* were isolated from tobacco by R. Delon (ITB) and from rape by H. Brun (INRA, Rennes). All fungal cultures were kept on malt agar at 24 °C.

Elicitor preparation

Elicitins were purified from *Phytophthora* culture filtrates following a modification of the method of Ricci *et al.* [1989]. The fungus was grown for 10 days in the dark at 24 °C in a liquid glucose/asparagine medium optimized for elicitin production. [1] The culture filtrate was concentrated five fold under vacuum at 35 °C, then extensively dialysed against deionised water at 4 °C. Sodium acetate (10 mM) was added and the pH adjusted to 5.5. Zeta-Prep 60 disks (SP for the basic elicitins cryptogein and cinnamomin, QAE for the acidic elicitins capsicein and parasiticein) were used according to the manufacturer's instructions (CUNO Inc, USA). Basic elicitins were eluted with 0.25 M NaCl, acidic elicitins with 50 mM sodium acetate pH 4.0. Elicitin-containing fractions were extensively dialysed against Ultrapure water and freeze dried. The yield was approximately 40 mg protein per liter. The purity of the preparations were assessed using SDS-PAGE, IEF and reverse-phase HPLC [Le Berre *et al.*, 1994].

Elicitor treatments and challenge inoculations

Fifty-day-old plants of tobacco, tomato, pepper and petunia were decapitated and treated with defined amounts of elicitin by applying 20 μl drops of aqueous solution on the fresh section. The necrotic areas appearing on the leaves were measured two days later and expressed in percent of the total leaf area. Fungal inoculation was performed two days after treatment (unless indicated otherwise) by applying a mycelium plug capped with a piece of aluminium foil either at the site of the treated decapitation, or on the section of a petiole of a leaf underneath, after the corresponding lamina had been cut off. One week after inoculation, the stem was dissected and the extent of stem invasion was estimated by the volume of discolored tissues (in cm^3). Percent of protection was computed as the relative reduction of invasion compared to water-treated and inoculated control plants. One-way or two-ways analysis of variance was used to test the significance of this reduction. In the case of rape, elicitins were applied to plants having 5 to 7 expanded leaves on the section of a petiole and the inoculation was performed two days later on the same petiole. Induction of necrosis on detached leaves of rape and *A. thaliana* was tested by dipping the petiole into 2 ml of cryptogein solution (0.5 to 4 μM).

Results

Acquired resistance of tobacco plants after elicitin treatment

As previously reported [Ricci *et al.*, 1989], tobacco plants (cv. Xanthi) treated with elicitin solutions on the fresh section of the stem developed necrotic symptoms locally (on the stem) and at a distance (on the leaves) during the following 24 h. The leaf area affected by necrosis varied with the amount of elicitin received by the plant and with the type of elicitin: leaf necrosis was much more extensive in response to the basic elicitin cryptogein than to the acidic elicitin capsicein, although the latter was applied in higher amounts (Table 1).

Two days after elicitin treatment, acquired resistance was tested by applying Ppn mycelium (isolate 183) either to the stem section, or to the petiole of the 2nd leaf beneath the section. During the following week, stem invasion was significantly reduced (up to more than 70%) in elicitin-treated plants compared to water-treated and inoculated control plants, whatever the mode of inoculation (Table 1). Fungal growth restriction was not dependent on the presence of a local necrosis. The stem part which had been treated with the elicitin solution and had turned necrotic was protected, but protection was even greater on tissues distant and different (petiole) from the site of elicitin application.

[1] 0.6 g H_2PO_4, 0.7 g KNO_3, 0.25 g $MgSO_4.7H_2O$, 0.125 g $K_2HPO_4.3H_2O$, 0.3 g $Ca(NO_3)_2$, 1 mg H_3BO_3, 1.5 mg $MnSO_4.H_2O$, 4 mg $ZnSO_4.7H_2O$, 0.1 mg $Na_2MoO_4.2H_2O$, 20 μg KI, 20 μg $CuSO_4.5H_2O$, 20 μg $CoCl_2.6H_2O$, 8 mg $FeNa_2EDTA$, 1 mg nicotinic acid, 1 mg pyridoxin, 1 mg calcium panthotenate, 1 mg thiamine hydrochloride, 1 g asparagine.H_2O and 20 g glucose in 1 l de-ionized water.

