Identification of Compatible and Incompatible Interactions Between *Arabidopsis thaliana* and *Xanthomonas campestris* pv. *campestris* and Characterization of the Hypersensitive Response

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Both compatible and incompatible interactions between *Arabidopsis thaliana* and *Xanthomonas campestris* have been identified and, for the first time, a strong hypersensitive response has been characterized. A highly reproducible mass spray inoculation protocol has been established and was used together with the more commonly used infiltration inoculation procedure to study the defense responses occurring in mature *A. thaliana* plants. A series of bacterial strains have been tested on *A. thaliana* ecotype Columbia (Col-O). *X. c.* pv. *campestris* was the most effective pathogen in these tests and was used for further detailed analysis. Several *X. c.* pv. *campestris* isolates were tested on *A. thaliana* Col-O, and one particular *X. c.* pv. *campestris* strain (147) was tested on 27 *Arabidopsis* ecotypes. Symptom development of compatible and incompatible interactions, including the hypersensitive response, was extensively characterized in *A. thaliana* Col-O. Lesion structure, bacterial distribution, accumulation of polyphenolic compounds, and the deposit of callose in inoculated leaves were documented by microscopic analysis. Activation of the defense-associated genes coding for phenylalanine ammonia lyase (PAL), β-1, 3-glucanases, chitinases, and peroxidases was evaluated by Northern blot analysis.

During the last 10 yr, considerable progress has been made in our understanding of the molecular events associated with plant defense responses (for review, see Lamb *et al.* 1989; Dixon and Harrison 1990). The activation of defense processes, both at the site of infection and systemically, is dependent on the recognition of a pathogen and the relay of this information throughout the plant.

A general model for the induction of plant defense responses, the gene-for-gene model, proposes that single dominant alleles in plants and pathogens determine whether the plant will be resistant or susceptible (Flor 1971; Ellingboe 1992). In the case of an incompatible interaction, where matching alleles of a pathogen avirulence gene and a plant resistance gene are present, a rapid recognition event occurs and in most cases the plant responds with a hypersensitive response (HR). The HR is characterized by rapid cell death in the immediate vicinity of the infection site, leading to the formation of a necrotic lesion and the inhibition of growth of biotrophic pathogens (Klement and Goodman 1967; Mansfield 1986). According to the model, in the case of compatible interaction where the dominant alleles are not present in both partners, then plant recognition does not occur, and the pathogen causes disease.

The genetic recognition and/or pathogenicity determinants of microorganisms are being identified and in some cases have already been isolated and characterized (Staskawicz *et al.* 1984; Daniels *et al.* 1984; Kobayashi *et al.* 1989; Boucher *et al.* 1987). Mainly as a result of deficiencies in mutation and cloning strategies in plants, no plant resistance gene has so far been isolated. The advent of *Arabidopsis thaliana* as a model plant for the study of plant/pathogen interactions might, however, resolve this gap in the present basic understanding of the plant defense response. Indeed, it has already been shown that *A. thaliana* is a host for various pathogens including bacteria, viruses, fungi, and nematodes (for a review, see Dangl 1992). Among these, *Xanthomonas campestris* pv. *campestris,* the causal agent of black rot of the cruciferous family, has been identified as a potential pathogen of *A. thaliana* (Simpson and Johnson 1990; Daniels *et al.* 1991). A differential responsiveness of *A. thaliana* towards two *Xanthomonas* races seems to be controlled by a single dominant nuclear plant gene (Tsuji *et al.* 1991). However, until now, no clear evidence has been given for the induction of an HR in *Arabidopsis* upon infection by *X. c.* pv. *campestris.*

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Better documented are the interactions between *Arabidopsis* and certain *Pseudomonas* strains (Bent *et al.* 1991; Dangl *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991) and recently, two loci determining resistance to different *P. syringae* pathovars have been identified (Debener *et al.* 1991; Kunkel *et al.* 1993) providing indications that at least certain *A. thaliana*/pathogen interactions conform to the gene-for-gene hypothesis.

To obtain a better insight into the orchestration of plant responses towards pathogens, we would like to apply a mutational analysis strategy based on the interaction of a bacterial pathogen with *A. thaliana*.

An initial screening for bacterial pathogens was performed by infecting *A. thaliana* ecotype Col-O with Brazilian isolates of *Xanthomonas, Pseudomonas,* and *Erwinia* species. A *X. c.* pv. *campestris* isolate was found to be the most effective inducer of necrotic lesions. Additional *X. campestris* isolates were tested on *A. thaliana* Col-O, and one particular isolate was tested on 27 different *Arabidopsis* ecotypes. Among these bacterium-plant combinations, incompatible interactions resulting in an HR and resistance, and compatible interactions resulting in disease were found, indicating a differential response of *Arabidopsis* to *X. c* . pv. *campestris* isolates. Since we are particularly interested in the HR, we established an inoculation procedure that allowed easy and efficient scoring for HR. Using this technique, we have been able, for the first time, to characterize a typical HR induced by *X. c.* pv. *campestris* on *A. thaliana.* This has been done by a detailed phenotypic, microscopic, and molecular analysis.

RESULTS

PHENOTYPIC CHARACTERIZATION OF *ARABIDOPSIS/X. C.* PV. *CAMPESTRIS* INTERACTIONS.

Establishment and identification of *Arabidopsis/X. c.* pv. *campestris* interactions.

Several taxonomically distant bacterial pathogens of the crucifer family were chosen and spontaneous antibiotic resistant mutants were isolated for most of the strains. We tested their ability to induce an HR by spraying them onto *A. thaliana* leaves, as described in Materials and Methods. As shown in Table 1, most of the bacteria were unable to induce an HR on *Arabidopsis*. However, it was found that the Spc^r mutants of both *X. c.* pv. *campestris* 147 and *X. c.* pv. *vitians* 394, like their wild-type parents, reproducibly induced dry necrotic lesions, typical of an HR. The lesions became visible within 24 hr after spraying and were dispersed over the upper leaf surface of all inoculated plants (Fig. 1C). The Rif^r and Rif^r Spc^r mutants of *X. c.* pv. *campestris* 147 lost the ability to induce an HR, therefore, the *X. c.* pv. *campestris* Spc^r strain was selected for all further experiments unless otherwise stated. To document the differential responsiveness of *A. thaliana* towards *X. c.* pv. *campestris,* 27 ecotypes of *A. thaliana* from different geographical locations were spray-inoculated with *X. c.* pv. *campestris* 147 Spc^r (Table 2). Similarly, we examined the interactions of *A. thaliana* Col-O inoculated with 28 different isolates of *X. c.* pv. *campestris* (Table 3). The latter analysis was performed using two different inoculation methods: spray inoculation and infiltration inoculation (see Materials and Methods).

Inoculating strain 147 on *Arabidopsis* ecotypes gave three possible responses: null responses (Chi-O, Mt-O, and Zu-O), incompatible (i.e., Col-O, Ha-O, Pr-O, La-er, and Pn-O), or compatible interactions (Sf-2) (Table 2). This differential response was similarly demonstrated by analyzing the interactions between 28 *X. c.* pv. *campestris* isolates and *A. thaliana* Col-O (Table 3).

In these latter experiments strong HR-like incompatible responses were observed with strains 147, 568, 7461, and 8116. Compatible interactions were observed with strains 8004 and 8088, and strain 8096 induced a "delayed" compatible response. All other interactions gave a weak HR-like incompatible response of varying intensity (i.e., 581, 8003, 7516, and 8035).

Macroscopic description of the symptoms of the interactions.

