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Incompatible Interactions Between Crucifers and *Xanthomonas campestris* Involve a Vascular Hypersensitive Response: Role of the *hrpX* Locus

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The *hrpXc* locus of *X. campestris* pv. *campestris* is required for pathogenicity on crucifers and for hypersensitivity on nonhost plants (S. Kamoun and C. I. Kado, J. Bacteriol. 172:5165-5172, 1990). Southern blot hybridizations of total DNA with a 334-bp *Pst*I probe internal to *hrpXc* was conducted on 14 independent *X. campestris* isolates from crucifers representing *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae*. All strains contained sequences homologous to *hrpXc* located in an 11-kb *Eco*RI fragment. Electroporation of integrative plasmids, that carry small insert fragments internal to the *hrpXc* transcriptional unit, allowed the construction of HrpX mutants in 10 strains representing *X. c.* pv. *campestris* (four races, eight strains) and *X. c.* pv. *armoraciae* (two strains). All mutants were unable to induce disease symptoms on crucifers or cause a hypersensitive response on nonhosts. Chromosomal transcriptional fusions of *hrpXc* to *gusA* (β-glucuronidase) and *cat* (chloramphenicol acetyltransferase) reporter genes were also constructed by marker-integration and used to confirm that *hrpXc* is expressed in *X. c.* pv. *campestris* only in plants. The response of crucifer plants to incompatible inoculations with *X. campestris* was also closely examined histochemically, and a localized and rapid vascular browning reminiscent of a hypersensitive response was found to correlate with incompatibility. Interestingly, all HrpX mutants induced this *v*ascular *h*ypersensitive *r*esponse (VHR), suggesting that the plant response to these *hrp* mutants is not null. Coinoculation of HrpXc mutants with the wild-type parental strains suppressed *in planta* growth defection and the vascular browning response, and rescued HrpXc mutants. Our observations lead us to propose the hypothesis that *hrpXc* may function in suppression of defense response(s) in the compatible host plant.

Additional keywords: black rot and bacterial leaf spots of crucifers, hypersensitive reaction, conserved pathogenicity genes, directed mutagenesis, *in planta* histochemical staining of *Xanthomonas*.

The genus *Xanthomonas* consists mainly of plant pathogenic bacteria that occur worldwide and cause a variety of diseases on diverse plants (Bradbury 1984; Leyns *et al.* 1984; Van den Mooter and Swings 1990). Members of this genus cause necrotic lesions on leaves, stems, or fruits (spots, streaks, and cankers), wilts (vascular), tissue maceration (rots), and hyperplasias (such as in citrus canker) on at least 124 monocotyledonous and 268 dicotyledonous plant species, including important crops such as rice, beans, manioc, cotton, crucifers, tomatoes, and citrus (Leyns *et al.* 1984). *Xanthomonas campestris* is by far the most complex species of the genus, and has been divided into 143 pathovars, i.e., groups of strains that are believed to be specific for certain host plants or diseases (Bradbury 1984; Dye *et al.* 1980; Vauterin *et al.* 1990).

X. c. pv. *campestris* causes the black rot disease of crucifers, a worldwide and economically important disease (Williams 1980). Natural infections begin with the bacteria entering the plant mainly at the leaf margin through the hydathodes, while secondary entry sites can develop in the roots or wounds (Cook *et al.* 1952; Shaw and Kado 1988b). These openings provide *X. c.* pv. *campestris* access to the vascular system, where it is usually confined to the xylem (Bretschneider *et al.* 1989). Subsequently, the bacteria multiply and eventually cause the distinct blackening of leaf veins, followed by the drying and death of panels of leaf tissue typical of the black rot disease.

X. c. pv. *armoraciae* causes the bacterial leaf spot disease of crucifers (McCulloch 1929; Moffett *et al.* 1976; White 1930). Disease lesions first appear on the undersurface of the leaf as small water-soaked spots which soon become apparent on both surfaces as necrotic spots with a distinct margin. Later, lesions develop into large necrotic areas limited by the large veins. *X. c.* pv. *armoraciae* is also able to penetrate the veinlets around the hydathodes where it induces a necrotic response with no subsequent invasion of the vascular system (Hunter *et al.* 1987; McCulloch 1929; Moffett *et al.* 1976). Therefore, in contrast to *X. c.* pv. *campestris*, *X. c.* pv. *armoraciae* is not a vascular but rather is a mesophyllic pathogen of crucifers (McCulloch 1929; White 1930). For this specific reason, the interaction between *X. c.* pv. *armoraciae* and the vascular tissue of crucifers is referred to in this paper as a tissue-specific incompatible interaction.

The plant-inducible *hrpXc* gene of *X. c.* pv. *campestris* is required for pathogenicity on crucifers as well as hypersensitivity on nonhost plants (Kamoun and Kado 1990a). HrpXc mutants of *X. c.* pv. *campestris* are growth impaired *in planta*, and can be complemented by coinoculation with wild-type strains, suggesting that this gene may encode for an exocellular component (Kamoun and Kado 1990a). Even though the function of most *hrp* genes, including *hrpXc*, is still unknown, it has been suggested

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Table 1. Bacterial strains, plasmids, and plant cultivars

