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Activation of defense-related genes in parsley leaves by infection with *Erwinia chrysanthemi*

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Abstract

Parsley leaves exhibited incomplete resistance following stomatal inoculation with the broad host range pathogen, *Erwinia chrysanthemi*, resulting in the development of local lesions in 58% of inoculated leaves. The responses of the same plant material to five mutants generated from the wild type bacterial strain varied from highly susceptible to completely resistant. While the susceptible phenotype was characterized by spreading maceration, hypersensitive-like necrotic lesions were typical for resistant leaves, with both types of symptom becoming visible two days after inoculation. Accumulation of transcripts of an entire set of defense-related genes and of furanocoumarin phytoalexins preceded visible onset of lesion development or maceration in the respective interactions. *Escherichia coli* did not elicit symptoms, gene activation or phytoalexin accumulation in parsley leaves. No correlation existed between the final extent of plant resistance on the one hand, and the timing or degree gene activation and phytoalexin accumulation on the other hand. Rapid activation of defense-related genes therefore appears to be insufficient, although it may be necessary, to protect parsley from attack by *Erwinia chrysanthemi*.

Abbreviations: BMT = *S*-adenosyl-L-methionine:bergaptol O-methyltransferase; 4CL = 4-coumarate:CoA ligase; *Ech wt* = *Erwinia chrysanthemi*, strain 3937; *Ech cel, Ech lps, Ech omp* and *Ech out* = mutant strains of *Ech wt*; PAL = phenylalanine ammonia-lyase.

Introduction

Plants respond to pathogen attack by activating a multicomponent defense reaction which in incompatible interactions provides efficient protection. Typical components of this response include local cell death, an oxidative burst, production of phytoalexins, reinforcement of the cell wall, secretion of hydrolytic enzymes and accumulation of pathogenesis-related proteins [Dixon and Lamb, 1990]. Most, but not all, of these responses involve transcriptional activation of specific defense-related genes [Scheel, 1992]. In incompatible plant/pathogen interactions, the individual components of the defense reaction appear to be activated in a coordinated manner, suggesting tightly linked regulation, whereas incomplete, delayed or no activation is observed in many compatible interactions [Bell *et al.*, 1984; Bell *et al.*, 1986; Jakobek *et al.*, 1993; Kiedrowski *et al.*, 1992; Meier *et al.*, 1993; Voisey and Slusarenko, 1989]. Accumulation of phytoalexins and transcripts of defense-related genes has also recently been reported to occur after infiltration of bean leaves with non-pathogenic bacteria and Hrp$^-$ mutants of *Pseudomonas syringae* pv. *tabaci*, indicating that the initiation of these defense responses is not necessarily associated with hypersensitive cell death [Jakobek and Lindgren, 1993]. It is unknown, however, if rapid, coordinated activation of defense-

related genes is sufficient for a successful resistance reaction.

The most comprehensive collection of plant defense-related genes has probably been described for parsley [Somssich *et al.*, 1989]. Differential screening of a cDNA library from cultured parsley cells with radioactively labelled run-on transcripts derived from nuclei of elicitor-treated and untreated cells resulted in the isolation of most of the 21 different types of elicitor-responsive genes known for parsley. Two of these encode enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), which are involved in the biosynthesis of a diverse pattern of secondary metabolites including lignin precursors and the furanocoumarin phytoalexins of parsley [Douglas *et al.*, 1987; Lois *et al.*, 1989]. Other genes code for *S*-adenosyl-L-methionine:bergaptol O-methyltransferase (BMT) which catalyzes the final methylation step in the synthesis of bergapten, a major component in the mixture of furanocoumarins [Hauffe *et al.*, 1986; Scheel *et al.*, 1987], and tyrosine decarboxylase converting tyrosine to tyramine which then appears to be incorporated into the cell wall [Kawalleck *et al.*, 1993a]. Two proteins of the parsley cell wall, a peroxidase and a hydroxyproline-rich glycoprotein, as well as two enzymes of the activated methyl cycle, *S*-adenosyl-L-methionine synthetase and *S*-adenosyl-L-homocystein hydrolase, are encoded by elicitor-responsive genes [Kawalleck *et al.*, 1992; Trezzini *et al.*, 1992]. The function of the remaining elicitor-responsive genes of parsley, two of which encode intracellular pathogenesis-related proteins, is unknown [Somssich *et al.*, 1986; Somssich *et al.*, 1988; van de Löcht *et al.*, 1990].

The transcripts of several elicitor-responsive genes have been shown to accumulate in cells surrounding infection sites on parsley leaves inoculated with zoospores of the Oomycete soybean pathogen, *Phytophthora megasperma* f.sp. *glycinea* [Schmelzer *et al.*, 1989; Somssich *et al.*, 1988]. Parsley leaves develop a non-host resistance reaction in response to *Phytophthora megasperma* f.sp. *glycinea* infection, which includes hypersensitive cell death as well as most of the components of a typical defense response outlined above [Jahnen and Hahlbrock, 1988]. We have now examined the expression of the elicitor-reponsive genes in parsley leaves infected with the bacterial pathogen, *Erwinia chrysanthemi*, the causal agent of soft rot disease in many crop and ornamental plants [Kotoujansky, 1987].