Table 1. Leaf necrosis and stem protection induced by elicitin treatments of tobacco plants. Fifty-day-old plants were decapitated, stem-treated with 20 μl of elicitin solution and inoculated 2 days later with Ppn 183 either on the stem or on the petiole of the second upper remaining leaf

Treatment[1]	Elicitin amount[2]	Leaf necrosis[3]	Inoculation on the stem		Inoculation on the petiole	
			Stem invasion[4]	Protection[5]	Stem invasion[4]	Protection[5]
Control	0	0%	4.32 ±0.60	–	4.60 ± 0.72	–
Cryptogein	100	30%	3.30 ±0.52	24%*	0.98 ± 1.36	79%**
Cryptogein	500	35%	2.27 ± 1.18	47%**	0.59 ± 0.84	87%**
Capsicein	1000	1%	1.28 ± 1.44	70%**	0.97 ± 1.05	79%**
Capsicein	5000	5%	1.12 ± 1.24	74%**	1.26 ± 1.11	73%**

[1] Six plants of tobacco cv. Xanthi per treatment.
[2] pmole elicitin per plant.
[3] Extent of necrosis in percent of the total leaf area.
[4] Volume of stem tissues (cm^3) showing symptom of fungal invasion.
[5] Relative reduction of stem invasion compared to controls.All protections are significant at the 5 (*) or 1(**) percent level. Protections are significantly higher in the case of petiole inoculations ($P \leq 0.01$).

On the stem section, the fungus was restricted in the cortex but occasionally colonized the pith to some extent. On the contrary, following petiole inoculation, the fungus was slowed down in the stem cortex and was usually arrested before reaching the vascular tissues.

The variation between repetitive experiments was not significantly different from the variation between replicates within a single experiment (Table 2). Among elicitin-treated plants, usually some were totally protected, most others exhibited limited colonization only, and a few occasional ones seemed to escape any protection at all (resulting in high standard deviation of stem invasion values). Nevertheless, a significant level of acquired resistance could always be demonstrated after treatment with appropriate amounts of elicitins.

The possibility of a direct antifungal effect of cryptogein on the mycelium of Ppn 183 was tested *in vitro* by applying filter paper disks soaked with various amounts of the protein (up to 5 nmole) in water solution on malt agar plates. No inhibition of the fungal growth was observed.

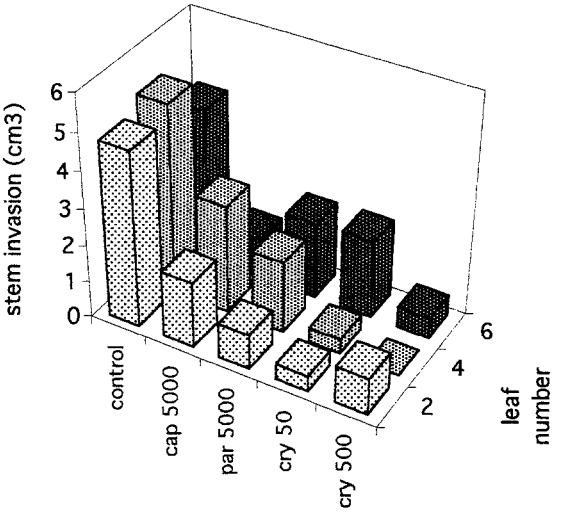

Fig. 1. Systemicity of acquired resistance triggered by elicitins. Tobacco plants (cv. Xanthi) were decapitated and stem-treated with water (control), capsicein (cap), parasiticein (par) or cryptogein (cry); amounts of elicitin in pmole per plant. Two days later, they were inoculated with *Phytophthora parasitica* var. *nicotianae* (Ppn 183) on the petiole of leaf 2, 4 or 6 beneath the stem section. The volume of infected stem tissue was measured 7 days post inoculation. Each treatment in triplicates.