Strain, plasmid or cultivar	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*), [F' *traD36, proAB, laclqZ*ΔM15]	Stratagene[b]
XL1-Blue	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac,* [F' *proAB, laclqZ*ΔM15, Tn*10* (tetr)]	Stratagene[b]
X. campestris pv. *campestris*		
2D541	Wild type, race 0	This study
SH9	2D541::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
SH11	2D541::pUCD2571, *hrpXc,* Ap^r	This study
SH61	2D541::pUCD3161, *hrpXc,* Km(Nm)^r	This study
2D543	Wild type, race 0	This study
SO9	2D543::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
2D540R	Wild type, race 1, Rm^r	Kamoun and Kado 1990a
SW9	2D540R::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
SW11	2D540R::pUCD2571, *hrpXc,* Ap^r	This study
SW61	2D540R::pUCD3161, *hrpXc,* Km(Nm)^r	This study
HasibH-MR	Wild type, race 1, Rm^r	Hunter *et al.* 1987
HM9	HasibH-MR::pUCD4109, *hrpXc,* Km(Nm), Ap^r	This study
HM11	HasibH-MR::pUCD2571, *hrpXc,* Ap^r	This study
HM61	HasibH-MR::pUCD3161, *hrpXc,* Km(Nm)^r	This study
NCPPB528R	Wild type, race 1, Rm^r derivative of type strain	This study
T61	NCPPB528R::pUCD3161, *hrpXc,* Km(Nm)^r	This study
2D518	Wild type, race 1, Rm^r	This study
SN9	2D518::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
2D520	Wild type, race 2, Rm^r	Shaw *et al.* 1988a
JS9	2D520::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
JS61	2D520::pUCD3161, *hrpXc,* Km(Nm)^r	This study
JS111	2D520::Tn*4431*, *hrpXc,* Tc^r	Shaw *et al.* 1988a
2D513R	Wild type, race 3, Rm^r	This study
SM9	2D513R::pUCD4109, *hrpXc,* Km(Nm)^r, Ap^r	This study
EEXC114R	Wild type, race 4, Rm^r	Hunter *et al.* 1987
171C	Wild type, race 4, Cm^r	Anne Alvarez
X. c. pv. *armoraciae*		
XLS10R	Wild type, Rm^r	This paper
XL9	XLS10R::pUCD4109, *hrpXa,* Km(Nm)^r, Ap^r	This paper
XL61	XLS10R::pUCD3161, *hrpXa,* Km(Nm)^r	This paper
756C	Wild type, Cm^r	Kamoun and Kado 1990b
HW9	756C::pUCD4109, *hrpXa,* Km(Nm)^r, Ap^r	This paper
417	Wild type	Hunter *et al.* 1987
A342R	Wild type, Rm^r	Hunter *et al.* 1987
Plasmids		
pTZ18R, pTZ19R	*ori*pUC, Ap^r	Mead *et al.* 1985
pUCD2550	pTZ18R::*hrpXc* 11.0-kb *Eco*RI fragment	Kamoun and Kado 1990a
pUCD2569	pTZ19R::pUCD2550 1.5-kb. *Eco*RI-*Nhe*I fragment	This study
pUC118	*ori*pUC, Ap^r	Vieira and Messing 1987
pUCD4100	pUC118::pUCD2569 0.34-kb *Pst*I fragment	This study
pUCD1738	pUC118::*gusA*	Koji Okumura
pUCD2571	pUCD4100::pUCD1738 1.8-kb. *Sal*I *gusA* fragment	This study
pBGS18	*ori*pUC, Km(Nm)^r	Spratt *et al.* 1986
pUCD3161	pBGS18::pUCD2571 2.1-kb *Eco*RI-*Hind*III fragment	This study
pCMKM4	*ori*pUC, *ctn* cassette	Close and Rodriguez 1982
pUCD4109	pUCD4100::pCMKM4 2.1-kb *Bam*HI *ctn* cassette	This study
Cultivars		
Brassica oleracea		
Early Jersey Wakefield cabbage (EJW)		Northrup King Co.[c]
Early Super Snowball cauliflower		Park Seed Co.[d]
Brassica campestris		
Purple Top White Globe turnip		Burpee & Co.[e]
Just Right turnip (TJR)		American Takii Co.[f]
Tokyo Turnip Hybrid (TTH)		Burpee & Co.[e]
Seven Top Green turnip (TST)		Northrup King Co.[c]
Brassica juncea		
Florida Broadleaf India Mustard (MFB)		Northrup King Co.[c]
Raphanus sativus		
White Icicle radish		Burpee & Co.[e]
Capsicum frutescens		
Pepper California Wonder (CW)		Burpee & Co.[e]
Datura stramonium		U.C. Davis cultivation

[a]Km(Nm) designated kanamycin resistance gene (acetylphosphotransferase gene) that also encodes resistance to neomycin; Rm, Cm, and Ap designate rifampin, chloramphenicol, and ampicillin resistance genes, respectively; *ori* and a plasmid name indicate the source of the origin of replication.
[b]La Jolla, CA.
[c]Minneapolis, MN.
[d]Greenwood, SC.
[e]Warminster, PA.
[f]Salinas, CA.

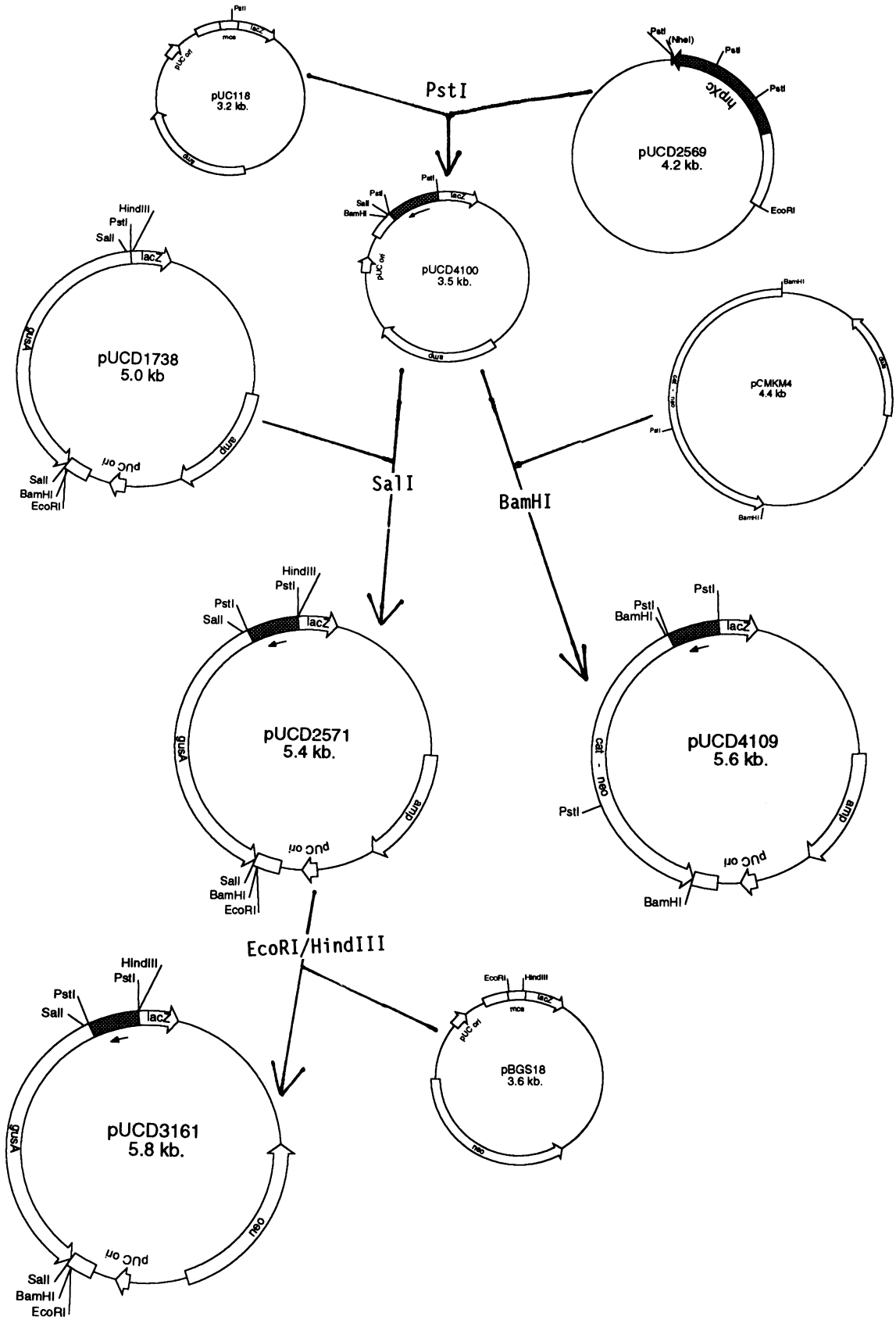

Fig. 1. Construction of integrative plasmids. The details of each cloning step are given in the text. All plasmids are drawn to scale. mcs refers to the multiple cloning site of pUC118. amp refers to the gene encoding for β-lactamase that confers ampicillin-resistance. neo refers to the gene encoding for aminoglucosidephosphotransferase that confers kanamycin and neomycin-resistance.

that these genes correspond to basic compatibility genes responsible for the primary interaction between the bacterium and the plant (Ellingboe 1982; Lindgren *et al.* 1988).