Components of the bacterial cell envelope, a functional iron uptake system and several extracellular enzymes that catalyze the degradation of plant cell wall constituents have been identified as pathogenicity factors of *Erwinia chrysanthemi* [Enard *et al.*, 1988; Expert and Toussaint, 1985; Kotoujansky, 1987; Schoonejane *et al.*, 1987]. Mutants affected in these pathogenicity factors were isolated and tested for their pathogenicity in different plants [Aymeric *et al.*, 1988; Beaulieu and Van Gijsegem, 1992; Expert and Toussaint, 1985; Expert *et al.*, 1993; Expert *et al.*, 1992]. Here we compare the pathogenicity of these mutants in parsley with their ability to activate the defense-related genes of this plant.

Materials and methods

Plant and fungal materials. Parsley (*Petroselimum crispum*) plants were grown in greenhouses for 5–6 months before use. Cell suspension cultures derived from these plants were grown in modified B5 medium as described previously [Kombrink and Hahlbrock, 1986]. *Phytophthora megasperma* f.sp. *glycinea*, race 1, was maintained as described by Kombrink and Hahlbrock [1986]. A crude cell wall elicitor was preared from fungal mycelium according to Ayers *et al.* [1976] and used for treatment of cultured parsley cells as described previously [Renelt *et al.*, 1993].

Bacterial strains. The bacterial strains used in this study were *Escherichia coli*, F^- W3110 [Bachmann, 1972]; *Erwinia chrysanthemi*, strain 3937 (*Ech wt*), which was isolated from a diseased *Saintpaulia ionantha* plant [Lemattre and Narcy, 1972] and the following 3937 mutants: R1456 (*Ech lps*) which has an altered lipopolysaccharide core structure [Expert and Toussaint, 1985; Schoonejans *et al.*, 1987]; RH7011 (*Ech omp*) which lacks one of the major outer membrane proteins (omp 19::Mu dIIPR13) [Beaulieu and Van Gijsegem, 1992]; RH7015 (*Ech out*) which is unable to secrete pectinases and cellulases (out457::Mu dIIPR13) [Beaulieu and Van Gijsegem, 1992]; E1005 (*Ech cel*), a $CelY^-$ mutant which also carries a regulatory mutation affecting pectate lyase synthesis [Aymeric *et al.*, 1988; F. Van Gijsegem, unpubl.]; and *cbr-2* (*Ech cbr*), a mutant carrying an insertion (Mu dII1734) in the *cbr* locus resulting in modified regulation of iron uptake and pectate lyase production [Expert *et al.*, 1992; Expert *et al.*, 1993].

Pathogenicity tests. Bacteria were grown on LB plates [Miller, 1972] for 24 hours at 26 °C and suspended in 10 mM $MgSO_4$ at a density of approximately 10^8 cfu ml^{-1} (0.1 OD at 600nm). Detached leaves were used for all experiments. The bacterial suspension was infiltrated through lower surface stomata of two basal first order leaflets with a disposable 1 ml syringe (stomatal infiltration). Alternatively, petioles were infected by wounding with a bacteria-coated toothpick (petiole infection). Control experiments utilized either untreated parsley leaves or leaves inoculated with 10 mM $MgSO_4$. Inoculated leaves were placed in water-filled vials which were incubated in small plastic greenhouses in growth chambers under a 16 h day/8 h night regime (26 °C day/24 °C night, relative humidity >90%, irradiation 320 μmol $m^{-2}s^{-1}$). Symptoms were scored six days after inoculation.

Determination of phytoalexins. Leaves were harvested 24 h after inoculation by stomatal infiltration and ground in water (1 ml g^{-1} fresh weight). The extract was cleared by centrifugation and coumarins in the supernatant were quantified by measuring the fluorescence at 410 nm after excitation at 350 nm in a Perkin Elmer LS-2B fluorimeter (Beaconsfield, England). The coumarins of the extracts were partitioned into dichloromethane and analyzed by thin-layer chromatography [Dangl *et al.*, 1987].

RNA isolation, separation and hybridization. For RNA isolation, the three upper first order leaflets were inoculated by stomatal infiltration until 20–40% of the parenchymal tissue was infiltrated. In addition, two basal leaflets were inoculated as described for pathogenicity tests, above. The plant material was harvested 4, 14, or 24 h after inoculation, frozen in liquid nitrogen and kept at −80 °C. Total RNA was prepared by the CsCl gradient method as described in Sambrook *et al.* [1989]. RNA was extracted from cultured parsley cells as described previously [Dangl *et al.*, 1987]. Total RNA (20 μg/lane) was denatured and electrophoresed on formaldehyde/agarose gels. Conditions of denaturation, electrophoresis and blotting onto nylon filters (Amersham Hybond N) were as those of Fourney *et al.* [1988]. cDNA probes were ^{32}P-labelled using an Amersham random-priming kit (cat. no. 1004760) according to the manufacturer's instructions. Filters were prehybridized for 3 h at 65 °C in hybridization buffer (1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg ml^{-1} denatured salmon sperm DNA). Hybridization was carried out in the same buffer for 20–24 h at 65 °C. Filters were washed twice in 2xSSC at room temperature and twice in 0.2xSSC, 0.5% SDS at 65 °C for 45 min. The cDNA probes used in this study are listed in Table 1.