After spray inoculation with a bacterial suspension of 10^8 cfu/ml, the incompatible interaction was characterized by rapid and localized hypersensitivity resulting in necrotic lesions after 24 hr (Fig. 1C,D). The different HR intensities were scored after 2 days (Tables 2 and 3). Sometimes, light and limited chlorosis was noticed in the immediate vicinity of the lesion after 48 hr, but in general, no further

Table 1. Bacterial strains tested for the ability to induce HR in *Arabidopsis thaliana*

Name	Antibiotic resistance	HR	Source
Xanthomonas campestris			
pv. *campestris* 147	none	+++	I.A.C.
	Spc	+++	This study
	Rif	0	This study
	RifSpc	0	This study
	Nal	++	This study
	Str	+	This study
pv. *vesicatoria* 5071	none	0	I.A.C.
pv. *vitians* 394	none	++	I.A.C.
	Spc	++	This study
pv. *vitians* 9001	Rif	0	M. Daniels
Erwinia carotovora			
subsp. *atroseptica* 0784	none	0	I.A.C.
	Spc	0	This study
	Rif	0	This study
	Cb	0	This study
subsp. *carotovora* 0771	none	0	I.A.C.
	Spc	0	This study
	Rif	0	This study
	Cb	0	This study
Pseudomonas cepacia			
C 398	none	0	F.T.A.
	Spc	0	This study
	Rif	0	This study
	Cb	0	This study
Pseudomonas cichorii			
C 356	none	0	F.T.A.
	Cb	0	This study

[a] Abbreviations: Cb, carbenicillin; Nal, nalidixic acid; Rif, rifampicin; Spc, spectinomycin; Str, streptomycin; I.A.C., Instituto Agronómico de Campinas SP (Brasil); F.A.T., Fundacão Tropical de Pesquisas e Technologia "André Tosello" São Paulo (Brasil); +++, very strong; ++, strong; +, weak; 0, none.

change or expansion of the lesions occurred after the second day. However, the tissue within the lesions became desiccated 36–48 hr after treatment. After infiltration inoculation with an incompatible strain at 10^6 cfu/ml, a strong chlorosis—limited to the infiltrated area and its immediate vicinity—was induced after 2 days (Fig. 1A). No further symptom development was noticed within 6 days (Tables 2 and 3). Infiltration at 10^8 cfu/ml, resulted in the transformation of the whole infiltrated area into one big necrotic lesion within 24 hr (data not shown).

In contrast, a compatible interaction was characterized by the appearance of spreading chlorosis several days after spray inoculation, extending from a few initial necrotic lesions (Fig. 1E). After inoculation by infiltration, a compatible interaction progressed beyond the infiltrated region, ending with the complete chlorosis of the infected leaf (Fig. 1B). A "delayed" compatible interaction was characterized by the appearance of a rapid and strong HR followed several days later by the observation of a spreading chlorosis (Table 3).

In vivo growth of *X. campestris* isolates in *A. thaliana*.

One of the most commonly used indicators of resistance or susceptibility of a plant towards a pathogenic bacterium is multiplication *in planta*. Since the establishment of the compatible interaction was not clearly visible until several days after spray inoculation, we decided to establish growth curves for the different interactions observed above, after infiltrating the bacteria into leaf tissue. The strain 147 typically gave three types of responses on *A. thaliana* ecotypes exemplified by infection on Chi-O, Col-O, and Sf-2 (Fig. 2A). In a null interaction (Chi-O), a residual bacterial population can be detected after brief initial growth. The incompatible interaction (Col-O) resulted in a rapid and strong increase in bacterial numbers followed by a decrease. The compatible interaction (Sf-2) induced rapid and sustained bacterial multiplication.

We subsequently studied the growth of various *X. c.* pv. *campestris* isolates on *A. thaliana* Col-O (Fig. 2B). We exemplified the results by selecting the following strains

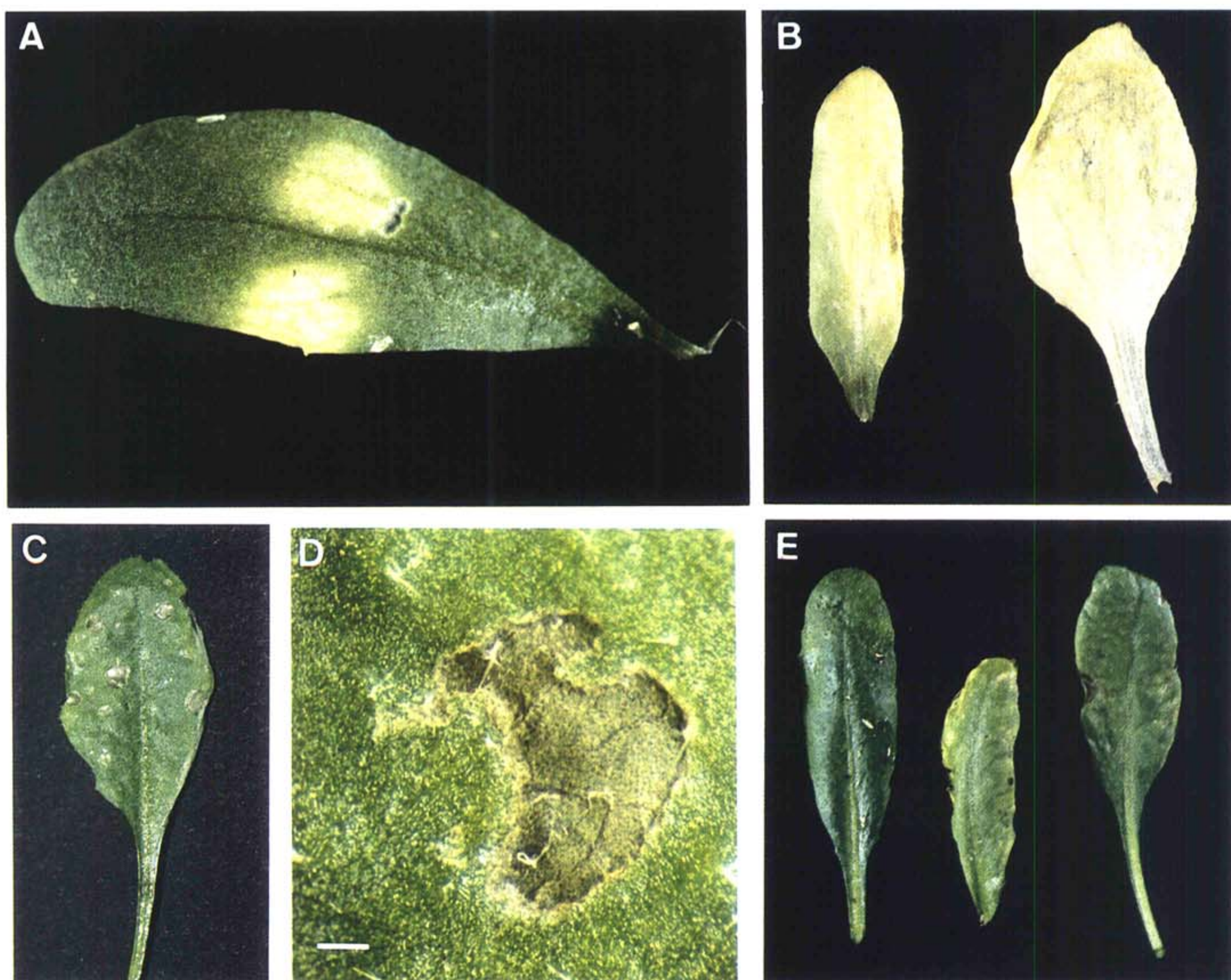

Fig. 1. Macroscopic observation of symptoms in *Arabidopsis thaliana* **A, B,** after infiltration or, **C–E,** spray inoculation with *Xanthomonas campestris* pv. *campestris*, strain 147 (A, C, and D) and strain 8004 (B and E). **D,** Close up of necrotic lesion. Scale bar = 0.4 mm.