In this paper, we demonstrate that homologous alleles of *X. c.* pv. *campestris* 2D520 *hrpXc* locus are present in all examined *X. campestris* crucifer strains. HrpX mutants were constructed by marker-integration mutagenesis in eight strains of *X. c.* pv. *campestris* and two strains of *X. c.* pv. *armoraciae*. *In planta* histochemical GUS assays of *X. campestris* strains bearing *hrpXc* transcriptional fusions to *gusA* (β-glucuronidase) were conducted. A close examination of the response of crucifer plants to inoculations with incompatible *X. campestris* strains indicated that a localized and rapid vascular browning plant response correlates with incompatibility. Interestingly, both HrpXc and HrpXa mutants induced this vascular hypersensitive response, suggesting that crucifer response to HrpX mutants is not null. Coinoculation of HrpXc with the wild type led to the suppression of the response and the rescue of the HrpXc mutants, suggesting that *hrpXc* may encode a function that suppresses plant defense mechanisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. Spontaneous rifampin- or chloramphenicol-resistant derivatives were constructed as previously described (Shaw and Kado 1986) and were indistinguishable from the wild-type isolates in all other aspects.

Plant cultivars. The plant cultivars used and their commercial sources are listed in Table 1.

Media and antibiotics. *Escherichia coli* was routinely grown in LB medium or on LB 1.5% agar plates (Miller 1972) at 37° C; *X. campestris* strains were grown in medium 523 broth or on 523 1.5% agar plates or on minimal medium 925 1.5% agar plates (Kado *et al.* 1972). The antibiotics used for *E. coli* were kanamycin (20 μg/ml) and ampicillin (100 μg/ml); the antibiotics used for *X. campestris* were rifampin (50 μg/ml), chloramphenicol (30 μg/ml), ampicillin (100 μg/ml), kanamycin (30 μg/ml), and neomycin (30 μg/ml).

Enzymes and chemicals. Enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), Du Pont Co. (Wilmington, DE), or Stratagene (La Jolla, CA) and used as recommended by the manufacturer. X-Gluc was purchased from Clonetech (Palo Alto, CA), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

DNA manipulations. DNA manipulations were performed as described elsewhere (Ausubel *et al.* 1987; Sambrook *et al.* 1989). DNA fragments were isolated from agarose gels, using a Geneclean kit (Bio101, La Jolla, CA). Total genomic DNA was isolated from liquid-grown cultures using the CTAB method (Ausubel *et al.* 1987). Alkaline DNA transfer to Nytran Hybond N+ (Amersham,

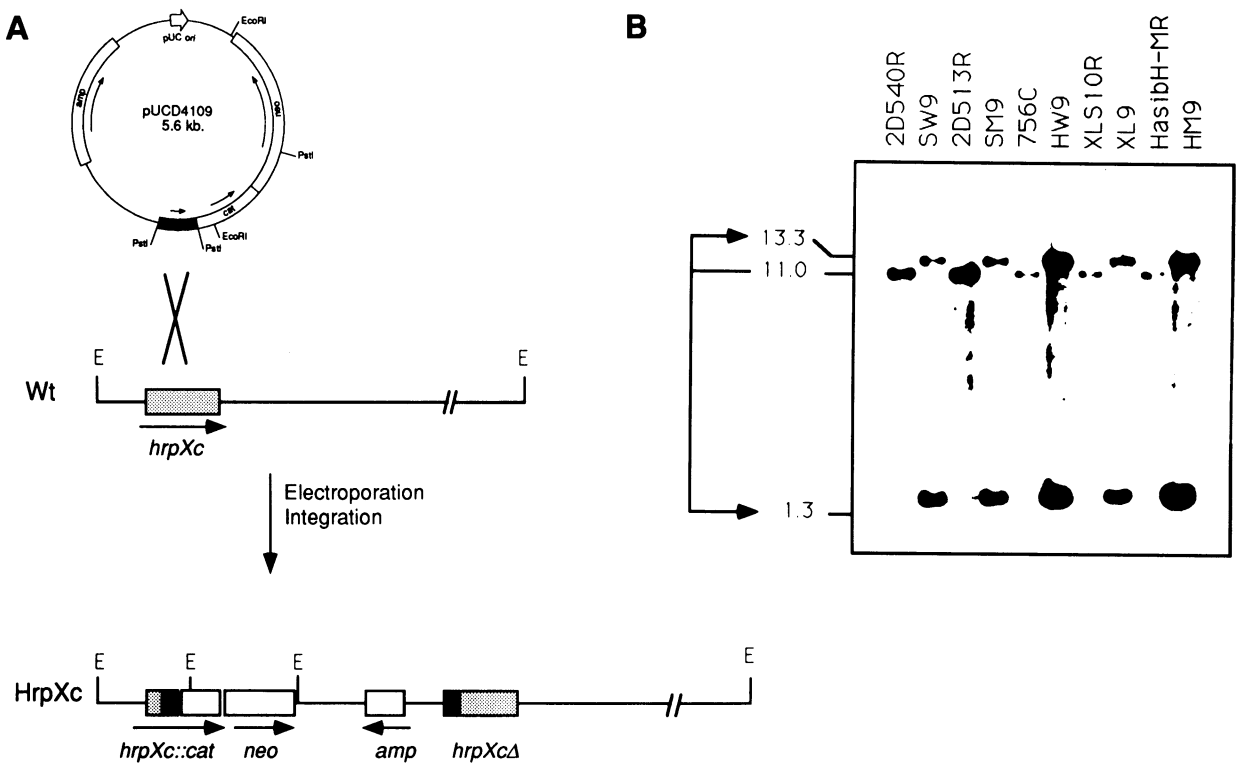

Fig. 2. A, Schematic representation of the integration of pUCD4109 into *Xanthomonas campestris* pv. *campestris hrpXc* locus. As shown, the resulting marker-integration mutant does not contain a functional complete copy of *hrpXc* but contains a *hrpXc::cat* fusion. *Eco*RI fragments size: Wild-type (11-kb) and HrpXc (1.3, 1.8, and 13.3 kb). **B,** Southern blot of wild-type and pUCD4109 integrative mutants of *X. campestris*. *Eco*RI digested total DNA of 2D540R, SW9, 2D513R, SM9 (*X. c.* pv. *campestris*), 756C, HW9, XLS10R, XL9 (*X. c.* pv. *armoraciae*), HasibH-MR, and HM9 (*X. c.* pv. *campestris*) were probed at high stringency with the 0.34-kb *Pst*I fragment of pUCD4109. Sizes are in kilobase.

Arlington Heights, IL) was performed as described elsewhere (Ausubel *et al.* 1987). Hybridizations were performed at 65° C in a PR800 hybridization chamber (Hoefer Scientific Instruments, San Fransisco, CA) as recommended by the manufacturer.