Results

Pathogenicity of Erwinia chrysanthemi *in parsley.* The symptoms provoked by wild type *Erwinia chrysanthemi*, strain 3937 (*Ech wt*), in parsley leaves were examined after inoculation of leaf parenchyma or petioles. Upon stomatal infiltration of a bacterial suspension, initial symptoms appeared after two days and were in most cases restricted to the inoculated area. Representative examples of symptom development are shown in Fig. 1. Initial slight maceration developed rapidly into a dry brown lesion frequently surrounded by a ring of chlorotic cells (Fig. 1B). In a few cases, tissue outside the inoculated area was also macerated (Fig. 1C), while petiole rotting (Fig. 1D) was only rarely observed.

When petioles of parsley leaflets were wound-infected, maceration of the petiole began after approximately 24 h and was complete within two days (results not shown). The maceration usually ended at the base of the second order leaflet without reaching the parenchymal tissue. In rare cases, second order leaflets were also affected along the vascular bundle. No symptoms developed when parsley leaves (Fig. 1A) or petioles (results not shown) were inoculated with $MgSO_4$ solution or a suspension of *Escherichia coli*.

From a collection of *Erwinia chrysanthemi* mutants, five which provoked distinct symptoms on parsley leaves were selected for detailed analysis. With the exception of *Ech lps*, the mutants were derived from the wild type strain by insertional mutagenesis. The pathogenicity of these mutants on parsley was analyzed by leaf infiltration and scored according to the symptom classification shown in Fig. 1. Initial symptoms were visible after two days. The *Ech out*, *Ech omp* and *Ech lps* mutants showed either no symptoms or localized lesions, but never spreading maceration (Table 2). In contrast, the *Ech cel* and *Ech cbr* mutants provoked predominantly spreading maceration and even petiole rotting. The titer of the wild type strain (*Ech wt*) as well as the *Ech out*, *Ech omp*, *Ech cel* and *Ech cbr* mutants increased by approximately two orders of magnitude within 24 h after inoculation, whereas the

Table 1. Parsley cDNA probes used in this study. References refer to the cloning or the functional identification of the cDNAs

cDNA	Function	mRNA size [bp]	Reference
pal	phenylalanine ammonia-lyase	2,600	Lois *et al.*, 1989
4cl	4-coumarate:CoA ligase	2,000	Douglas *et al.*, 1987
bmt	*S*-adenosyl-L-methionine:bergaptol O-methyltransferase	1,600	Scheel *et al.*, 1987
pr2	intracellular pathogenesis-related protein 2	850	Somssich *et al.*, 1986
eli3	cinnamyl alcohol dehydrogenase	1,550	Somssich *et al.*, 1986; Knight *et al.*, 1992
eli5	tyrosine decarboxylase	1,900	Kawalleck *et al.*, 1993a
eli6	unknown	1,500	Somssich *et al.*, 1989
eli7	unknown	1,500	Somssich *et al.*, 1989
eli9	hydroxyproline-rich glycoprotein	4,300	Trezzini *et al.*, 1993
eli10	unknown	1,300	Somssich *et al.*, 1989
eli11	anionic peroxidase	2,000	Trezzini *et al.*, 1993
eli12	unknown	1,650	Somssich *et al.*, 1989
eli13	unknown	1,300	Somssich *et al.*, 1989
eli14	*S*-adenosyl-L-homocysteine hydrolase	2,000	Kawalleck *et al.*, 1992
eli16	unknown	1,900 and 1,700	Somssich *et al.*, 1989
eli17	unknown	1,800	Somssich *et al.*, 1989
eli18	*S*-adenosyl-L-methionine synthetase	1,700	Kawalleck *et al.*, 1992
con2	polyubiquitin	2,000	Kawalleck *et al.*, 1993b

Table 2. Pathogenicity of *Erwinia chrysanthemi* in parsley. Parsley leaves were inoculated with bacterial suspensions (10^8 cfu ml^{-1}) by stomatal infiltration. The symptoms were scored six days after inoculation and grouped into the four categories shown in Fig. 1. Results are averages with standard deviations of at least three experiments

Strain	Symptoms [% of infected leaves]			
	Non-invasive		Invasive	
	None	Localized necrosis	Spreading maceration	Petiole rotting
Ech wt	24 ± 17	68 ± 17	7 ± 2	1 ± 1
Ech out	9 ± 3	91 ± 3	0	0
Ech omp	78 ± 25	22 ± 25	0	0
Ech lps	90 ± 9	10 ± 9	0	0
Ech cel	3 ± 3	21 ± 9	37 ± 17	39 ± 18
Ech cbr	4 ± 6	29 ± 15	32 ± 16	35 ± 22

Ech lps mutant did not multiply at all within the plant tissue (results not shown). With this latter mutant, the number of bacteria declined to 10% of the input by 9 h after inoculation.