Effectiveness of acquired resistance against Ppn and Sclerotinia sclerotiorum

Acquired resistance triggered by three elicitins was challenged by stem inoculation with three Ppn isolates from diverse origins (Table 3). AR was similarly effective against Ppn 183 (from the US) and Ppn 308 (from Cuba) which are both highly aggressive isolates devoid of elicitin production [Ricci *et al.*, 1992]. Remarkedly, Ppn 310 (from Australia) which is less pathogenic to tobacco [Bonnet *et al.*, 1994] and produces parasiticein *in vitro* [Mouton-Perronnet *et al.*, 1995] was affected by pretreatment with elicitins, including parasiticein. In order to test its specificity, elicitin-induced resistance was also challenged with the unrelated tobacco stem pathogen *S. sclerotiorum*. As indicated in Table 3, this fungus was able to invade control plants as rapidly

Table 2. Variability within and between protection experiments with cryptogein

Experiment number	Stem invasion following petiole inoculation[1]		Protection[2]	
	Control	Treated with 100 pmole per plant[3]		
1	4.40 ± 1.01	1.16 ± 1.64	74%	
2	4.80 ± 0.81	0.42 ± 1.36	83%	
3	4.55 ± 0.78	0.71 ± 0.18	83%	
4	3.74 ± 0.76	0.91 ± 1.31	76%	
5	4.62 ± 1.38	0.90 ± 1.30	80%	
6	4.50 ± 0.99	0.29 ± 0.34	93%	
7	5.87 ± 0.13	0.56 ± 0.66	91%	
Two-way analysis of variance:				
Source of variation	Deg. of freedom	Mean square	*F*	*P*
Treatment	1	156.90	169.09	≤0.01
Experiment	6	0.52	0.60	>0.05
Interaction	6	0.93	1.09	>0.05
Error	28	0.86		
Total	41			

[1] Inoculation performed with Ppn 183 on the petiole of the second upper leaf 2 days after treatment. Invasion measured as in Table 1.
[2] Relative reduction of stem invasion compared to controls.
[3] Stem treatment of tobacco cv. Xanthi performed as in Table 1

as the highly aggressive Ppn isolates. In elicitin-treated plants, growth of *S. sclerotiorum* was restricted to a similar extent as that of Ppn.

Systemicity and durability of acquired resistance

As reported above, induced resistance was not restricted to the site of elicitin application. The extent of the protection was examined by inoculating the petiole of the 2nd, 4th or 6th leaf under the stem section, corresponding to an average of 40, 120 or 230 mm respectively from the site of elicitin application (Fig. 1). In control plants, infection via petiole 6 was slightly slower than via petiole at upper levels, possibly due to higher tissue lignification. After elicitin treatment, stem invasion was significantly reduced at all three levels, whatever the elicitin used. In other experiments, tobacco rooted stem cuttings which had been treated at the base with cryptogein or capsicein demonstrated acquired resistance when challenged by inoculation with Ppn on the upper part of the stem (not shown), confirming the systemic induction of resistance.

Durability of induced resistance was analyzed by inoculations on petiole 2 performed at various delays after treatment. Protection was as effective after 8 days as after 48 h, and plants treated once with 100 pmole of

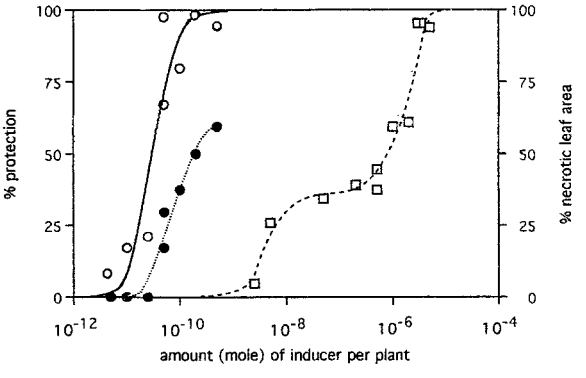

Fig. 2. Responses of tobacco to cryptogein (circles) and INA (squares) as a function of the amount of inducer applied per plant. Extent of leaf necrosis (solid labels) was recorded 2 days after stem treatment. A challenge inoculation with *Phytophthora parasitica* var. *nicotianae* (Ppn 183) was then performed on petiole of leaf number 2, and the level of protection relative to control plants (open labels) was determined 7 days later.

cryptogein were still partially protected after 2 weeks (Table 4). It should be noted that the necrotic symptoms induced by elicitin treatment are fully developed by 48 h and do not evolve significantly afterwards.