Electroporation. Electroporation of *E. coli* and *X. c.* pv. *campestris* was performed essentially as we described (Kamoun and Kado 1990a), except that the cells were grown in liquid media to an OD_{600} of 1.2, and washed twice with an ice-cold 10% glycerol solution. Electroporation was performed at 2.5 kV cm^{-1} and 200 ohms, using a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA). The cuvettes used for electroporation were routinely sterilized and reused as described elsewhere (Cooley *et al.* 1991).

Enzymatic assays. Assays for chloramphenicol acetyltransferase (CAT), and β-glucuronidase (GUS) were conducted as described elsewhere (Jefferson *et al.* 1986; Kamoun *et al.* 1989).

Plant inoculations. Plant pathogenicity and hypersensitivity assays of *X. c.* pv. *campestris* were conducted as previously described (Kamoun and Kado 1990a; Shaw and Kado 1988b) using the plants listed in Table 1. To obtain the virulence curves, plants at the six-leaf stage were needle inoculated on leaves three, four, and five, and the total number of leaves with black rot was scored daily for 20 days following the inoculation. For each combination, a minimum of four plants (12 leaves) was used, and virulence was estimated as the percentage of inoculated leaves that showed disease symptoms. Inoculations on each host were performed simultaneously for all strains to minimize environmental effects. Incompatible interactions represent combinations that failed to show black rot symptoms even after repeated inoculations. We define compatible or susceptible interactions as a full expression of the black rot disease and not simply the water-soaking and tissue collapsing symptoms observed when two-leaf seedlings and detached leaves are infiltrated (Shaw and Kado 1988b). Assays for bacterial leaf spot of crucifers were conducted by spraying to wetness crucifer plants (six-leaf stage) with a distilled water solution containing 10^9 cfu of *X. c.* pv. *armoraciae* per milliliter.

Seedling assay. A rapid seedling assay was developed to test for the vascular response of crucifers to incompatible *X. campestris* inoculations. Turnip seedlings were grown in soil, in 5- × 5- × 5-cm pots, under continuous light, at 29° C. Seven- to 10-day-old seedlings were inoculated by pricking the hypocotyl with a 25-gauge syringe needle that was dipped into a bacterial colony. The vascular response was usually visible about 16 hr after inoculation as a necrotic lesion approximately 5 mm on the hypocotyl around the point of inoculation, and usually results in the collapse of the seedling. Compatible interactions resulted in no response at the inoculation site, but disease symptoms (black rot) usually start appearing on the leaves as early as 2 days after inoculation. Coinoculation experiments were conducted with 1:1 mixtures of the two strains.

***In planta* growth.** Growth of *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* in cabbage mesophyll was determined

Table 2. Host range of wild-type and *hrpX* mutants of representative *Xanthomonas campestris* pv. *campestris* and *X. c.* pv. *armoraciae* strains

	Turnip			Mustard	Cabbage	Pepper
Race/strain	**TJR**	**TTH**	**TST**	**MFB**	**EJW[a]**	**CW[b]**
X. campestris pv. *campestris*						
Race 0						
2D541	+	+	+	+	+	HR
2D541*hrpXc*	−	−	−	−	−	−
2D543	+	+	+	+	+	HR
2D543*hrpXc*	−	−	−	−	−	−
Race 1						
2D540R	+	+	+	−	+	HR
2D540R*hrpXc*	−	−	−	−	−	−
HasibH-MR	+	+	+	−	+	HR
HasibH-MR*hrpXc*	−	−	−	−	−	−
2D518	+	+	+	−	+	HR
2D518*hrpXc*	−	−	−	−	−	−
NCPPB528R	+	+	+	−	+	HR
NCPPB528R*hrpXc*	−	−	−	−	−	−
Race 2						
2D520	+	+	−	+	+	HR
2D520*hrpXc*	−	−	−	−	−	−
Race 3						
2D513R	−	−	+	−	+	HR
2D513R*hrpXc*	−	−	−	−	−	−
Race 4						
171C	−	−	−	−	+	HR
EEXC114R	−	−	−	−	+	HR
X. campestris pv. *armoraciae*						
XLS10R	NA	+	NA	NA	+	HR
XLS10R*hrpXa*	NA	−	NA	NA	−	−
756C	NA	+	NA	NA	+	HR
756C*hrpXa*	NA	−	NA	NA	−	−

[a]Other general hosts include: Early Super Snowball cauliflower, Purple Top White Globe turnip, and White Icicle radish.
[b]+ Refers to black rot or leaf spots induction, HR refers to Hypersensitive Response, NA to not available. − Refers to the absence of disease symptoms.

as follows. The first true leaf of 2-wk-old cabbage plants were infiltrated with a bacterial suspension to approximately 10^2 colony-forming units (cfu)/cm^2. At different time points 0.636-cm^2 leaf disks were sectioned, mascerated in distilled H_2O, serially diluted, and plated on 523 medium supplemented with the appropriate antibiotics. Each time point corresponds to three independent measurements. Statistical analysis was performed by Student's paired *t* test (Little and Hills 1978) using StatView software (Brain Power, Inc., Calabasas, CA).

Histochemical localization of GUS. Histochemical localization of GUS activity in *Xanthomonas*-infected cabbage leaves was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) as a chromogenic substrate (Jefferson *et al.* 1986, 1987). Infected cabbage leaves were vacuum infiltrated for 1 min with 10 ml of GUS staining buffer (100 mM $NaPO_4$, pH 7.0, 1% Triton X, 1% DMSO, 10 mM EDTA, and 0.5 mg/ml X-Gluc). Then, the leaves were incubated for 24 hr at 37° C and thoroughly washed with 70% ethanol to clear out the chlorophyll. Stained leaves were photographed with Kodak Ektachrome 160 tungsten film using a Carl Zeiss Iena bright field microscope.

RESULTS

Construction of integrative plasmids. Using complementation analysis, we have previously localized the *hrpXc* locus to a 2.1-kilobase (kb) fragment of *X. c.* pv. *campestris* genome (Kamoun and Kado 1990a). A 1.4-kb *Eco*RI-*Nhe*I fragment containing the 5′ half of the *hrpXc* gene was subcloned from pUCD2550 to the *Eco*RI-*Xba*I sites of