Activation of plant defense-related genes following infection with Erwinia chrysanthemi. The activation of plant defense-related genes was analyzed in parsley leaves within the first 24 h after inoculation with bacterial suspensions of wild type or mutant *Erwinia chrysanthemi* in comparison to untreated, mock-inoculated or *Escherichia coli*-infected tissue. Total RNA was extracted from the relevant leaf tissue, separated on formaldehyde agarose gels, blotted onto nylon membrances and hybridized with cDNAs of elicitor-responsive and constitutively expressed genes from parsley (Table 1). This response of parsley leaves to bacterial infection was compared to the correspond-

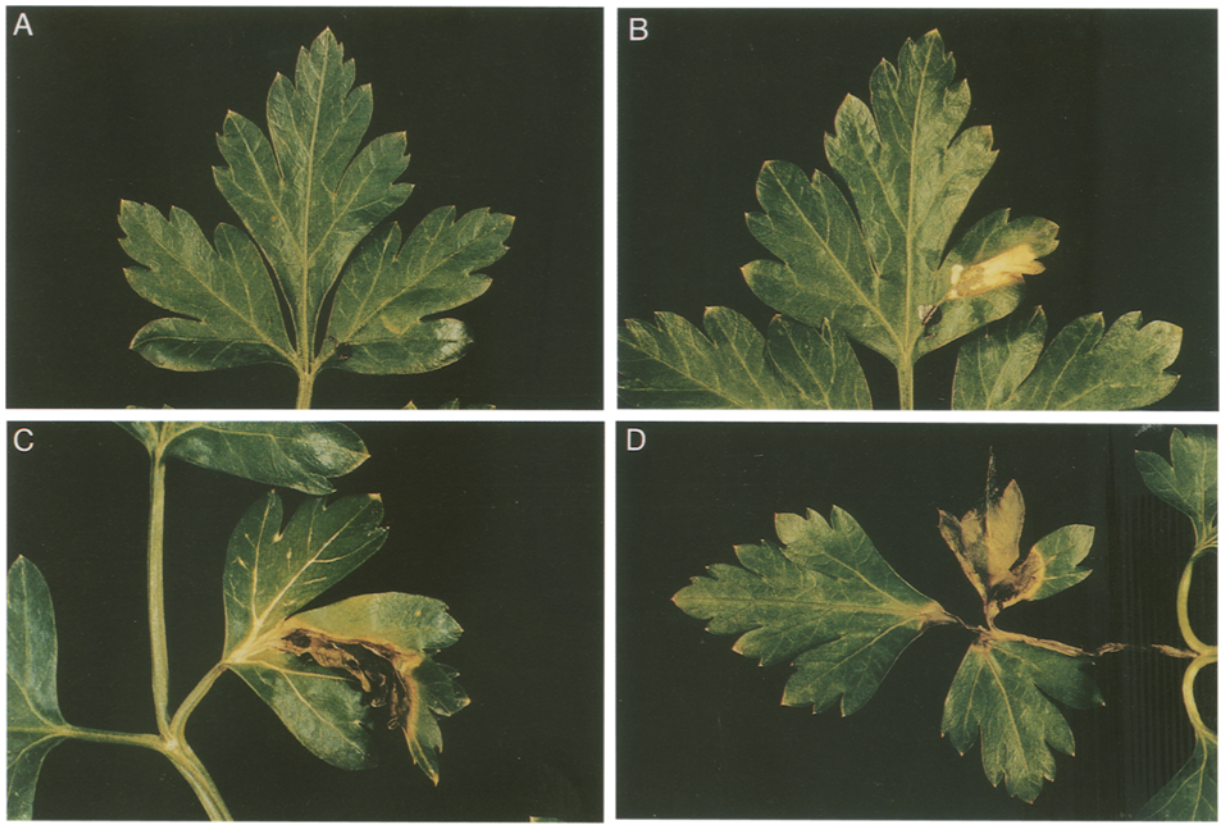
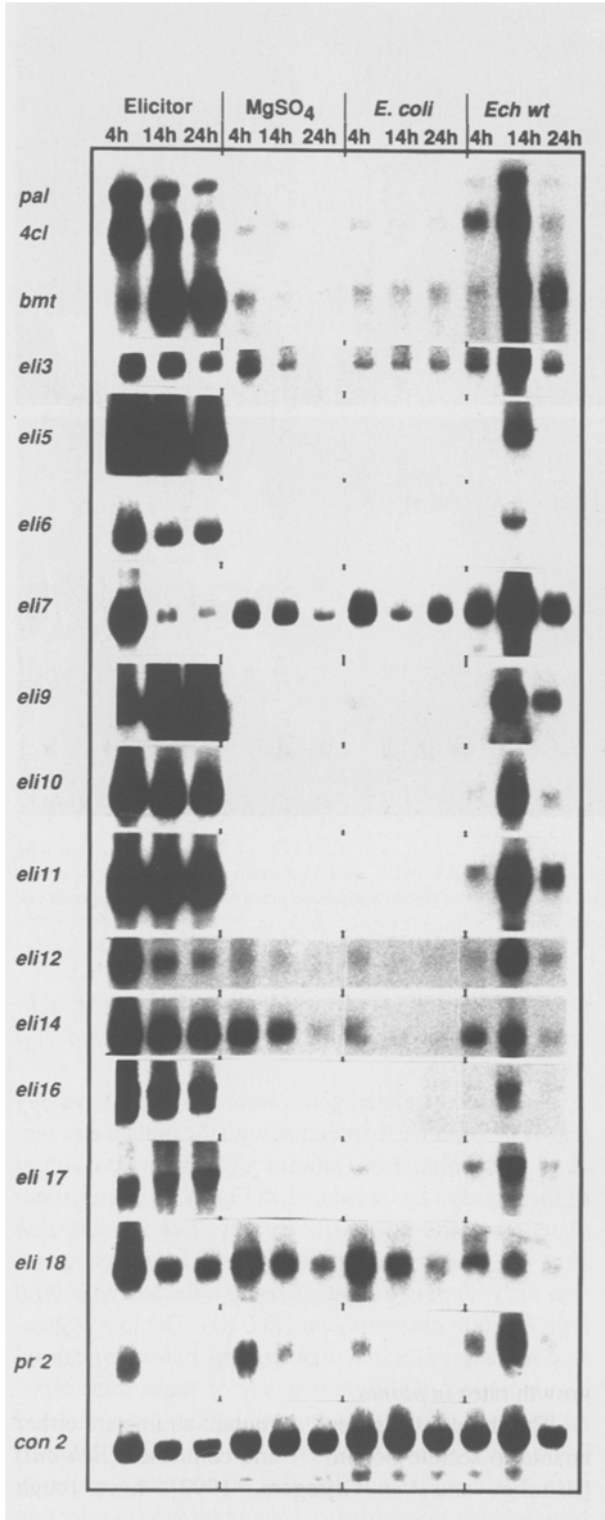