Table 3. Protection induced by elicitin treatments in tobacco plants challenged with different pathogens. Fifty-day-old plants (cv. Xanthi) were decapitated, stem-treated with 20 μl of elicitin solution and stem-inoculated 2 days later

Treatment	Elicitin amount[1]	Leaf necrosis[2]	Ppn 183[3]		Ppn 308[3]		Ppn 310[3]		*Sclerotinia sclerotiorum*[4]	
			Stem invasion[5]	Protection[6]	Stem invasion[5]	Protection[6]	Stem invasion[5]	Protection[6]	Stem invasion[5]	Protection[6]
Control	0	0%	3.28 ± 0.22	–	2.73 ± 0.93	–	1.69 ± 0.53	–	2.95 ±0.63	–
Cryptogein	100	11%	nt[7]		nt		nt		0.85 ± 0.47	71%
Cryptogein	500	33%	0.32 ± 0.45	90%	0.59 ± 0.06	79%	0.26 ± 0.25	84%	0.84 ± 0.12	72%
Capsicein	1000	2%	nt		nt		nt		0.48 ± 0.50	84%
Capsicein	5000	1%	0.11 ± 0.16	97%	0.22 ± 0.31	92%	0 ±0	100%	0.32 ± 0.26	89%
Parasiticein	5000	1%	0.95 ± 1.03	71%	0.29 ± 0.21	89%	0.71 ± 0.07	58%	nt	

[1] pmole elicitin per plant.
[2] Extent of necrosis in percent of the total leaf area.
[3] Two plants per treatment.
[4] Five plants per treatment.
[5] Volume of stem tissues (cm^3) showing symptom of fungal invasion.
[6] Relative reduction of stem invasion compared to controls. All protections are significant at the 1 percent level.
[7] nt: not tested.

Table 4. Durability of elicitin-induced resistance of tobacco plants. Fifty-day-old plants (cv. Xanthi) were decapitated and stem-treated with 20 μl of elicitin solution, prior to inoculation with Ppn183

Treatment	Elicitin amount[1]	Delay between treatment and inoculation[2]					
		2 days		8 days		14 days	
		Stem invasion[3]	Protection[4]	Stem invasion[3]	Protection[4]	Stem invasion[3]	Protection[4]
Control	0	4.97 ± 0.66	–	3.81 ± 0.28	–	3.90 ± 0.53	–
Cryptogein	50	1.88 ± 1.73	62%	1.19 ± 0.66	69%	nt[5]	
Cryptogein	100	0.95 ± 0.36	81%	0.85 ± 0.89	78%	2.15 ± 0.86	45%
Capsicein	5000	3.27 ± 1.15	34%	2.25 ± 0.68	41%	nt	
Parasiticein	5000	2.68 ± 0.70	46%	2.02 ± 0.97	47%	nt	

[1] pmole elicitin per plant.
[2] Five plants per treatment.
[3] Volume of stem tissues (cm^3) showing symptom of fungal invasion.
[4] Relative reduction of stem invasion compared to controls.
[5] nt: not tested.
All protections are significant at the 1 percent level; no significative difference is observed between 2 and 8 days; protection by 100 pmole of cryptogein is significantly reduced after 14 days.

Comparison of resistance induced by various doses of elicitins and of INA

The chemical 2,6-dichloroisonicotinic acid (INA) is known to induce resistance in plants [Ward *et al.*, 1991]. We have compared the dose-effect of INA to that of elicitins in the same experimental procedure, i.e. applying the inducer on the decapitated stem and challenging the resistance after 48 h by inoculation of petiole 2 with Ppn.

Because of individual variations (see above), accurate dose-effect curves were difficult to establish for acquired resistance (Fig. 2). With cryptogein, little or no resistance was induced when less than 50 pmole per plant was applied, and 70 to 100% protection was achieved with 100 pmole or more. Over the same range of doses, the extent of leaf necrosis increased from zero to nearly 50% of the total leaf area. For comparison, other elicitins were included in the tests (Table 5). At 50 and 100 pmole per plant, protection and necrosis induced by cinnamomin, another basic elicitin, did not differ from those incited by cryptogein at the same doses. With the acidic elicitins capsicein and parasiticein, leaf necrosis was undetectable or limited to a few small flecks when up to 5 nmole per plant was applied. However, partial protection was already induced by 1 nmole of acidic elicitin.