Table 2. Interactions between 27 *Arabidopsis* ecotypes and the *X. c.* pv. *campestris* isolate 147

Ecotype	HR (2 days)	Chlorosis (6 days)
Be-O	+	0
Bu-2	+	0
Bur-O	+	0
Chi-O	0	0
Col-O	++++	1
C 24	++	1
Edi-O	+	1
Ha-O	++++	1
Hs-O	++	1
La-er	+++	1
Ll-O	++	1
Mt-O	0	0
Nd-O	++	1
No-O	+++	1
Ob-O	++	1
Oy-O	+	1
Pn-O	+++	1
Po-1	+	1
Pr-O	++++	1
Rsch-O	+	0
Sei-O	++	0
Sf-2	++	2
Ts-1	+	0
Uk-2	++++	1
Uk-4	+	0
Ws	++++	1
Zu-O	0	0

Table 3. Interactions between *Arabidopsis* ecotype Col-O and 28 *X. c.* pv. *campestris* isolates[a]

Isolate	Spray HR 2 days	Spray chlorosis 6 days	Infiltration chlorosis 6 days
147	++++	1	1
568	+++	1	1
581	++	1	1
583	++	1	1
584	++	1	1
7461	+++	1	ND
7516	+	1	1
7662	+	1	ND
8002	++	1	1
8003	++	0	1
8004	+	2	2
8007	+	0	1
8034	+	1	ND
8035	+	0	1
8050	++	1	1
8088	+	2	2
8090	+	1	1
8096	+++	2	2
8100	+	1	1
8101	+	1	1
8102	++	1	ND
8104	++	1	1
8106	++	1	1
8109	+	1	1
8114	++	1	1
8116	+++	1	1
8238	++	1	1
2D520	++	1	1

[a] Abbreviations: 0, symptomless (no chlorosis); +, very weak HR (<25% of plants show necrotic lesions); ++, weak HR (25-75% of plants show necrotic lesions); +++, strong HR (75-100% of plants show necrotic lesions) ++++, very strong HR (100% of plants and >50 of all leaves show necrotic lesions); 1, limited chlorosis around the necrotic lesions; 2, spreading chlorosis; ND, not determined.

8003 and 147 (incompatible), 8004 (compatible), and 8096 (delayed compatible response-interaction). The growth profiles of the different isolates are a reflection of the observed phenotypical symptoms and are comparable to the classes described above, except for strain 8096, whose typical compatible growth curve does not correspond with the phenotypic symptoms induced (a strong HR followed several days later by a spreading chlorosis).

MICROSCOPIC CHARACTERIZATION OF INCOMPATIBLE/COMPATIBLE BACTERIAL INTERACTIONS WITH *ARABIDOPSIS*.

Scanning electron microscopy analysis.

Scanning electron microscopy allowed a detailed screening of the infected leaf epidermis and an evaluation of the spatial distribution of the pathogen following spray inoculation. In an early stage of the HR (12 hr), epidermal cells had already lost their turgor and bacterial clusters were scattered over the necrotic area (Fig. 3A). Based on where bacteria were found, stomata appeared to be potential penetration sites (Fig. 3B). At a later stage (48–72 hr), bacteria almost completely covered the necrotic area (Fig. 3C–E). This local, but dense, epiphytic bacterial population was observed until several days after inoculation.

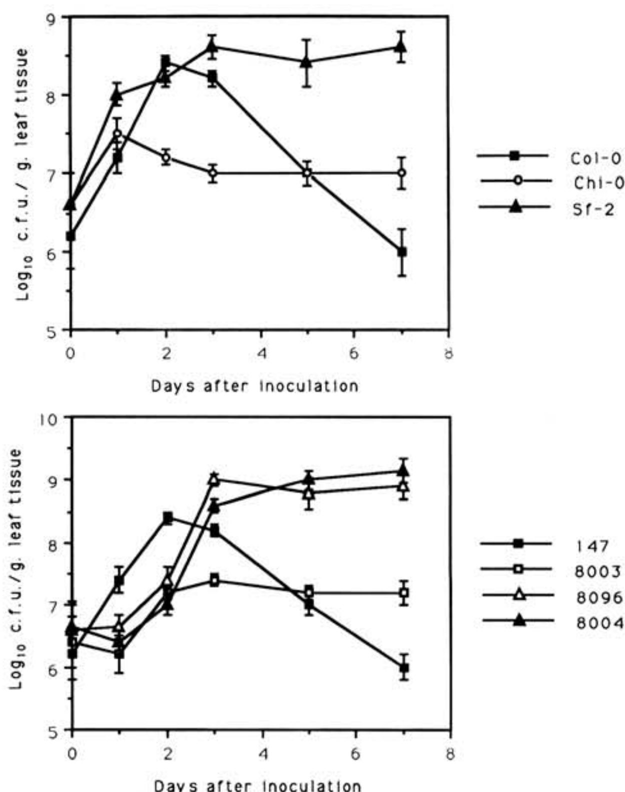

Fig. 2. *In planta* bacterial growth curves. **A,** Leaves of mature plants of different *Arabidopsis* ecotypes Chi-O, Col-O, and Sf-2 were infiltrated with *X. c.* pv. *campestris* 147 (see text). Leaves were harvested at the indicated time points and the concentration of the *in planta* growing bacteria was measured. **B,** Leaves of mature *A. thaliana* Col.O plants were infiltrated with *X. c.* pv. *campestris* isolates 147, 8003, 8004, and 8096 (see text). The Col-O leaves were harvested at the indicated time points and the concentration of the *in planta* growing bacteria was measured.

In the incompatible interaction, only a few scattered bacteria could be found outside the necrotic areas. The same pattern occurred on the whole leaf with compatible interactions, except for a few necrotic lesions, where bacterial distribution similar to that for incompatible interactions was noticed.

Fluorescence microscopy analysis.

Col-O leaves were infiltrated with *X. c.* pv. *campestris* 147, 8004, and 8420 at 10^6 and 10^8 cfu/ml. The mutant strain 8420 is a derivative of 8004, lacking the complete *hrp* locus (M. Daniels, personal communication), was used as a negative control. No visible lesions, chlorosis, or fluorescence were observed on the leaves 40 and 72 hr after infiltration with *X. c.* pv. *campestris* 8420 (Fig. 4A,B), although bacterial multiplication was comparable to that of the compatible *X. c.* pv. *campestris* 8004 strain (data not shown). Forty hours after inoculating strain 147 at 10^6 cfu/ml an intense fluorescence was found in the infiltrated areas and at the borders of the already necrotic tissues (Fig. 4C); only weak fluorescence was observed in the areas infiltrated with 8004 (Fig. 4E). From the third day on, there was a stronger level of fluorescence in the leaf areas infiltrated with strain 8004 (Fig. 4F) compared with those infiltrated with the strain 147 (Fig 4D). At 10^8 cfu/ml, the temporal and quantitative differences in fluorescence between leaves infiltrated with *X. c.* pv. *campestris* 8004 and 147 were less apparent.