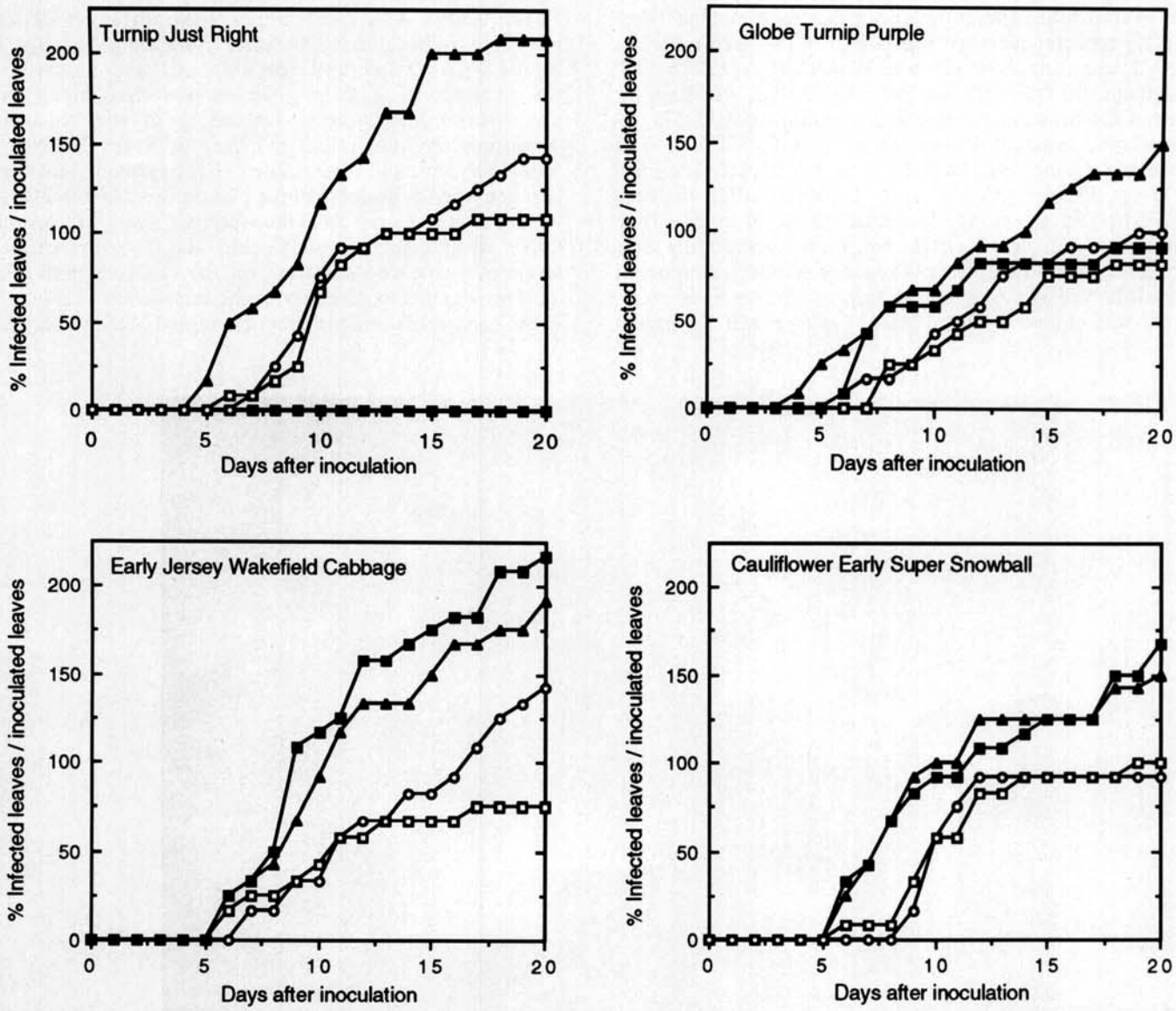

Fig. 3. Virulence of *Xanthomonas campestris* pv. *campestris* strains on four crucifer cultivars. *X. c.* pv. *campestris* 2D540R (filled triangles), 171C (filled squares), 2D518 (open circles), and 2D520 (open squares) were inoculated on the following cultivars: Turnip Just Right, Globe Turnip Purple, Early Jersey Wakefield Cabbage, and Cauliflower Early Super Snowball. Virulence is indicated as the percentage of infected leaves over inoculated leaves. Virulence levels higher than 100% reflect systemic infections.

pTZ19R (Mead *et al.* 1985), yielding pUCD2569. (The overall cloning strategy along with the maps of the various plasmids are shown in Fig. 1.) Based on our previous mapping of *hrpXc* (Kamoun and Kado 1990a), a 338-bp *Pst*I fragment of pUCD2569 was determined to be promoterless and contained within the boundaries of *hrpXc*. To generate mutagenic integrative plasmids, the 338-bp, internal, and promoterless *Pst*I fragment of pUCD2569 was then cloned into the *Pst*I site of pUC118 (Vieira and Messing 1987). The resulting plasmid, pUCD4100, was determined by DNA sequencing to contain the *Pst*I fragment in an orientation opposite to that of the *lacZ* promoter.

Subsequent cloning pursued two goals: 1) Construction of integrative plasmids that can generate transcriptional fusions of the *hrpXc* promoter to reporter genes (Fig. 2A). 2) Construction of integrative plasmids with resistance to kanamycin, to mutagenize strains that are naturally resistant to ampicillin. Therefore, a promoterless β-glucuronidase (*gusA*) reporter gene from pUCD1738 (K. Okumura and C.I. Kado, unpublished) was cloned as a 1.8-kb *Sal*I fragment in the *Sal*I site of pUCD4100, yielding the ampicillin-resistant integrative plasmid pUCD2571. A kanamycin-resistant derivative of pUCD2571 was also obtained by cloning the 2.2-kb *Eco*RI-*Hin*dIII fragment (338-bp *Pst*I::*gusA*) into the *Eco*RI-*Hin*dIII digested pBGS18 (Spratt *et al.* 1986), resulting in pUCD3161. Similarly, a 2.1-kb *Bam*HI *ctn* cassette, consisting of a promoterless chloramphenicol acetyltransferase (*cat*) reporter gene followed by a complete acetylphosphotransferase (*apt*) gene, was cloned from pCMKM4 (Close and Rodriguez 1982) into the *Bam*HI site of pUCD4100. The resulting plasmid, pUCD4109, encodes for resistance to both ampicillin and kanamycin, and generates through integration into *X. campestris* chromosome an *hrpXc::cat* fusion.

Virulence and host specificity of *X. c.* pv. *campestris* strains. Genetic, serotypic, and phenotypic analyses showed that *X. c.* pv. *campestris* is composed of a heterogenous group of strains (Alvarez and Lou 1985; Hunter *et al.* 1987; Lazo and Gabriel 1987; Lazo *et al.* 1987; Van den Mooter and Swings 1990; Vauterin *et al.* 1990). To group these strains with respect to their host specificity, we examined 22 crucifer cultivars for resistance against several representative strains of *X. c.* pv. *campestris* (S. Kamoun, unpublished data). Four cultivars, Turnip Just Right, Tokyo Turnip Hybrid, Turnip Seven Top, and Mustard Florida Broadleaf, were completely immune (asymptomatic) to some but not all of the strains examined, whereas all other cultivars tested were susceptible to all strains. Subsequently, *X. c.* pv. *campestris* strains were divided into five races relative to their host range (Table 2). Similarly, a considerable variation was also observed in the virulence of *X. c.* pv. *campestris* strains along with the severity and systemic spreading of the black rot symptoms. As illustrated in Figure 3, strain 2D540R is highly virulent, and often causes severe systemic infections that can lead to the death of the plant, in contrast to 2D520, which usually causes mild nonsystemic black rot lesions. Other strains, such as 171C, and 2D518, show variable levels of virulence depending on the cultivars used (Fig. 3). This significant diversity in the interaction between *X. c.* pv. *campestris* and crucifers prompted the investigation