Fig. 1. Symptoms provoked by *Erwinia chrysanthemi* in parsley. Leaves were inoculated with the wild type strain of *Erwinia chrysanthemi* (10^8 cfu ml^{-1}) by stomatal infiltration and incubated in a growth chamber for six days. The phenotypes were grouped into four categories: A, no symptoms; B, localized necrosis; C, spreading maceration; D, petiole rotting.

ing reaction of cultured parsley cells to treatment with fungal elicitor.

Upon infection of parsley leaves with wild type *Erwinia chrysanthemi (Ech wt)* the same transcripts accumulated as after treatment of cultured cells with fungal elicitor. Likewise, the *con2* gene encoding polyubiquitin was constitutively expressed (Fig. 2). Although higher amounts of defense gene-encoded RNA were detectable in cultured cells, the time course of accumulation of the individual transcripts was similar in infected leaves and elicitor-treated cells. The amount of transcript encoding BMT, for example, increased and decreased significantly later than did those of PAL and 4CL. Transcripts of the elicitor-responsive genes *eli3, eli7, eli14, eli18* and *pr2* also accumulated in response to infiltration of parsley leaves with $MgSO_4$ solution, indicating wound-responsiveness of these genes (Fig. 2), as they were not expressed in healthy, unwounded tissue (Fig. 3). Only these wound-responsive genes appear to be activated in plant tissue infected with *Escherichia coli* (Fig. 2).

All defense-related genes were activated in parsley leaves in response to infection with the mutant *Erwinia chrysanthemi* strains. Data for a representative subset of these genes are presented in Fig. 3. In leaves inoculated with the aggressive strains, *Ech cel* and *Ech cbr*, the activation pattern of defense-related genes was very similar to that of tissue infected with wild type *Erwinia chrysanthemi (Ech wt)*. The less aggressive mutants, *Ech out*, *Ech lps* and *Ech omp*, caused accumulation of different levels of these transcripts. Although only small amounts of defense gene-encoded RNA were found to accumulate in leaves infected with the *Ech lps* mutant, the plant cells apparently responded more rapidly to this mutant than to infection with any other strain. Relatively high levels of BMT RNA were already detectable 4 h after inoculation and



low amounts of PAL and 4CL transcripts were found only at this early time point, indicating that maximum levels of these mRNAs probably accumulated even earlier. The responses of parsley leaves to the *Ech out* mutant and the wild type strain, however, were almost identical. In *Ech omp*-infected tissue as well, defense gene transcripts initially accumulated to levels similar to those accumulating in leaves inoculated with the wild type strain. However, 24 h after inoculation, even higher levels of these transcripts were detectable. The responses to all other strains were more transient and had begun to dissipate at this time point.

Similarly high levels of furanocoumarin phytoalexins accumulated in leaves 24 h after inoculation with *Ech omp* and *Ech cel* mutants, whereas lower amounts were detectable in *Ech cbr*-infected leaves (Fig. 4). The phytoalexin contents of leaves inoculated with the less aggressive *Ech out* and *Ech lps* mutants were approximately five times the background level of untreated, mock-inoculated or *Escherichia coli*-infected plants. The same pattern of phytoalexins accumulated in infected leaves and elicitor-treated cell cultures, as shown by thin-layer chromatography of extracts from leaves infected with *Ech wt, Ech cel* and *Ech out* (Fig. 5).

Discussion

Parsley plants are able to discriminate between *Escherichia coli*, which is not a phytopathogen, and the broad host range pathogen, *Erwinia chrysanthemi*. In contrast, bean leaves have been found to accumulate phytoalexins as well as transcripts encoding chitinase or enzymes involved in phytoalexin synthesis in response to infiltration with *Escherichia coli* [Jakobek and Lindgren, 1993], indicating the existence of different levels of selectivity in non-self recognition in these two plants. In both plants, however, neither phytoalexin synthesis nor transcription of genes encoding the corresponding enzymes were found to be induced by

Fig. 2. Accumulation of defense gene-encoded RNAs in parsley leaves infected with *Erwinia chrysanthemi* or *Escherichia coli*. Leaves were inoculated with bacterial suspensions (10^8 cfu ml^{-1}) by stomatal infiltration and harvested 4, 14 or 24 h later. Total RNA was isolated and analyzed by Northern blotting and hybridization with the cDNA probes listed in Table 1. Each lane contained 20 μg of RNA isolated from elicitor-treated cell suspension cultures (elicitor) or from leaves infiltrated with $MgSO_4$ solution, *Escherichia coli* (*E. coli*) or wild type *Erwinia chrysanthemi (Ech wt)* suspensions in $MgSO_4$ solution. Results obtained with untreated plants are shown in Figure 3.