We did not observe necrotic lesions when *Arabidopsis* Col-O leaves were spray inoculated with *X. c.* pv. *campestris* 147 at low densities ($<10^6$ cfu/ml); this agrees with previous observations (Turner and Novacky 1974; Essenberg *et al.* 1979; Pierce and Essenberg 1987). However, small autofluorescent areas dispersed over the leaf surface were seen under the fluorescent microscope (Fig. 4G). In addition to the collapsed dead cells inside the

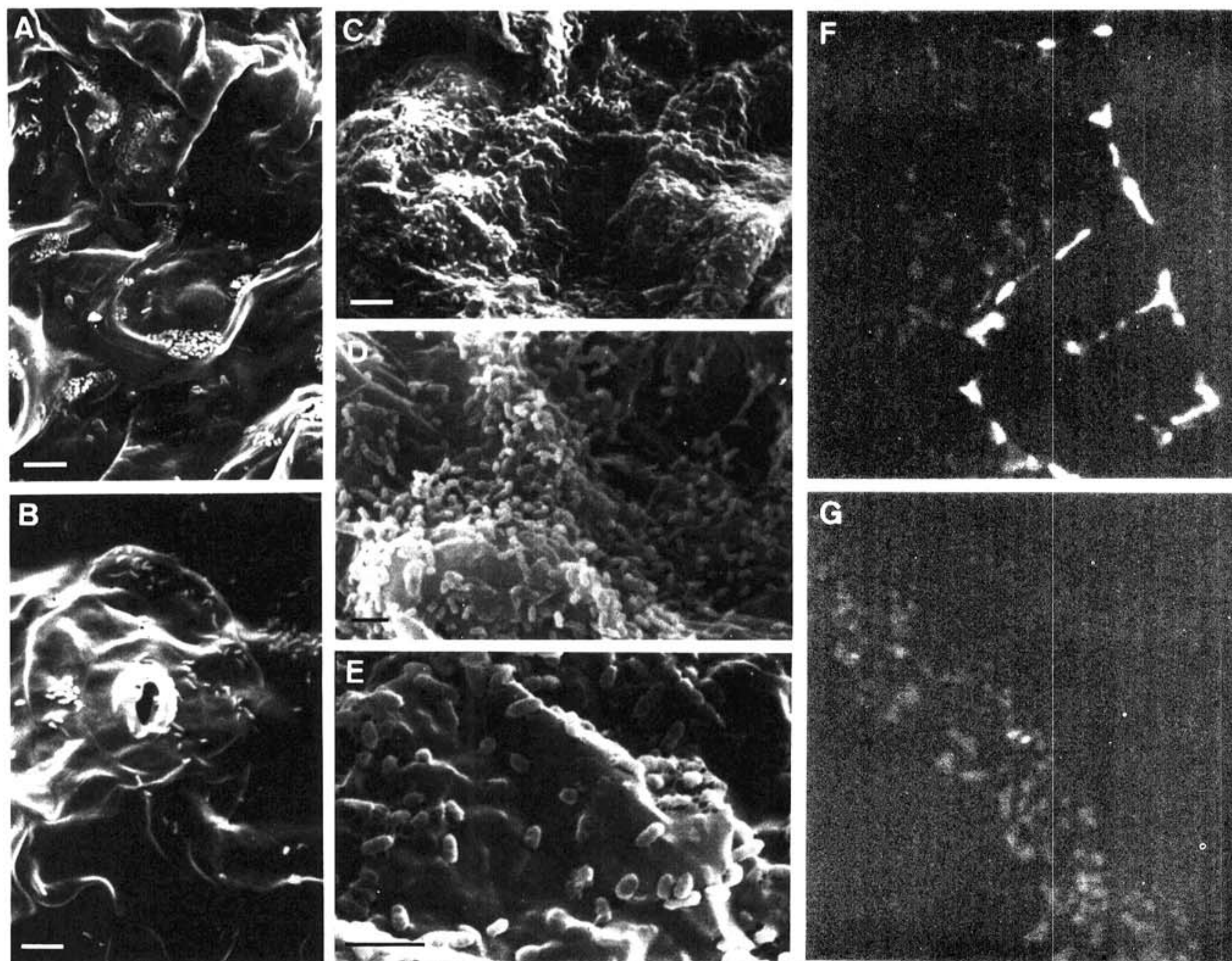

Fig. 3. Analysis of necrotic lesions in *Arabidopsis. thaliana* Col-O, after spray inoculation with *X. c.* pv. *campestris* 147. **A–E,** Pictures taken by scanning electron microscope. **A,** Central part of a lesion on the abaxial epidermis, 12 hr after inoculation with *X. c.* pv. campestris 147. All cells are plasmolyzed. The bacteria are dispersed over the lesion area. **B,** A preferential bacterial site at early infection stage: stomata surrounded and invaded by several bacterial cells. **C–E,** Similar lesion as shown in A, but 72 hr after inoculation. The bacteria are covering major parts of the necrotic domain. **F,G,** Cross section of a necrotic lesion (F) or a control leaf (G) treated with sirofluor under fluorescent microscope. The white areas correspond to the presence of callose plugs; the little gray dots indicate the presence of starch in chloroplasts. Scale bars: A, 15 μm, B, 10 μm, C, 15 μm, D, 2 μm, and E, 2 μm.

necrotic areas, the viable cells surrounding the lesions also revealed yellow fluorescent compounds (Fig. 4H). Confocal microscopy demonstrated that these small areas corresponded to single cells (Fig. 4I–K); these bright spots might represent microscopic HRs that do not coalesce to form necrotic lesions visible to the naked eye. Twenty-four hours after spray inoculation with *X. c.* pv. *campestris* 147 at 10^8 cfu/ml, fluorescence microscopy revealed bright spots on the leaves reacting hypersensitively. The fluorescent areas were similar to those described above and corresponded to the necrotic lesions as seen under white light (data not shown).

Callose is a typical component of a structural barrier formed early at infection sites (Kauss 1987). We found that callose accumulated rapidly in Col-O after spray inoculation with *X. c.* pv. *campestris* 147. In fact, fluorescence microscopy of leaf sections 24 hr after inoculation revealed specific deposition of callose plugs on cell walls at the site of the necrotic lesion (Fig. 3F). No specific deposition of callose was found outside the immediate vicinity of the necrotic areas (Fig. 3G).

MOLECULAR CHARACTERIZATION OF THE INTERACTIONS BETWEEN *X. C.* PV. *CAMPESTRIS* AND *ARABIDOPSIS.*

We investigated whether different genes known to be associated with plant defense mechanisms (Hahlbrock and Scheel 1989; Kauffman *et al.* 1987; Legrand *et al.* 1987; Van Loon 1986) were also activated as a part of the *Arabidopsis* defense response to *X. campestris*. Northern analyses, 0, 12, 24, 48, and 96 hr after inoculation of *Arabidopsis* Col-O with *X. c.* pv. *campestris* 147, 8004, and 8420 and water were performed. The levels of transcripts of genes encoding phenylalanine ammonium lyase (PAL), a key enzyme in phytoalexin biosynthesis (Hahlbrock and Scheel 1989); two β-glucanases (BG1 and BG2), recently isolated from *A. thaliana* (Dong *et al.* 1991); a bean chitinase, whose nucleotide sequence shows 70% homology with an *Arabidopsis* chitinase nucleotide sequence (Broglie *et al.* 1986; Samac *et al.* 1990); and an ascorbic peroxidase (Kubo *et al.* 1991), were analyzed. The levels of PAL mRNA increased after inoculation with an incompatible strain of *X. c.* pv. *campestris*. The strongest PAL mRNA accumulation was noticed 48 hr after the incompatible interaction was established (Fig. 5). In contrast, PAL was very weakly induced by the compatible bacterial strain 8004. No PAL mRNA accumulation could be observed after infiltration with the bacterial mutant, *X. c.* pv. *campestris* 8420 or water. In parallel, mRNA corresponding to BG2 seemed also to be preferentially induced by the strain 147. The strain 8420 did not induce BG2 (neither did water), and we were unable to detect expression of BGl at any time (data not shown). These data agree with a number of reports indicating that these genes are induced in plants to a similar or higher level and much more rapidly by avirulent strains than by virulent strains (Dixon and Lamb 1990).