In contrast to the almost threshold effect of cryptogein, the level of resistance induced by INA increased

Table 5. Comparison of leaf necrosis and stem protection induced by basic and acidic elicitins. Fifty-day-old plants (cv. Xanthi) were decapitated, stem-treated with 20 μl of elicitin solution, and inoculated 2 days later with Ppn 183 on the petiole of the second upper remaining leaf

Treatment	Elicitin amount[1]	Leaf necrosis[2]	Protection[3]
Cryptogein	50	25%	42%
Cryptogein	100	17%	89%
Cinnamomin	50	8%	67%
Cinnamomin	100	24%	75%
Capsicein	1000	0%	38%
Capsicein	5000	5%	74%
Parasiticein	1000	2%	45%
Parasiticein	5000	6%	75%

[1] pmole elicitin per plant.
[2] Extent of necrosis in percent of the total leaf area.
[3] Relative reduction of stem invasion compared to controls.

progressively with doses in the μmole range (Fig. 2). Between 0.25 to 2.5 μmole per plant, only a partial reduction of infection (about 50% of the controls) was observed; near-total resistance was achieved with amounts above 2.5 μmole. Toxic symptoms on leaves were not detected, except on plants treated with amounts of INA exceeding 5 μmole. Another difference between cryptogein and INA was noted when the systemicity of the acquired resistance was examined: effectiveness of the INA treatment decreased significantly when the inoculation was performed farther from the site of the treatment (not shown).

Plant specificity of elicitin effects

We compared four cultivars of *Nicotiana tabacum* and six other species belonging to the three sections of the genus *Nicotiana* [Darlington *et al.*, 1955] for their response to cryptogein. All of them developed leaf necrosis after stem application of 0.5 nmole of cryptogein, although with large quantitative differences (Table 6). Tobacco cv. Samsun produced larger necrosis than cv. Xanthi (an observation that has been frequently repeated); all other cultivars and species appeared less reactive. The same test was applied to three other cultivated Solanaceous genera: petunia, pepper and tomato. In contrast to the universal reaction in the genus *Nicotiana*, none of them developed any leaf necrosis in response to cryptogein.

The investigation was further expanded to the representatives of various plant families: Acanthaceæ (*Thunbergia* sp.), Aristolochaceæ (*Aristolochia* sp.), Balsaminaceæ (*Balsamina* sp.), Campanulaceæ (*Campanula* sp.), Compositeæ (*Senecio petasites*), Geraniaceæ (*Geranium* sp.), Labiaceæ (*Ocimum basilicum*, *Pogestemon* sp.), Loganiaceæ (*Buddleya* sp.), Malvaceæ (*Hibiscus* sp., *Pavonia* sp.), Onagraceæ (*Fuchsia* sp., *Crotalaria pallida*, *Mucuna* sp.), Passifloraceæ (*Passiflora* sp.), Rosaceæ (*Rosa indica*), Rutaceæ (*Citrus* sp.), Sapindaceæ (*Greyia* sp.), Umbelliferaceæ (*Petroselinum sativum*), Urticaceæ (*Parietaria* sp.) and Verbenaceæ (*Lantana* sp.). Responsiveness to cryptogein was determined by application of 2 nmole to the petiole of detached leaves, a very sensitive test in tobacco [Bonnet *et al.*, 1985]. None of the tested plants developed any necrosis, with the exception of *Diplotaxis erucoïdes* and *Raphanus sativus*, two Brassicaceæ.

A set of cultivars of rape (*Brassica napus*), a species of Brassicaceæ of large economical importance, was therefore examined in the same way. In contrast to the situation in tobacco, out of the 30 cultivars tested, only five (Cobra, Lirabon, Lirajet, Liberator and Jantar) exhibited clear leaf symptoms in response to cryptogein (1 and 4 nmole): the lamina turned yellow with pin-point black dots in 48 h. Three additional cultivars (Maxol, Doublol and Samourai) developed occasional and irregular leaf discolouration or wilting, while the others (such as Shogun and Jet Neuf) remained unaffected. Treatment with 1 nmole of cryptogein induced no symptom on detached leaves of three accessions of *A. thaliana* (Columbia, Wassilieska and Landsberg) and four Landsberg mutants.