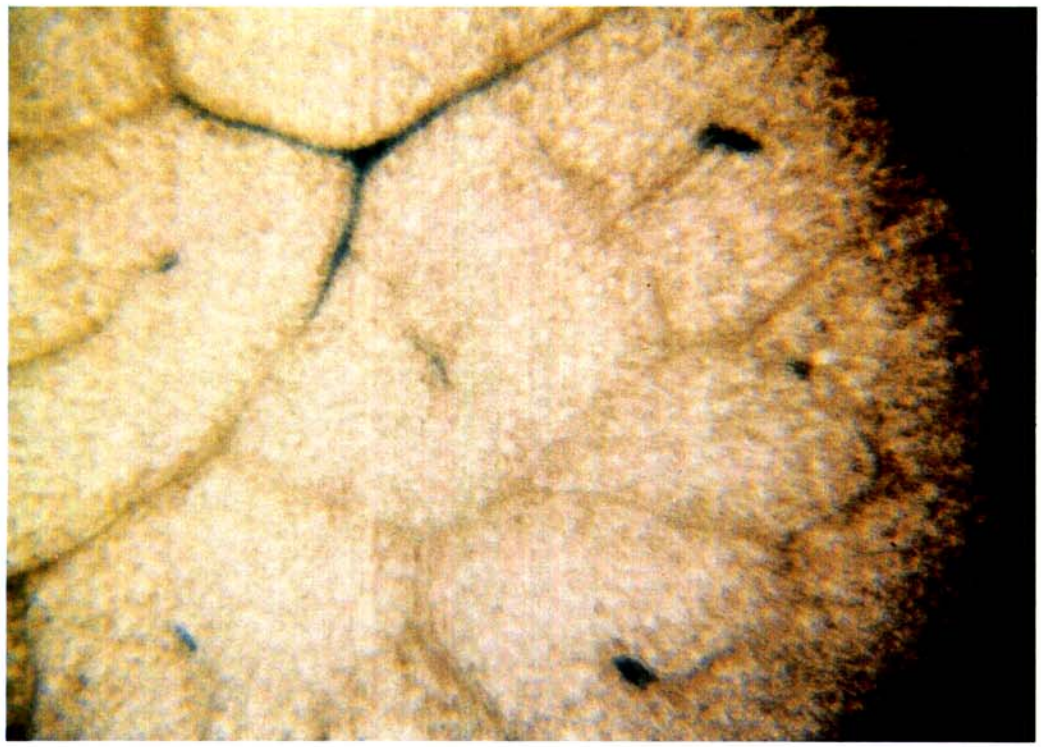

Fig. 4. Histochemical staining of β-glucuronidase activity in cabbage leaves coinfected with *X. campestris* pv. *campestris* 2D541 and SH61 (*hrpXc::gusA*). Tissue was cleared of chlorophyll by washing in 70% ethanol, and visualized by using a bright field microscope (magnification ×81).

of the conservation and role of the *hrpXc* gene in strains other than 2D520.

Construction of HrpX mutants in heterologous *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* strains. Southern blot hybridizations of total DNA with a 338-bp *Pst*I probe internal to *X. c.* pv. *campestris* 2D520 *hrpXc* gene was conducted on 14 independent *X. campestris* isolates from crucifers. All 10 strains of *X. c.* pv. *campestris* (2D541, 2D543, 2D540R, HasibH-MR, NCPPB528R, 2D518, 2D520, 2D513R, EEXC114R, and 171C) along with four strains of *X. c.* pv. *armoraciae* (756C, XLS10R, 417, and A342R) possess a sequence homologous to *hrpXc* located in an 11.0-kb *Eco*RI fragment (Kamoun *et al.* 1991a; Kamoun *et al.* 1991b) (Fig. 2B). This suggests that *hrpXc* is conserved in pathogenic crucifer isolates of *X. campestris.* The *hrpXc* allele present in *X. c.* pv. *armoraciae* is designated *hrpXa.*

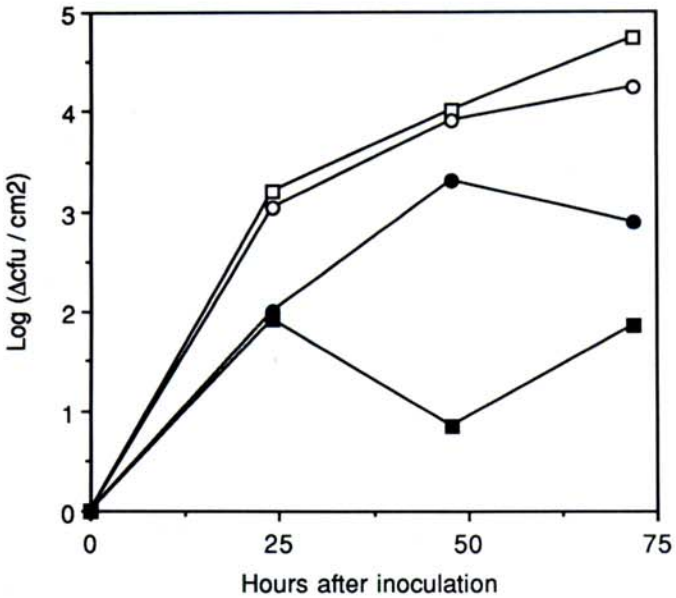

Fig. 5. Time course of the growth of *Xanthomonas campestris* pv. *campestris* and *X. c.* pv. *armoraciae* strains in separate inoculations in cabbage mesophyll. *X. c.* pv. *campestris* 2D540R (open squares), HrpXc SW61 (closed squares), *X. c.* pv. *armoraciae* XLS10R (open circles), and HrpXa XL61 (closed circles) are represented. Each point represents the average of three independent replicas.

To construct HrpXc mutants, *X. c.* pv. *campestris* 2D541, 2D543, 2D518R, 2D540R, HasibH-MR, NCPPB528R, and 2D513R were electroporated with the integrative plasmids pUCD4109, pUCD3161, and/or pUCD2571 (Table 1). The resulting mutant derivatives were obtained at a frequency of 10^2 transformants per microgram of DNA, and were subsequently confirmed by Southern blot hybridizations (Fig. 2B). All the mutants obtained displayed the typical *hrp* phenotype regardless of the race or aggressiveness of the parental strain (Table 2). The integrative plasmids pUCD4109 and pUCD3161 were also electroporated into *X. c.* pv. *armoraciae* XLS10R and 756C to generate integration mutants XL9, XL61, and HW9 (Table 1, Fig. 2). In contrast to the wild-type isolates, all mutants were unable to induce a hypersensitive response on the nonhosts *Datura stramonium* and pepper and were also unable to cause leaf spots on several crucifer cultivars, thus bearing a typical *hrp* phenotype (Table 2). However, all HrpX mutants were

Fig. 6. Tokyo Turnip Hybrid 7-day-old seedlings 16 hr after hypocotyl inoculation with *X. c.* pv. *campestris* 2D540R (three plants on the left showing no immediate response) and HrpXc SW61 (three plants on the right showing a vascular hypersensitive response). Arrows designate the points of inoculation. The distinct area of necrosis is caused by the mutant around the inoculation site.

identical to wild-type isolates in several other aspects including *in vitro* growth rate, colony morphology, and soft rot of potato tuber (data not shown).