In contrast, the bean chitinase probe revealed a basal expression of the basic chitinase genes in *Arabidopsis,* under our experimental conditions (Fig. 5). However, after inoculation with strain 147, the level of chitinase transcripts strain reached a maximum at 48 hr (Fig. 5).

Similarly, the levels of ascorbic peroxidase transcripts were found to increase, both in *X. c.* pv. *campestris* 147 and 8004 infiltrated leaves (Fig. 5). It therefore appears that the chitinase and peroxidase gene families are activated by both virulent and avirulent bacterial strains, whereas the PAL and BG2 genes appear to be induced preferentially by avirulent strains. No accumulation of ascorbic peroxidase mRNA transcripts was seen after infiltration with *X. c.* pv. *campestris* 8420 or water.

DISCUSSION

Understanding of plant defense responses is still elementary, partly because, until recently, there was no appropriate model system amenable to molecular genetics and which had easily studied pathogens. Our results confirm that *A. thaliana* constitutes a useful model for analysis of a range of plant/pathogen interactions of many sorts (for a review, see Dangl 1992).

We have tested *A. thaliana* as a host for infection by a variety of strains of the cosmopolitan phytopathogen *X. campestris*. Interactions between *X. c.* pv. *campestris* isolates and *Arabidopsis* ecotypes generated different responses (incompatible, compatible, and null), suggesting a specific recognition of host and pathogen. After spray inoculation, we identified incompatible interactions which resulted in the appearance of an HR, classified as strong (+++ or ++++) when 75% to 100% of all the plants tested, showed necrotic lesions. In contrast, the compatible interactions were usually phenotypically characterized by the appearance at a later stage (several days after the inoculation) of a spreading chlorosis which gradually covered the whole infected leaf. However, up to 48 hr after spraying with a virulent strain, which subsequently leads to a compatible interaction, an HR classified as weak (+ or ++) when only less than 25% of the leaves per plant and less than 75% of all plants tested bear some scattered necrotic lesions—could be observed. The necrotic points that constituted this HR were quickly surrounded by extensive spreading chlorosis. Similar observations were made by Whalen *et al.* (1991), in a compatible interaction between *Arabidopsis* ecotype Col-O and a virulent *Pseudomonas syringae* strain. In addition, Boucher *et al.* (1985), described a virulent *P. solanacearum* strain inducing HR-like symptoms within 24 hr on tobacco; but in 50% of the cases these symptoms led to total wilting of the inoculated plants within 2 wk. Furthermore, we found that in a "delayed compatible" interaction, an HR classified as strong could be seen 24 hr after inoculation, although some days later it appeared that the initial resistant response was overruled and was followed by the appearance of phenotypical symptoms typical of a compatible interaction.

At least for the *X. c.* pv. *campestris-A. thaliana* interactions, different hypotheses could explain the various phenotypes observed. For example, they could reflect a genetically determined variability of host or pathogen which has already been observed in other extensively studied plant/pathogen models (Flor 1971; Clarke *et al.* 1987; Whalen *et al.* 1991; Debener *et al.* 1992); and/or

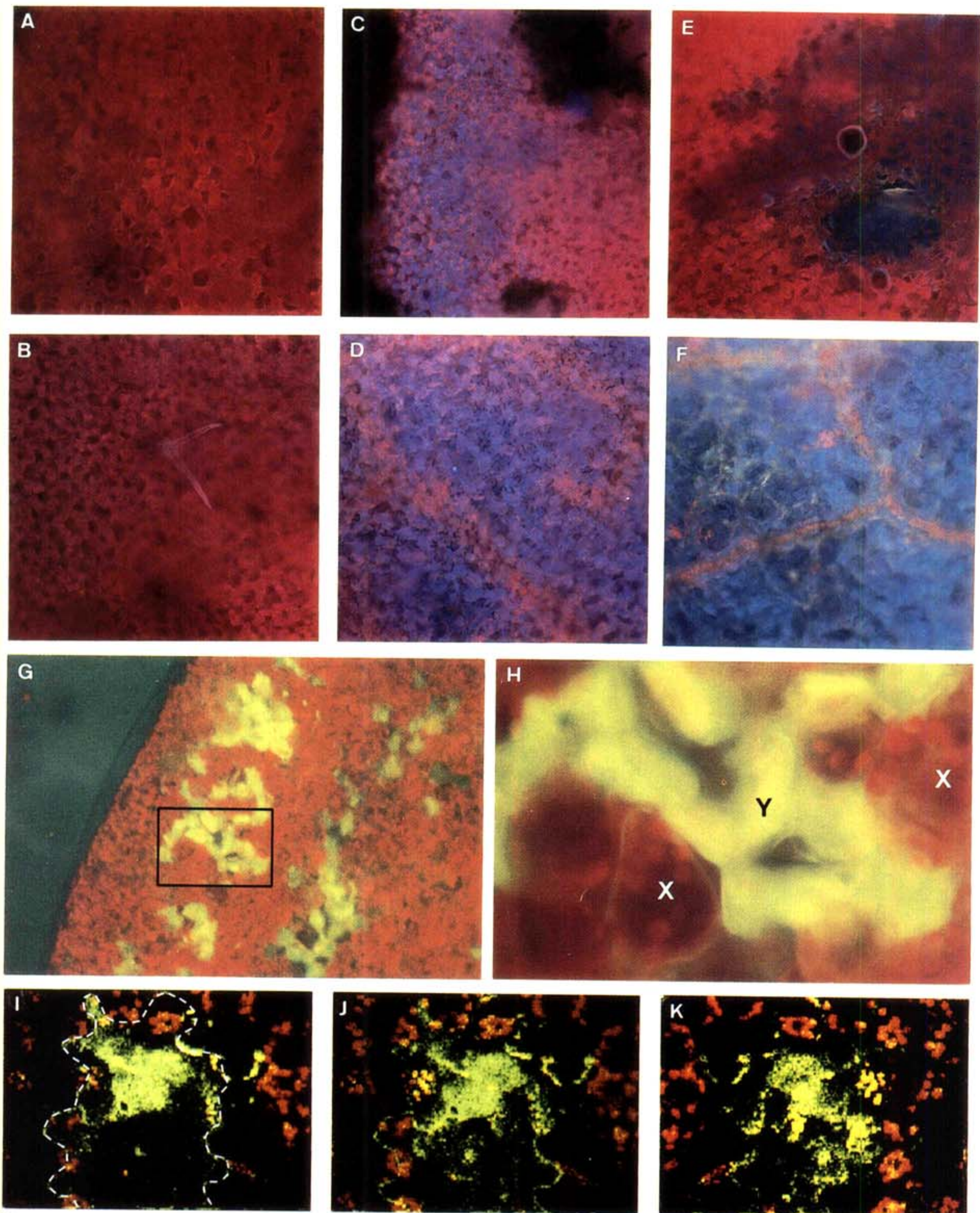

Fig. 4. Fluorescence microscopic analysis of *Arabidopsis thaliana* leaves inoculated with *X. c.* pv. *campestris*. Pictures were taken after infiltration inoculation (A–F) or spray inoculation (G—K) with *X. c.* pv. *campestris*, 40 hr after infiltration (A, C, E) and 72 hr after infiltration (B, D, F). Autofluorescence of areas of inoculated leaves was observed: Red corresponds to chlorophyll autofluorescence, and yellow-green or blue (depending on the filters used, as described in text) fluorescence corresponds to polyphenolic compounds. **A, B,** Infiltration with strain 8420. **C, D,** Infiltration with strain 147. **E, F,** Infiltration with strain 8004. **G,** Spray inoculation with strain 147: an area of dispersed small necrotic lesions. **H,** Detail of the area shown in G. Yellow-fluorescent compounds accumulated in and arround cell walls of living cells (X) neighboring dead collapsed cells emitting bright yellow-green fluorescence (Y). **I–K,** Section of 10 μm through one cellular necrotic lesion after spray inoculation with strain 147. Pictures taken by confocal microscope. Red dots, chlorophyl pigments; green dots, polyphenolic compounds; yellow dots, same point containing both chlorophyll and polyphenolics. Dotted line schematic drawing of the presumed border of the necrotic cell.