As cryptogein appears to be a very specific elicitor for necrosis induction, we compared its ability to induce resistance in some of the plants that had exhibited or not a necrotic response. The infection potential of Ppn 183 was sufficient on all the tobacco cultivars and other *Nicotiana* species examined to provide the basis of a protection test. The exception was *N. plumbaginifolia* harbouring the R1 resistance gene to black shank [Chaplin, 1962] which was therefore inoculated with Ppn 181, an isolate belonging to the r1 physiological race. As shown in Table 6, a pretreatment with 0.5 nmole of cryptogein significantly reduced stem invasion in all these plants, except *N. rustica* and *N. plumbaginifolia*. Although the highest protection was obtained with cv. Samsun in this experiment, no general relationship appeared between the extent of necrosis and the level of acquired resistance. Similar protection tests were performed in tomato, petunia and pepper by stem inoculation with the respective pathogenic Phytophthoras, belonging to *P. para-*

Table 6. Response of tobacco cultivars and *Nicotiana* spp. to cryptogein

Plant species and cultivar	Leaf necrosis[1]	Stem invasion following petiole inoculation[2]		Protection[3]
		Control	Cryptogein	
N. tabacum[4]				
Xanthi	35%	3.89 ± 0.69	1.48 ± 1.41	62%**
Samsun	60%	3.37 ± 0.46	0.26 ± 0.23	92%**
Hicks	9%	4.44 ± 0.61	2.51 ± 1.03	43%**
ZZ 100	12%	3.80 ± 0.68	1.57 ± 1.24	59%**
Other *Nicotiana* spp.[5]				
sect. *tabacum*				
N. glutinosa	19%	3.02 ± 0.68	1.69 ± 0.13	44%*
sect. *rustica*				
N. glauca	6%	1.67 ± 0.34	1.07 ± 0.11	36%*
N. rustica	5%	0.87 ± 0.11	0.48 ± 0.73	44% NS
sect. *petunioïdes*				
N. alata	20%	1.64 ± 0.69	0.15 ± 0.13	91%*
N. plumbaginifolia[6]	16%	3.48 ± 0.88	2.42 ± 0.53	30% NS
N. sylvestris	18%	nt[7]	nt	–

[1] Stem treatment with 500 pmole of cryptogein per plant performed as in Table 1.
[2] Inoculation performed with Ppn 183 on the petiole of the second upper leaf 2 days after treatment. Invasion measured as in Table 1.
[3] Relative reduction of stem invasion compared to controls, significant at the 5 (*) or 1(**) percent level, or non significant (NS).
[4] Five plants per treatment.
[5] Four plants per treatment.
[6] Inoculation with Ppn 181 (race 1).
[7] nt: not tested.

sitica and *P. capsici*. These plants, which exhibited no necrotic response at all, did not acquire any resistance either (Table 7).

An attempt was made to compare rape cultivars for the effect of cryptogein on their resistance to *S. sclerotiorum*, a serious pathogen of rape. Young plants treated with a drop of cryptogein or parasiticein solution (4 nmole) on a petiole developed distal leaf necrosis on the other leaves in the case of cvs. Cobra and Lirabon, and not of cvs. Shogun and Jet Neuf. However, when these plants were inoculated with *S. sclerotiorum* on the treated petiole, one or three days after treatment, no difference in the rate of invasion could be observed between control and elicitin-treated plants in any of the cultivars (not shown).

Discussion

Descriptions of induced resistance triggered by the interaction with a living pathogen have led to the basic concept of SAR [Ryals *et al.*, 1994]. The synthetic chemical INA has effects which share common features with SAR and has been used as a convenient tool to study SAR induction [Ward *et al.*, 1991]. A unique opportunity to study the effects of a defined natural trigger of acquired resistance is provided by elicitins which are involved in non-host *Phytophthora*-tobacco interactions [Devergne *et al.*, 1992] and which can be easily purified in large amounts [Billard *et al.*, 1988].