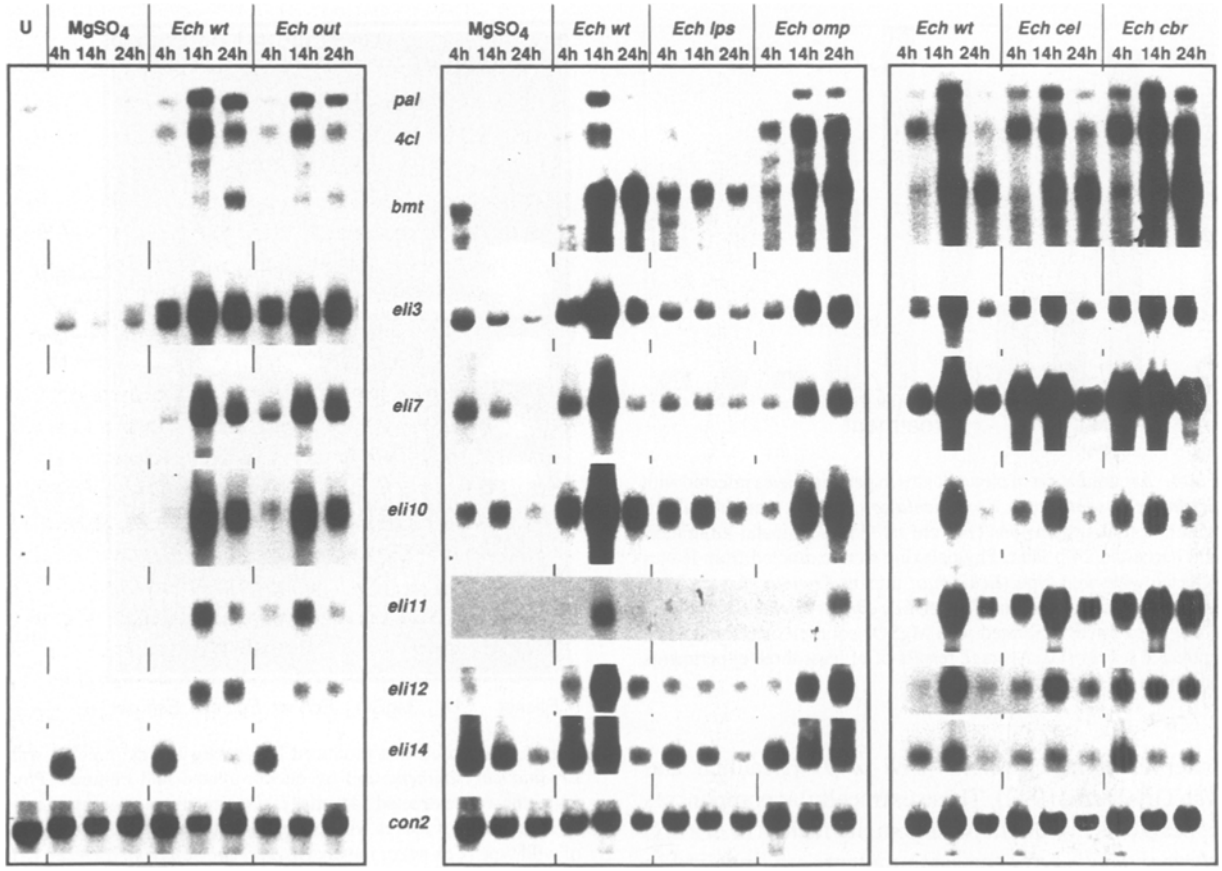


Fig. 3. Accumulation of defense gene-encoded RNAs in parsley leaves infected with wild type or mutant strains of *Erwinia chrysanthemi*. Leaves were inoculated with bacterial suspensions (10^8 cfu ml^{-1}) by stomatal infiltration and harvested 4, 14, or 24 h later. Total RNA was isolated and analyzed by Northern blotting and hybridization with a representative subset of the cDNA probes listed in Table 1. Each lane contained 20 μg of RNA isolated from healthy, untreated leaves or from leaves infiltrated with $MgSO_4$ solution, wild type (*Ech wt*) or mutant *Erwinia chrysanthemi* (*Ech out, Ech lps, Ech omp, Ech cel, Ech cbr*) suspensions in $MgSO_4$ solution. Each block contains results obtained with leaves from a single plant.

mock inoculation, whereas a subset of other defense-related genes was transiently activated in $MgSO_4$-infiltrated parsley leaves, indicating that these genes are wound-responsive.