they could reflect the existence of several layers of responses required for resistance expression. The large phenotypic variability of lesion densities observed within the HR could be attributable to the qualitative differences in bacterial aggressiveness observed in *X. campestris* (Davis *et al.* 1991; this study). The null responses (no induction of necrotic lesions or chlorosis) exemplified by the interaction between *X. c.* pv. *campestris* 147 and *A. thaliana* ecotype Chi-O could only be observed after spray inoculation; after infiltration, however, Chi-O responded to the bacterial infection with a strong, but very localized chlorosis reminiscent of a "nonhost" resistance.

A definitive causal relationship between HR and resistance remains to be established. However, in most incompatible interactions, an HR is observed and a growing number of data are in favor of a positive correlation between HR and resistance (Klement 1982; Collinge *et al.* 1987; Keen *et al.* 1990; Davis *et al.* 1991; Debener *et al.* 1991).

To date, no *X. c.* pv. *campestris* strain had been isolated that gave a clear HR on any *A. thaliana* ecotype (Dangl 1992). We presume that this is because some or all of the conditions required to reproduce an HR are absent. To our understanding, these critical factors are; 1) preconditioning of the plants for 24 hr at 100% humidity. This increases stomatal opening and thus may facilitate bacterial entry and, in the case of spray inoculation, provides more potential penetration sites. 2) Preparation of the inoculum based on a bacterial smear (and not a liquid culture) (see Materials and Methods). 3) For spray inoculation, the density and the volume of bacterial suspension applied (10^8 cfu/ml, 1 ml per plant). In the *Arabidopsis*/*P. syringae* system, Whalen *et al.* (1991) were not able to detect an HR when inoculating *Arabidopsis* with virulent or avirulent strains of *P. syringae* at a bacterial concentration of 10^6 cfu/ml. As a result of inoculations of pepper with *X. c.* pv. *vesicatoria* or soybean with *P. s.* pv. *glycinea*, resistant plants only showed an HR when avirulent bacteria were inoculated at a density above 10^7 cfu/ml (Dahlbeck and Staskawicz, unpublished results). 4) The addition of Tween 20 (used at 0.05% required for spray inoculation) to the bacterial suspension. This surfactant improves the spreading of the bacterial droplets uniformly over the leaf surface and probably facilates the movement of bacteria through the stomata into the intercellular space. A similar strategy using another surfactant L-77 (at 0.01 or 0.02%), a silicon-based polymer, has been previously described by Whalen *et al.* (1991) and Bent *et al.* (1992), respectively. Toxicity of L-77 was visually apparent at 5–10 times the above-mentioned concentration, therefore, we opted for Tween 20 whose toxicity was apparent only at 50 times that concentration.

There are two particular features of the HR which we have characterized that are typical of all HRs so far described in plant/bacteria interactions: 1) the rapid appearance of dry, necrotic lesions, and 2) the restriction of bacterial growth in an incompatible interaction in comparison with a compatible interaction. Indeed a 100-fold difference in bacterial multiplication between compatible and incompatible interactions was observed 7 days after infiltration, although, until 3 days after inoculation, with both the susceptible ecotype Sf-2 and the resistant ecotype Col-O with *X. c.* pv. *campestris* 147, no important difference between the bacterial growth curves could be observed. However, between the different isolates tested on Col-O, earlier and more marked differences in symptom expression as well as in pathogen multiplication could be observed.

No other report concerning the *Arabidopsis*/*X. c.* pv. *campestris* system has so far found differences greater than

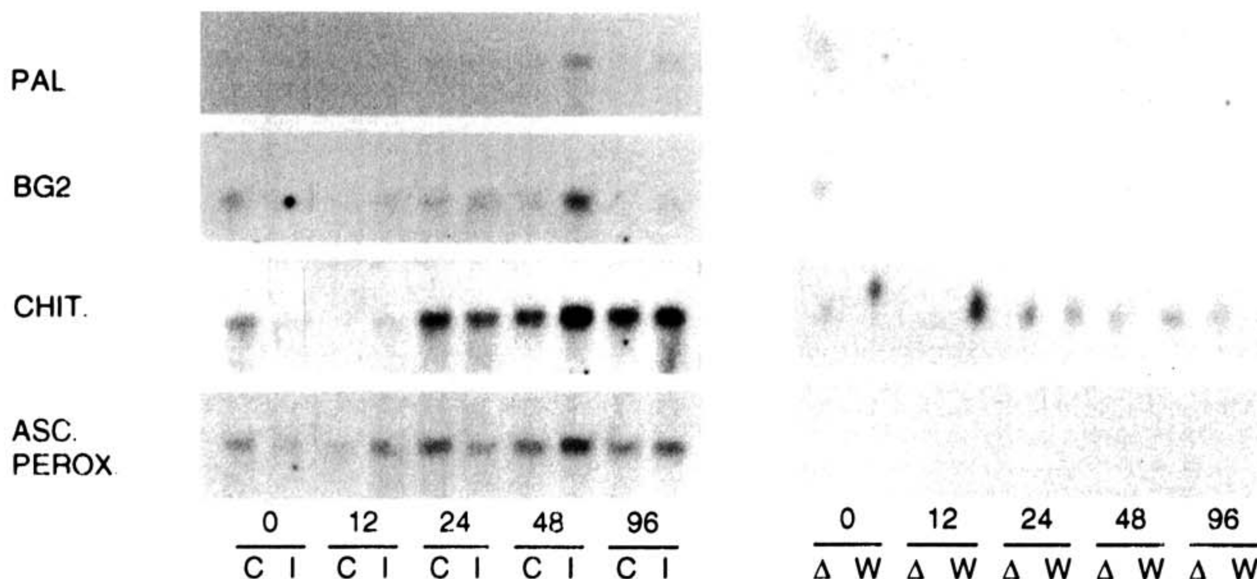

Fig. 5. Northern analysis of RNA isolated from *Arabidopsis thaliana* leaves at different times after infiltration with the incompatible strain (I) (strain 147), the compatible strain (C) (strain 8004), the *hrp*$^-$ bacterial mutant (Δ) (strain 8420) of *X. c.* pv. *campestris*, or water (W). Equal amounts of total RNA (20 μg) were separated on 1% agarose gel. Northern blot hybridization methods and specific gene probes were as described in text.

those presented here between growth *in planta* of virulent and avirulent bacteria (Dangl 1992). In contrast, a 10^3- to 10^5-fold difference is observed in the *Pseudomonas/Arabidopsis* system (Debener *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991) thus growth of incompatible *X. campestris* strains on *Arabidopsis* seems to be restricted less efficiently than that of incompatible *Pseudomonas* strains. In addition, 1 wk after the establishment of both incompatible and null interactions, we found a residual bacterial level of 10^6 to 10^7 cfu/g leaf tissue in Col-O and Chi-O leaves, which correlates with the observations made by scanning electron microscopy. Other groups have also reported that Col-O supports reasonably high bacterial growth levels *in planta* (Tsuji *et al.* 1991; Debener *et al.* 1992).