In previous studies, elicitin-induced resistance to Ppn in tobacco had been demonstrated locally, at the place where the elicitin was applied [Ricci *et al.*, 1989]. As most of the protein remains bound at the site of application which turns rapidly necrotic (even with acidic elicitins) [Devergne *et al.*, 1992], the inhibition of the challenge inoculum could have resulted from a direct antifungal effect of the elicitin or from the extensive alteration of the host tissues before inoculation. In this study, we have checked that cryptogein does not inhibit Ppn growth *in vitro*. Furthermore, our results

Table 7. Response of three Solanaceous genera to cryptogein

Plant species and cultivar	Treatment[1]		Inoculation[2]		Stem invasion[3]
	Elicitin	Amount[4]	Species	Isolate	
Lycopersicon esculentum[5]					
Marmande VF	Control		*P. parasitica*	179	3.08 ± 0.34
...	Cryptogein	500	...	...	2.33 ± 0.69
...	Capsicein	5,000	...	...	2.65 ± 0.48
Petunia hybrida[5]					
Hybride à grandes fleurs	Control		*P. parasitica*	237	1.06 ± 0.20
...	Cryptogein	500	...	...	1.07 ± 0.16
...	Capsicein	5,000	...	...	0.86 ± 0.18
Capsicum annuum[6]					
Yolo Wonder	Control		*P. capsici*	147	1.17 ± 0.40
...	Cryptogein	1,000	...	...	1.25 ± 0.23
Phyo 636	Control		*P. capsici*	147	0.29 ± 0.09
...	Cryptogein	1,000	...	...	0.27 ± 0.03

[1] Stem treatment as in Table 1. No leaf necrosis observed after treatment in any plant.
[2] Stem inoculation two days after treatment.
[3] Volume of stem tissues (cm^3) showing symptom of fungal invasion. No significant difference between treated and untreated plants.
[4] pmole elicitin per plant.
[5] Four plants per treatment.
[6] Three plants per treatment.

demonstrate that elicitin-induced resistance is also effective when the trigger and the challenge are located at different sites, and that this resistance extends to the whole stem. It could therefore be expected to operate at the collar level, the natural route of infection. Other experiments have shown that young tobacco plants stem-treated with elicitins are able to resist infection by Ppn inoculated to the soil as zoospores [Keller *et al.*, 1995].

Systemicity is a classical feature of acquired resistance. Kloepper *et al.* [1992] distinguish the trigger, produced locally by the inducing organism, and the signal(s) released by the plant under the effect of the trigger, and which must account for the systemic modification of the plant resistance. In the case of elicitins, the situation is complicated by the ability of the trigger itself to move rapidly upwards and downwards for long distances in the plant through the vascular system [Devergne *et al.*, 1992]. Necrosis induction by cryptogein seems therefore not to require an additional systemic signal. The same may not be true for cryptogein-induced resistance which, in contrast to necrosis, involves endogenous salicylic acid [Keller *et al.*, 1994]. Also relevant to this distinction is the fact that tissue necrotization in the affected areas is completed between 24 and 48 h whereas the state of resistance persists for more than one week.

Another general feature of SAR is that it is effective against a wide range of pathogens [Kuc 1982]. We show here that the resistance induced by proteinaceous elicitors from *Phytophthora* spp. is not specifically directed towards *Phytophthora* infection, but extends to the unrelated pathogen *Sclerotinia sclerotiorum*. Kamoun *et al.* [1993] have also reported that elicitins protect radish against bacterial infection. The protection observed in our experiments was rarely complete, often allowing the challenge pathogen to develop to some extent. This is related to the fact that only highly aggressive pathogens (*P. parasitica* or *S. sclerotiorum*) were used by application of a heavy inoculum to a wound area. These conditions were selected to provide 100% infections in control plants. Different is the fact that a few treated plants seemed to escape protection, especially when relatively low amounts of elicitins were used. This might result from some plant-to-plant variation in the reactivity to elicitins; indeed the dose-response curve for resistance induction by cryptogein exhibits a rather threshold effect.