The level of sensitivity of parsley leaves to *Erwinia chrysanthemi* depended on the inoculation mode. Plants were susceptible when bacterial suspensions of the wild type strain were injected into petioles, whereas stomatal infiltration into leaves resulted in a moderate level of resistance. When the latter method was used to inoculate parsley leaves with mutant strains of *Erwinia chrysanthemi*, the plant responses to the various mutants differed drastically. In comparison to the incomplete resistance elicited by wild type *Erwinia chrysanthemi*, leaves of the same parsley plant showed complete resistance to *Ech out, Ech omp* and *Ech lps* mutants, but were highly suscpetible to *Ech cel* and *Ech cbr*. With the possible exception of the *Ech lps* mutant, these differences could not be explained by differences in the abilities of the bacteria to multiply *in planta*. Insertional mutagenesis reduced (*Ech out, Ech omp*) as well as drastically increased (*Ech cel, Ech cbr*) bacterial pathogenicity on genetically identical plant material without affecting initial bacterial growth rates *in planta*.

The three less aggressive mutant strains are either unable to secrete pectinases and cellulases (*Ech out*) [Beaulieu and Van Gijsegem, 1992], have rough lipopolysaccharides with an altered core structure (*Ech lps*) [Schoonejans *et al.*, 1987] or lack one major

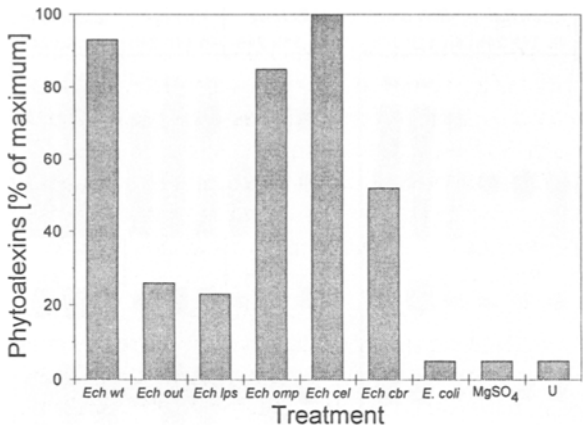


Fig. 4. Accumulation of phytoalexins in parsley leaves infected with *Erwinia chrysanthemi* or *Escherichia coli*. Leaves were inoculated with bacterial suspensions (10^8 cfu ml^{-1}) by stomatal infiltration and harvested 24 h later. Phytoalexins were extracted from leaves infected with wild type (*Ech wt*) or mutant *Erwinia chrysanthemi* (*Ech out, Ech lps, Ech omp, Ech cel, Ech cbr*), or *Escherichia coli* (*E. coli*), from leaves infiltrated with $MgSO_4$ solution or from healthy, untreated leaves (U). Average results of at least three experiments are shown.

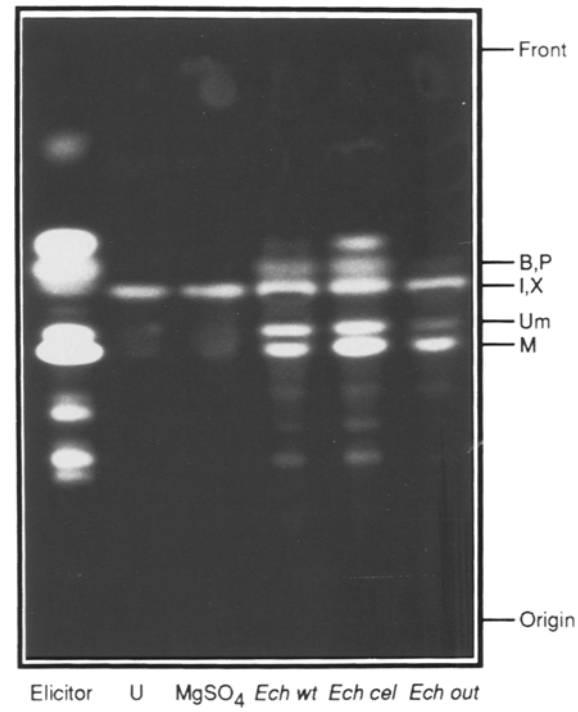


Fig. 5. Phytoalexins produced by parsley leaves infected with *Erwinia chrysanthemi* and by elicitor-treated cell cultures. Phytoalexins were extracted 24 h after elicitor treatment of cultured cells or inoculation of leaves with bacterial suspensions (10^8 cfu ml^{-1}) of wild type (*Ech wt*) or mutant *Erwinia chrysanthemi* (*Ech cel, Ech out*) and separated by think-layer chromatography. Cochromatography with authentic standard compounds is indicated (B, bergapten; I, isopimpinellin; M, marmesin; P, psoralen; Um, umbelliferone; X, xanthotoxin). No phytoalexins were detectable in extracts from healthy, untreated (U) or $MgSO_4$ solution-infiltrated leaves.

outer membrane protein (*Ech omp*) [Beaulieu and Van Gijsegem, 1992]. These extracellular components appear to be important pathogenicity factors in *Erwinia chrysanthemi*, since the same mutants are impaired in pathogenicity on other plants as well [Beaulieu and Van Gijsegem, 1992; Schoonejans *et al.*, 1987]. The formation of localized brown lesions by parsley leaves in response to infiltration with the *Ech out, Ech omp* and *Ech lps* mutants, and to some extent, with wild type *Erwinia chrysanthemi*, closely resembles a hypersensitive reaction which many plants develop during host-incompatible or non-host interactions with pathogenic bacteria [Klement, 1982].

The two strains with increased pathogenicity, *Ech cel* and *Ech cbr*, harbor regulatory defects [Aymeric *et al.*, 1988; Expert *et al.*, 1993]. *Ech cel* was found to be more aggressive than the wild type strain in *Saintpaulia ionantha* [Aymeric *et al.*, 1988], whereas *Ech cbr* is less aggressive due to a lag during the primary phase of disease development [Sauvage *et al.*, 1994].