Microscopic analyses at the cellular level confirm the observed phenotype and the *in planta* bacterial growth curves of the different interactions. We observed, under the scanning electron microscope, the proliferation on the leaf surface of *X. c.* pv. *campestris* 147 covering the whole necrotic area corresponding to the HR. This dense bacterial population remained several days after the formation of necrotic lesions, raising the question of the epiphytic growth of *X. c.* pv. *campestris*, and, in general, of all phytopathogenic bacteria. Very little is known about this phase of growth compared with the pathogenic growth mode. Further proliferation of the incompatible strain was, however, inhibited after the third day.

As described for other plant species, the observed fluorescence in living cells close to the inoculation site probably indicates the biosynthesis and accumulation of phenolic compounds, including phytoalexins that are compounds commonly produced during the defense response of the plant (Holliday *et al.* 1981; Jahnen and Hahlbrock 1988). No temporal correlation could be found between the fluorescence observed and PAL mRNA accumulation after infiltration with the compatible or the incompatible strains. This suggests that PAL mRNA accumulation might be a consequence of the HR and might be responsible for only a small part of the fluorescence observed. Deposition of callose plugs, which is a common HR-associated response was observed exclusively in the immediate vicinity of the necrotic lesions, another indication that what we have reported is a typical HR.

The onset of activation of four plant genes (PAL, β-glucanase, chitinase, and peroxidase) known to play roles in plant defense against pathogenic organisms (Davis *et al.* 1991; Kauffman *et al.* 1987; Legrand *et al.* 1987; Van Loon 1986) was determined by Northern blot analysis. Of these, both PAL and BG2 genes are preferentially activated during the incompatible interaction, 48 hr after inoculation. This is comparatively late for an HR; several possible explanations can be formulated concerning this delay. PAL and BG2 gene expression could respond to a bacterial signal induced by the plant or to a signal generated by substantial bacterial growth (which at day 2 was 10 times higher in strain 147 than in strain 8004).

Besides the basal expression of the chitinase under our experimental conditions, we observed in plants infected by virulent and avirulent strains an activation of this gene which is both stronger and earlier than the one observed for BG2 and which resembles the induction profile obtained with the gene encoding ascorbic peroxidase.

The levels of peroxidase expression have been shown in several plant systems to be altered by stress and infection (Bruce and West 1989), with a set of peroxidases that are induced during plant defense responses and a different set that is induced during senescence or aging (Farkas and Stahmann 1966; Solymosy *et al.* 1967; Van Loon and Geelen 1971). Moreover, peroxidase activation has been demonstrated to occur earlier and more markedly in resistance reactions that involve tissue necrosis than in compatible interactions (Van Loon 1986). In our experiments, ascorbate peroxidase transcripts were found to accumulate early (12 hr) during the compatible as well as the incompatible interaction, although the highest induction was observed at 48 hr after inoculation with the avirulent strain. No induction could be detected after infiltration with water or the bacterial *hrp*$^-$ mutant strain. This seems to indicate that the described ascorbate peroxidase induction is not stress related, in contrast to some other tested peroxidase isozymes that were strongly induced after infiltration with water (data not shown).

In conclusion, all four genes analyzed exhibit the highest level of activation 48 hr after the establishment of the incompatible interaction. However, it must be emphasized that these general defense genes are not truly specific for incompatible interactions, since their activation usually differs only in terms of kinetics and magnitude during compatible/incompatible interactions. This emphasizes the need for genes that are induced earlier than the known defense genes and whose activation is specific for the HR; such genes could be used as efficient genetic markers for the differentiation of incompatible/compatible interactions.

Although no genetic basis is presented for the establishment of an HR (e. g., involvement of bacterial avirulence genes), our data clearly provide evidence for the establishment of an HR in *Arabidopsis* when infected by *X. c.* pv. *campestris*. These results were partly obtained using a mass spray inoculation method. This inoculation technique is a very suitable screening protocol, because it is applicable for large numbers of plants by eliminating the need for time-consuming hand inoculation of individual plant leaves and by being relatively easy. Spray inoculation mimics the normal infection process, including normal recognition and/or exclusion of the pathogen, while infiltration is more applicable for measuring bacterial growth *in planta* and monitoring development of the interactions at later stages. When evaluating the sensitivity of 27 *A. thaliana* ecotypes to *X. c.* pv. *campestris* 147 or the ability of 28 *X. c.* pv. *campestris* isolates to induce an HR on ecotype Col-O, we were able to distinguish at least four different HR intensities, indicating the more subtle nature of the interaction established after spray inoculation compared to infiltration inoculation. Therefore, spray inoculation together with the more routinely used infiltration inoculation can be recommended as complementary tools for exhaustive plant-pathogen interaction studies.

Having established the conditions needed to induce an HR, we evaluated the sensitivity of *A. thaliana* to *X. c.* pv. *campestris* infection at different stages of plant devel-

opment. Using spray inoculation, a reliable HR could best be obtained once the plants developed fully grown rosettes (data not shown). Necrotic lesions also appeared on stem leaves and seed pods of senescent plants, which could aid in large-scale screening of mutated Col-O plants. The possibility of screening at this stage offers the ideal way of rescuing seeds from those plants where the appearance of the HR is blocked as a consequence of the mutation. The techniques we have presented in this paper and that we have used to characterize the HR in *A. thaliana* at the phenotypical, microscopical, and molecular levels will be used as tools for further characterization of such mutants.

MATERIAL AND METHODS

Plant growth conditions.

A. thaliana ecotype Col-O seeds were provided by the Lehle Seeds company (Tucson, AZ). Seeds from the ecotypes Rsch-O, Be-O, Zü-O, Ts-1, Ll-O, and Chi-O were a kind gift of M. Jacobs from the Vrije Universiteit Brussel (Brussels, Belgium). Accessions C 24, La-er, and Ws were a gift from M. Van Lijsebettens from the Universiteit Gent (Gent, Belgium). All other ecotypes (Bu-2, Bur-O, Edi-O, Ha-O, Hs-O, Ll-O, Mt-O, Nd-O, No-O, Ob-O, Oy-O, Pn-o, Pr-O, Sei-O, Sf-2, Uk-2, and Uk-4) were obtained from the *Arabidopsis* Information Service Seed Bank (F. Kranz, Botanical Institute, J. W. Goethe-Universität, Frankfurt, Germany).

Seeds were wet-vernalized for 4 days, and grown in culture chambers with a 12-hr photoperiod, 16°-23° C dark to light temperature shift, and about 75% humidity. For the evaluation of Col-O/*X. c.* pv. *campestris* isolate interactions, Col-O seeds were sown at a density of 1 plant/2 cm^2 in 44- × 40- × 8-cm plastic trays containing 3:2 mix of potting and sandy soil. For all the other experiments, seeds were sown at a density of one plant per 25 cm^2 in multipot flats. After sowing, the trays were covered with plastic film for 4 days to prevent desiccation. Six-week-old plants (at the flowering stage) were used for all experiments, unless otherwise stated.

Bacterial strains.

The names and sources of the different bacterial strains tested are mentioned in Table 1. *X. c.* pv. *campestris* 8004 (Spc^r) (Turner *et al.* 1984), as well as the bacterial mutant 8420 (Kan^r), which is a derivative of 8004 and has been deleted for the *hrp* gene cluster, were a kind gift of M. Daniels (John Innes Institute, Norwich, UK). All the other *X. c.* pv. *campestris* isolates (568, 573, 581, 583, 584, 4761, 7516, 7662, 8002, 8003, 8007, 8034, 8035, 8050, 8088, 8090, 8096, 8100, 8101, 8102, 8104, 8106, 8109, 8114, 8116, and 8238) came from the LMB collection at the Laboratory of Microbiology (Universiteit Gent, Belgium).