***In planta* expression of *hrpXc* and histochemical localization of *X. c.* pv. *campestris* in crucifers.** HrpX mutants constructed with plasmid pUCD4109 and pUCD3161 contain chromosomal transcriptional fusions of the *hrpX* promoter with *cat* and *gusA*, respectively. We have previously observed with a *lux* fusion that the *hrpXc* promoter is not expressed in bacteriological media (Kamoun and Kado 1990a). This was confirmed since no CAT or GUS activity could be detected from all mutants growing in rich or minimum media. To analyze the expression of *X. c.* pv. *campestris hrpXc* in crucifers, cabbage leaves were wound inoculated with HrpXc mutant SH61 (*hrpXc::gusA*) alone or coinoculated with the wild-type strain 2D541, and were subjected to histochemical staining with X-Gluc to detect areas of GUS activity. Coinoculated leaves showed blue staining strictly confined to the veins of the cabbage leaves and often concentrated in the vein endings (Fig. 4). Control leaves inoculated solely with the wild-type strain 2D541 or the HrpXc mutant SH61 did not show any blue staining (data not shown).

Growth of *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* in cabbage mesophyll. We previously showed that an HrpXc mutant displays impaired growth in crucifer xylem (Kamoun and Kado 1990a). To determine the growth of the crucifer strains in the mesophyll of crucifers, *X. c.* pv. *campestris* 2D540R and HrpXc mutant SW61, along with *X. c.* pv. *armoraciae* XLS10R and HrpXa mutant XL61 were infiltrated into leaves of EJW cabbage. At various times the bacterial population was enumerated, and both representative wild-type pathovars appeared to grow at similar rates to reach at day 3 population levels about four to five orders of magnitude higher than at inoculation (Fig. 5). Therefore, it appears that even though *X. c.* pv. *campestris* is restricted to the xylem in natural infections and does not cause leaf spots on crucifers (Bretschneider *et al.* 1989; Moffett *et al.* 1976), it is able to grow in crucifer mesophyll at least as well as a pathogenic *X. c.* pv. *armoraciae* strain. On the other hand HrpXc mutant SW61 was unable to significantly grow in cabbage mesophyll, whereas HrpXa XL61 showed some degree of growth that is still significantly lower than the wild type ($P < 0.05$ by Student's paired *t* test) (Fig. 5).

Vascular hypersensitive response (VHR) induction by incompatible strains and HrpX mutants. Crucifer resistance to *X. campestris* is often associated with a localized browning reaction that could be the vascular analog of the hypersensitive response (Bretschneider *et al.* 1989; Robeson *et al.* 1989; Cook and Robeson 1986; Staub and Williams 1972; Williams *et al.* 1972; Daniels *et al.* 1984). We refer to this plant reaction as the vascular hypersensitive response (VHR). VHR induction on the differential cultivar TTH turnip by several strains and mutants of *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* was determined by scoring wound-inoculated six-leaf plants for a localized browning response 48 hr after inoculation (data not shown). All *X. c.* pv. *campestris* incompatible strains and HrpXc mutants, along with *X. c.* pv. *armoraciae* XLS10R, 756C, and their HrpXa mutants were able to induce a localized browning response, confirming that the VHR appears to be associated with vascular incompatibility in crucifers.

To rapidly test for the vascular response, the hypocotyls of Tokyo Turnip Hybrid seedlings were prick inoculated with bacteria (Materials and Methods). As shown in Figure 6, an incompatible vascular response is characterized by a localized necrosis spanning about 5 mm around the inoculation site (seedling VHR), whereas compatible interactions are characterized by no immediate response (null), disease symptoms appearing later on the cotyledons or the leaves. With this assay at least 75% of all seedlings inoculated with HrpXc or HrpXa mutants or wild-type *X. c.* pv. *armoraciae* displayed a typical seedling VHR, whereas compatible *X. c.* pv. *campestris* race 0 and race 1 strains did not cause any response. Incompatible *X. c.* pv. *campestris* strains representing race 3 and race 4 consistently failed to induce a strong necrotic response on the seedlings and often resulted in a null reaction.

Suppression of VHR in coinoculation experiments. We showed previously that the *in planta* growth deficiency of HrpXc mutants can be rescued by coinoculation with the wild-type strain (Kamoun and Kado 1990a). To investigate whether this exocellular complementation occurs for the VHR, wild-type *X. c.* pv. *campestris* 2D540R and 2D541 were coinoculated with their respective HrpXc mutants on Tokyo Turnip Hybrid hypocotyl and petioles. The VHR induced by HrpXc mutants on seedlings or whole plants was suppressed in the presence of the wild-type strains. On the other hand, wild-type *X. c.* pv. *campestris* 2D540R and 2D541 were unable to suppress the VHR induced by incompatible *X. c.* pv. *campestris* strains 171C and EEXC114R or *X. c.* pv. *armoraciae* XLS10R. Also, coinoculation of HrpXc mutants SW61 and SH61 with strains 171C, EEXC114R, or XLS10R did not result in any complementation or suppression of VHR (data not shown).

DISCUSSION

***hrpX* is required for black rot and bacterial leaf spot induction by *X. campestris*.** By using marker-integration mutagenesis with plasmids containing a 338-bp fragment internal to *hrpXc*, HrpX mutants were constructed in 10 crucifer isolates representing *X. c.* pv. *campestris* (four races, eight isolates) and *X. c.* pv. *armoraciae* (two isolates). All mutants displayed typical *hrp* phenotypes, nonpathogenic on crucifer hosts and noninducer of hypersensitivity on nonhosts (Table 2), and were indistinguishable from the wild-types in all tested *in vitro* features. However, representative mutants of *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* were unable to grow to wild-type levels in crucifer leaves. In contrast to wild-type isolates, *X. c.* pv. *campestris* HrpXc mutants appeared unable to grow either in the xylem or the mesophyll of cauliflower and cabbage (Fig. 5) (Kamoun and Kado 1990a), and *X. c.* pv. *armoraciae* HrpXa mutant XL61 displayed some level of growth in cabbage mesophyll that is lower than the wild type (Fig. 5). It appears, therefore, that *hrpX* is essential for pathogenicity and growth of *X. campestris* on crucifers regardless of the genetic background of the strain. It should be noted that we have recently constructed

by marker-integration mutagenesis, *hrpX* mutants in *X. oryzae* pv. *oryzae*. These mutants were unable to cause leaf blight on rice, or induce a hypersensitive response on pepper and *Datura*, suggesting that *hrpX* is highly conserved and may be essential for phytopathogenicity of all *Xanthomonas* (H. Kamdar, S. Kamoun, and C. Kado, unpublished).