Macroscopically, the first symptoms of infection of parsley leaves by *Erwinia chrysanthemi* wild type or mutant strains only became visible more than 24 h after inoculation. However, within the initial 24 h of infection, RNAs encoding defense-related genes accumulated rapidly and transiently in parsley leaves infected with wild type or mutant strains of *Erwinia chrysanthemi*, clearly preceding visible onset of lesion development or maceration. The lack of correlation between the degree or timing of activation of defense-related genes and the final extent of plant resistance could have at least four different explanations. First, we are aware of the inherent limitations of our analyses caused by the lack of a phenotype within the time period when RNA was isolated from the various infiltrated plant tissue and the variability in symptom development observed later on. We cannot completely exclude the possibility, even in the case of the most aggressive strain, *Ech cel*, that the expression kinetics of the defense-related genes detected by RNA Northern hybridizations may be biased towards a particular symptom category depicted in Table 2. However, this appears unlikely, since similar results were obtained in a number of independent experiments. Secondly, defense gene transcripts accumulate but are not prop-

erly translated in the susceptible leaves. Thirdly, no causal relationship exists between defense gene activation and resistance. Fourthly, defense gene activation is necessary but not sufficient for the initiation of hypersensitive resistance.

Three of the defense-related genes found to be activated in parsley leaves by infection with *Erwinia chrysanthemi* encode enzymes involved in the formation of furanocoumarin phytoalexins, namely PAL, 4CL and BMT [Scheel *et al.*, 1987]. The production of phytoalexins elicited by the different strains of *Erwinia chrysanthemi* correlated well with the accumulation of transcripts encoding these enzymes, indicating that at least these genes are transcribed and translated at levels corresponding to the amount of transcripts detected by RNA hybridization. Assuming that this holds true for the complete set of defense-related genes, a lack of proper expression of these genes does not appear to be the cause of susceptibility of parsley leaves to specific *Erwinia chrysanthemi* strains.

Pathogen or elicitor-induced biosynthesis of furanocoumarins in parsley involves rapid (PAL, 4CL) as well as delayed gene activition (BMT) and represents a rather late defense response [Scheel *et al.*, 1987]. The accumulation of phytoalexins within 24 h of inoculation of parsley leaves with different *Erwinia chrysanthemi* strains consequently implies that the products of other, more rapidly activated genes should also have been present in the infected tissue hours before onset or failure of necrotic lesion development.

Genetical analysis of the linkage between hypersensitive resistance and defense gene activation has been performed in a gene-for-gene pathosystem consisting of *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *maculicola* [Kiedrowski *et al.*, 1992]. Here, the *Arabidopsis* homologue of the parsley *eli3* gene was found to be under strict regulatory control of the *RPM 1* disease resistance locus which renders this plant genotype hypersensitively resistant to bacterial strains carrying the corresponding avirulence gene, *avrRpm 1*. Although acccumulation of *eli3* RNA always preceded lesion development in these interactions, a functional role of *eli3* expression in hypersensitive resistance has yet to be demonstrated. Moreover, rapid activation of the *eli3* gene was also observed in compatible interactions with bacteria non-isogenic to those carrying the *avrRpm 1* gene [Kiedrowski *et al.*, 1992]. It might therefore be concluded that rapid and massive activation of defense-related genes is not sufficient, although it may be necessary, for establishing a hypersensitively resistant phenotype.

The rapid and complete activation of defense-related genes in parsley leaves inoculated with the aggressive mutants, *Ech cel* or *Ech cbr*, is indicative of the efficient operation of a pathogen recognition mechanism. This recognition event, however, did not trigger local lesion development in the infected tissue. Similar observations have recently been reported for compatible interactions between *Arabidopsis* and *Pseudomonas syringae* pv. *maculicola* [Kiedrowski *et al.*, 1992], as outlined above, and for bean plants inoculated with a Hrp^- mutant of *Pseudomonas syringae* pv. *tabaci*, which in contrast to the corresponding wild type strain, did not elicit hypersensitive necrosis in bean [Jakobek and Lindgren, 1993]. In bean, defense-related genes were found to be activated with almost identical timing and intensity after infection with the Hrp^- and wild type strains, indicating separate regulation of the two processes. These results, on the whole, suggest the existence of distinct signalling mechanisms for the activation of plant defense-related genes and the initiation of the hypersensitive response. Under specific conditions, however, both responses can apparently be regulated in a coordinate manner by the product of a single gene or locus, such as *RPM 1*. Genetically well defined bacteria in combination with uniform plant genetic backgrounds were required to unequivocally demonstrate this strictly coordinated regulation [Kiedrowski *et al.*, 1992], indicating that multiple signals can regulate defense genes in a resistance gene-independent manner.

In conclusion, analyses of defense responses of different plants to various pathogenic and non-pathogenic bacteria suggest that activation of defense-related genes is insufficient for plant protection and is not necessarily followed by lesion development. Formation of local lesions, however, generally appears to be preceded by defense gene activation in interactions of plants with particular bacterial pathogens. Whether this correlation indicates a causal role of defense gene products in hypersensitive resistance of plants to bacterial pathogens remains to be elucidated.

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