All strains and isolates were routinely cultivated on Kado medium (Kado and Heskett 1970) at 28° C. Spontaneous antibiotic-resistant mutants were selected using a gradient plate technique (Szybalski 1952).

The concentrations of antibiotics in the media were: 100 μg/ml rifampicin (Rif); 20 μg/ml streptomycin (Str); 50 μg/ml spectinomycin (Spc); 50 μg/ml nalidixic acid (Nal), and 100 μg/ml carbenicillin (Cb).

Inoculation procedures.

Plants were kept for 24 hr at 100% humidity in a moist chamber prior to inoculation. Overnight liquid cultures of bacteria derived from a single colony were plated on solid medium and cultivated for 72 hr at 28° C. For spray inoculation, the smear of bacteria covering the surface of four plates was removed with a sterile spatula and resuspended in 100 ml of 0.05% Tween 20. A bacterial suspension of 10^8 colony-forming units (cfu) per milliliter was used as an inoculum. One hundred milliliters of bacterial suspension was applied to each tray of plants with a hand pump spray bottle. For infiltration inoculation, the same bacterial suspension used for the spray inoculation was diluted with sterile water to 10^6 cfu/ml and was infiltrated, through the stomata, into the intercellular spaces of fully expanded leaves of plants grown on multi pot flats. This was done by pressing the blunt end of a disposable plastic 1-ml syringe to one side of each leaf undersurface and applying pressure to the plunger (Collinge *et al.* 1987). Approximately 10 μl of the bacterial suspension was applied per site. Excess inoculum was blotted from the surface of the leaves. The infiltrated bacterial suspension formed small water-soaked areas extending outside the area covered by the syringe. They disappeared after approximately 1 hr; afterward the leaves appeared normal. The plants were kept at high humidity until the end of the experiment. Spray inoculation of *X. c.* pv. *campestris* and other bacteria was repeated three to five times with approximately 100 plants per strain or isolate. Infiltration inoculation was repeated at least twice, with a minimum of 20 leaves per experiment. Spray inoculation of different ecotypes was repeated two to three times with at least 50 plants. The ability to induce an HR was determined after 24 hr with the Col-O/*X. c.* pv. *campestris* 147 Spc^r interaction and after 2 days for the other ecotype/isolate interactions.

Col-O fully grown leaves were collected for Northern analysis 0, 12, 24, 48, and 96 hr after infiltration with *X. c.* pv. *campestris* and stored at −70° C until use.

Bacterial growth curve *in planta.*

Bacterial growth in spray-inoculated *A. thaliana* leaves showing a compatible phenotype is difficult to measure because spraying is never homogenous and no clear symptoms can be observed until 2 days after inoculation. Moreover, *X. c.* pv. *campestris* is not able to colonize *Arabidopsis* plants through the vascular system (Daniels *et al.* 1991) and is restricted even in a compatible interaction to the initially inoculated leaves, which in the case of spray inoculation is arbitrary. For these reasons, harvesting inoculated leaves at early stages after spray inoculation can give very unreliable results. In contrast, after infiltration, bacterial growth in susceptible leaves can easily be followed. The bacterial growth *in planta* was measured at 0, 1, 2, 3, 5, and 7 days after bacterial infiltration. The leaves of ecotype Col-O were rinsed three times in distilled water and blotted dry. They were then macerated in 1 ml of liquid bacterial growth medium, ground in a small mortar and the cfu/ml were counted by plating the serial dilutions of the extracts onto solid bacterial growth medium. Three representative rosette leaves from six

separate plants were harvested twice for each time point and each experiment was repeated three times. The bacterial concentrations *in planta* were calculated per gram of fresh leaf tissue.

Bright-field light microscopy.

Intact and sectioned leaves were examined on a Zeiss Universal microscope. Color photographs were taken with Ektachrome 100 ASA day light film (Kodak, Rochester, NY). Black and white photographs were taken with Ilford FF P4 125 ASA film.

Fluorescence microscopy.

Control and infected intact leaves were examined after spray inoculation by epifluorescent illumination on a Zeiss Universal microscope with a HB0200 mercury lamp (excitation wavelengths 450-490 nm) and FR510/LP520 filters and after infiltration inoculation by an Olympus Microscope with a HBO200 Mercury lamp (excitation wavelenghts 450-490 nm), and LBD-2N/KB-4 filters. Color photographs were taken with Ektachrome 100 ASA daylight film (Kodak, Rochester, NY).

Callose detection.

Transverse sections of infected and control leaves were prepared using a freezing microtome. After staining for 30 min with 0.03 mg/ml sirofluor (aniline blue fluorochrome, Biosupplies, Victoria, Australia) at room temperature, the sections were washed several times with distilled water and blotted dry. Samples were examined by bright-field and fluorescence microscopy. The bright spots seen in and around the necrotic area are due to the presence of an intensive fluorescent complex formed by the applied fluorochrome sirofluor, a purified aniline blue, and β-1,3-glucan, the major component of callose. The small gray dots are due to the weaker binding of sirofluor to starch present in the chloroplasts.

Confocal microscopy.

Images from unprocessed infected and control leaves were recorded with a Bio-Rad MRC-500 Confocal scanning laser microscope (CSLM) system. The CSLM was fitted to a Leitz Orthoplan microscope. Excitation and dual wavelength detection were performed with the A1/A2 filter block combinations. The A1 filter combination consists of a 514 DF 10 filter for excitation with the 514-nm line of an Ar^{+}-ion laser (used at approximately 23% of maximum intensity) and a DR 527 LP dichroic reflector. The A2 filter combination consists of a DR 565 LP dichroic reflector for the separation of the red propidium iodide, and the green FITC fluorescence light, and two barrier filters, an EF 600 LP filter (red channel) and a 540 DF 30 filter (green channel). An Olympus 60x, NA 1.40, oil immersion lens (Olympus, Japan) was used.

Digital-image processing was performed by the software provided with the CSLM system. Color photographs were taken from a NEC multisync II video screen (NEC, Japan) with Ektachrome 64 daylight film (Kodak, Rochester, NY).

Scanning electron microscopy.

Leaves from normal and infected plants were fixed for 2 hr in a solution containing 4.0% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) or 1.25% Pipes buffer (pH 7.0), washed in the same buffer and postfixed for 1 hr in 1.0% osmium tetroxide in the same buffer. Samples were critical point dried in carbon dioxide after dehydration in acetone and fragments were positioned on a stub prior to gold sputtering in a Balzer's apparatus, where a coat of 20 nm thickness was deposited. Micrographs were taken with a JEOL 25-S-II SEM.

RNA isolation and hybridization.

RNA was isolated in small quantities according to Verwoerd *et al.* (1985). Northern blots were made from total RNA denatured with glyoxal and dimethyl sulfoxide as previously described (McMaster and Carmichael 1977), fractionated by agarose gel electrophoresis, and transferred to Hybond N membranes (Amersham). (^{32}P)-labeled probes were synthesized using random-primer labeling. Hybridization of probes to RNA blots was performed in 50% formamide, 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA, 5× SSC buffer, and 5× Denhardt's solution. The filters were prehybridized for at least 1 hr and hybridized overnight at 42° C (or 37° C for heterologous probes). The RNA blots were washed twice for 15 min with each of the following solutions: 2× SSC, 0.1% SDS, at room temperature and twice for 20 min with 0.1× SSC, 0.1% SDS at 50° C. Subsequently, the filters were blotted and autoradiographed with XAR-films (Kodak) using intensifying screens.

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