Vascular incompatible interactions between crucifers and *X. campestris* involve a localized browning response. Incompatible *X. c.* pv. *campestris* strains and several *X. c.* pv. *armoraciae* isolates were all found to induce a rapid and localized necrotic response in the vascular system of the race differential cultivar Tokyo Turnip Hybrid. This response (referred to as *v*ascular *h*ypersensitive *r*esponse or VHR) is characterized by the browning and necrosis of the tissue surrounding the inoculation point and is similar to the response of cabbage plants to incompatible *X. campestris* (Bretschneider *et al.* 1989; Cook and Robeson 1986; Robeson *et al.* 1989; Staub and Williams 1972; Williams *et al.* 1972). Several points suggest that the VHR shares some resemblance to the mesophyllic hypersensitive response (Klement 1964; Klement 1982). 1) The VHR of crucifers has been proposed to be associated with vascular resistance (or incompatibility) to *X. campestris* crucifer strains, whereas compatible pathogenic strains do not induce any localized reaction (Bretschneider *et al.* 1989; Cook and Robeson 1986; McCulloch 1929; Robeson *et al.* 1989; Staub and Williams 1972; White 1930; Williams *et al.* 1972) (Fig. 6). 2) The VHR is associated with severe cell aberrations such as degradation and deformation of cell walls, plasmolysis, and swelling of chloroplasts of cells surrounding the vascular system (Bretschneider *et al.* 1989). 3) The VHR is induced as early as 16 hr after inoculation (Bretschneider *et al.* 1989; Cook and Robeson 1986). 4) The VHR is localized and no spread of the symptoms occurs (Bretschneider *et al.* 1989; Cook and Robeson 1986; Daniels *et al.* 1984). 5) Another vascular xanthomonad, the rice pathogen *X. o.* pv. *oryzae* was shown to induce a localized browning reaction in incompatible interactions (Kaku and Hori 1977; Reimers and Leach 1991). This response was recently found to correlate with an accumulation of ligninlike phenolic compounds throughout the inoculation site and was also likened to a hypersensitive reaction (Reimers and Leach 1991).

***hrpX* may function in suppression of plant defense response(s).** Using both seedling and whole plant assays, we demonstrated in this study that several HrpXc mutants of *X. c.* pv. *campestris* induce a VHR on crucifers, which is similar to the response induced by incompatible *X. c.* pv. *campestris* races or by *X. c.* pv. *armoraciae* (the data are summarized in Table 3). Interestingly, VHR induction was suppressed by coinoculation of the mutants with their wild-type counterparts, and HrpXc *in planta* growth deficiency was subsequently rescued in coinoculated leaves (Kamoun and Kado 1990a). This observation suggests that wild-type compatible *X. c.* pv. *campestris* may have the ability to suppress plant defense responses in order to grow and invade plant tissue, whereas incompatible strains induce a VHR reminiscent of an HR (Bretschneider *et al.* 1989; Cook and Robeson 1986; McCulloch 1929; Robeson *et al.* 1989; Staub and Williams 1972; White 1930; Williams *et al.* 1972). It has been hypothesized that basic compatibility genes of phytopathogens may function in suppression, toleration, and/or detoxification of specific plant molecular defenses (Gabriel and Rolfe 1990). Moreover, several necrotrophic phytopathogenic fungi possess pathogenicity genes that encode for phytoalexin detoxification enzymes (VanEtten *et al.* 1989), and there is both biochemical and genetic evidence for the occurrence of plant defense suppressor genes in *X. c.* pv. *campestris* and *P. solanacearum* (Daniels *et al.* 1984; Sequeira 1976). Our observations lead us to speculate that the *hrpXc* gene encodes a product that directly or indirectly functions in suppressing plant defense response(s) to *X. c.* pv. *campestris*. The inability of *X. c.* pv. *campestris* wild-type strains to suppress the VHR induced by incompatible *X. c.* pv. *campestris* or *X. c.* pv. *armoraciae* suggests that additional *X. campestris* loci to *hrpX* are involved in VHR suppression and/or induction on crucifers.

Similarly to all Hrp mutants, HrpX mutants of *X. c.* pv. *campestris* and *X. c.* pv. *armoraciae* fail to induce an HR on nonhost plants (Table 2) (Willis *et al.* 1991). However, they gain (or retain) the ability to induce a VHR on turnip vascular tissue, suggesting that the plant response to HrpX mutants is not null (Table 3). It has been shown that similarly to their wild-type counterpart, *P. syringae* pv. *tabaci* Hrp mutants rapidly induce four major plant defense genes (phenylalanine ammonium-lyase, chalcone synthase, chalcone isomerase, and chitinase) when inoculated on the nonhost bean plant, even though no visible HR is observed (Jakobek and Lindgren 1990; Willis *et al.* 1991). Also, bean leaves preinoculated with *P. syringae* pv. *tabaci* Hrp mutants were more resistant to *P. s.* pv. *phaseolicola* than uninoculated leaves, confirming that the plant actively responds to the Hrp mutants although they do not induce a visible HR (Lindgren and Jacobek 1990). Based on these observations, our interpretation is that Hrp mutants might be unable to overcome one of the early plant defense barriers, which would lead to incompatibilty without the expression of a confluent visible HR. Such

Table 3. Summary of the interaction between crucifer pathovars of *Xanthomonas campestris* and plants[a]

Pathovar/race/genotype	Susceptible crucifers[b]		Tokyo turnip		Nonhost plants[c]
	Veins	Mesophyll	Veins	Mesophyll	Mesophyll
X. c. pv. *campestris*					
Race 0, 1, 2 Wt	C	C	C	C	HR
Race 0, 1, 2 HrpXc	VHR	N	VHR	N	N
Race 3, 4 Wt	C	C	VHR	N	HR
Race 3 HrpXc	VHR	N	VHR	N	N
X. c. pv. *armoraciae*					
Wt	VHR	C	VHR	C	HR
HrpXa	VHR	N	VHR	N	N

[a] C indicates compatible interactions or disease induction. HR and VHR indicate incompatible interactions characterized by respectively the mesophyllic hypersensitive reaction and the vascular hypersensitive reaction described in the text. N indicates incompatible reactions characterized by the absence of a visible macroscopic response by the plant. Wt refers to wild-type strains.

[b] Susceptible crucifers are cultivars that show no race-specific resistance to *X. campestris* pv. *campestris*, such as Early Jersey Wakefield cabbage.

[c] Pepper California Wonder and *Datura stramonium*.

an interpretation is also supported by the inability of several Hrp mutants to sustain normal growth *in planta* (Bonas *et al.* 1991; Kamoun and Kado 1990a; Willis *et al.* 1991).

Similarly to the hypothetical anti-plant defense genes (Gabriel and Rolfe 1990), *hrp* genes have been proposed to correspond to basic compatibility (or pathogenicity) genes (Ellingboe 1982; Lindgren *et al.* 1988). It should be emphasized that even though plant defense suppressor genes have been extensively described in fungi (VanEtten *et al.* 1989), such genes have remained poorly understood in bacteria even though there is considerable evidence for their existence (Daniels *et al.* 1984; Sequeira 1976). We feel that circumstantial evidence along with the VHR induction on crucifers by HrpX mutants and the exocellular complementation of these same mutants by wild-type *X. campestris* argue in favor of a role for *hrpX* and possibly other *hrp* genes in overcoming early plant defense response(s) to bacterial infections.

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