INA, an artificial inducer of resistance to several pathogens in tobacco and other plants [Ward *et al.*, 1991], was able to protect tobacco against Ppn with the experimental procedure used in this study. However, the dose-response curve was very different from that of cryptogein, increasing progressively over a large dose range (0.05 to 5 μmole per plant). These doses are in agreement with those reported in the literature [Ward *et al.*, 1991]. Although the resistance induced by INA was formally similar to that resulting from elicitin treatments, the modes of action of the two molecules are probably different. Indeed, the typical physiologial responses of tobacco cell suspension cultures to elicitins – i.e. extracellular pH increase [Blein *et al.*, 1991] and oxidative burst [Viard *et al.*, 1994] – were not observed upon INA treatment [Blein unpubl.], and it has recently been shown that resistance induction by INA in tobacco does not involve salicylic acid [Vernooij *et al.*, 1995].

Resistance induced by a biological agent can be tested only on those plants which develop some kind of interaction with this agent. Using elicitins, it was possible to investigate the degree of specificity of a trigger molecule itself, without such limits. For necrosis induction, cryptogein appears as a specific elicitor whose activity is limited to the genus *Nicotiana* and to some Brassicaceæ. The four *N. tabacum* cultivars and the six other *Nicotiana* spp. that were tested all proved reactivity. Response to cryptogein was independant from the presence of the N gene, which is responsible for the HR to TMV, and of the R1 gene which determines a specific resistance to Ppn. Also included was cultivar Hicks which is insensitive to a *Trichoderma* endoxylanase, another fungal proteinaceous elicitor of necrosis [Bailey *et al.*, 1990]. Among Brassicaceæ, cryptogein activity was found cultivar-specific in rape, as was reported for radish [Kamoun *et al.*, 1993]. While most radish cultivars were reported to exhibit symptoms in response to elicitins, only a minority of rape genotypes did so. None of the several *A. thaliana* ecotypes and mutants which were tested with cryptogein developed any necrotic symptom.

Large variation in necrosis intensity was observed among species and cultivars of *Nicotiana*, but this is different from the total lack of response of other plant species. For instance, multiple attempts were made with amounts up to 2.5 nmole of cryptogein, cinnamomin, capsicein and parasiticein applied to the stem of decapitated plants and to the petiole of detached leaves to produce necrosis on the tomato cultivar Marmande previously reported to be reactive to elicitins [Pernollet *et al.*, 1993]: no symptom was obtained whatever the doses and conditions. Similar observations were made on petunia and on two cultivars of pepper. Our results are therefore rather in agreement with those of Kamoun *et al.* [1993] suggesting a strict specificity for elicitins. This specificity might be related to the occurrence of a recognition between elicitins and plant cells. The observation of a specific reversible binding of cryptogein to tobacco cells in suspension culture supports this hypothesis [Blein *et al.*, 1991]. Under similar conditions, no such binding could be detected in cultured tomato cells [Blein unpubl.].

Regarding the association of elicitin-induced resistance with necrosis formation, our results are apparently contradictory. On the one hand, we observed acquired resistance only in the genus *Nicotiana*, but could not detect it with similar tests in tomato, petunia and pepper, all Solanaceous species which did not produce any necrosis in response to elicitins. Kamoun *et al.* [1993] could protect the radish reactive cultivar Early Mino against the bacterial pathogen *Xanthomonas campestris* pv. *armoraciae* with parasiticein, but not non-reactive cultivars. These observations suggest the existence of a link between necrosis and SAR. On the other hand, with plant species and cultivars which demonstrated a potential to produce a necrotic response to elicitins, there was no general quantitative relationship between necrosis and SAR intensities when different plant genotypes and different elicitins were compared. For instance, acidic elicitins induced resistance in tobacco even in the absence of necrosis, and we could not detect rape protection against *S. sclerotiorum* in plants exhibiting obvious symptoms after cryptogein treatment. This indicates that, in this system, necrosis and SAR may not be causally related.

In conclusion, we can propose a model in which necrosis and acquired resistance are two distinct responses to elicitins which develop with their own quantitative and kinetic parameters, but are both dependent on a single initial specific recognition event of the fungal protein as a trigger. This model is coherent with that of a branched signalling pathway suggested by recent attempts to genetically dissect the events leading to SAR in *A. thaliana*. [Cameron *et al.*, 1994]. Elicitin-induced resistance is systemic, being expressed at all levels along the stem; whether this involves a plant systemic signal or results from the migration of the elicitin itself awaits further investigation. It is also durably established following a single

treatment and is not specific to a single pathogen. It therefore presents all the features classically recognized for SAR.

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