Functional Analysis of the Early Chlorosis Factor Gene

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Submitted 28 June 2004. Accepted 17 January 2005.

Chlorosis is one of the symptoms of bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria*, which induces chlorosis before any other symptoms appear on tomato. We report characterization of a 2.1-kb gene called early chlorosis factor (*ecf*). The gene *ecf* encodes a hydrophobic protein with similarity to four other proteins in plant pathogens, including *HolPsyAE*, and uncharacterized gene products from *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*, and, at the tertiary structure level, to colicin Ia from *Escherichia coli*. We demonstrate that the associated phenotype is *hrp* dependent, and that the *ecf* gene product appears to be translocated to host cells. The gene *ecf* has no impact on electrolyte leakage or on bacterial growth in planta in response to infection. Concentrated culture filtrates do not produce chlorosis. Study of its role in *Xanthomonas* spp.–tomato interactions will forward our understanding of symptom production by plant pathogens and allows further investigation into the mechanisms of bacterial virulence and production of symptoms.

Plant-pathogenic bacteria enter the plant intercellular space or apoplast through natural or wound openings. Ingress may require the expression of diffusible factors secreted by the type II system, whose activation may require an environmental signal such as sensing of a nutrient-poor environment or a specific plant factor. Once bacteria have entered the apoplast, activation of hypersensitive response and pathogenicity (*hrp*) genes encoding the type III secretion system (TTSS) occurs rapidly and is triggered by contact with host cells (Brito et al. 2002) or sensing of the intercellular environment (Alfano and Collmer 1996). Early activation of the *hrp* system is the essential event of infection. The *hrp* system elaborates a proteinaceous secretion apparatus capable of delivering proteins both into the extracellular environment and across the plant cell wall and membrane (Jin et al. 2003). Proteins delivered into the extracellular environment may participate in the alkalization of the apoplast (which occurs in part through a K⁺ efflux or H⁺ influx exchange response) (Alfano and Collmer 1996) and may be significant in creating or anchoring the TTSS to the plant cell wall. Numerous proteins called effectors are delivered into the plant cell via the TTSS (Buttner and Bonas 2002; Collmer et al. 2002; Cornelis

and Gijsegem 2000; Greenberg and Vinatzer 2003), some of which are targeted to the nucleus (Szurek et al. 2002). These proteins are thought to suppress plant defenses or alter the plant metabolic machinery to promote nutrient synthesis or release. As a result, bacteria multiply in the intercellular space. The critical component of parasitism is the organism's ability to evade or subvert plant defense responses. The *hrp*-dependent factors are likely the key players in this process (Jin et al. 2003; Ponciano et al. 2003), and certain toxins which are also *hrp* dependent may suppress defense gene expression as well (Bender et al. 1999; Fouts et al. 2002; He et al. 2004; Mittal and Davis 1995). Genomic sequence data suggest that pathogenicity and virulence in plant pathogens result from the contribution of well over a hundred bacterial factors, some of which are unique to and conserved among pathogens, some of which are specific to individual pathogens, and some of which are possessed by pathogen and nonpathogen alike (Buttner et al. 2003; Buell et al. 2003; da Silva et al. 2002; Greenberg and Vinatzer 2003; Petnicki-Ocweija et al. 2002; Salanoubat et al. 2002; Simpson et al. 2000).

Bacterial spot disease of tomato and pepper is caused by *Xanthomonas campestris* pv. *vesicatoria* (also called *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*) (Jones et al. 1998) and is most prevalent in hot, humid climates (Stall 1995). In the field, the bacteria are disseminated through aerosols, enter small wounds or stomata, and produce small watersoaked lesions that become necrotic. When leaves have numerous lesions, they often coalesce and are accompanied by a general chlorosis. Chlorosis is the visible result of the loss of chlorophyll which may occur by a variety of mechanisms, including a response to the activity of toxins (Gross 1991) and ethylene (Stall and Hall 1984). This article reports the identification and physical characterization of a novel locus from *X. campestris* pv. *vesicatoria* that induces early chlorosis in tomato and several nonhosts. The isolation of this locus affords the opportunity to further investigate the mechanisms of bacterial virulence and production of symptoms.

RESULTS

Physical and molecular characterization of early chlorosis factor (*ecf*) gene.

To examine the factors that induce the response of bean to *X. campestris* pv. *vesicatoria* tomato races, a library of DNA from *X. campestris* pv. *vesicatoria* 75-3 (Minsavage et al. 1990) was screened in the bean pathogen *X. campestris* pv. *phaseoli* 85-6. One cosmid clone, pEC432, converted the host response from a normal watersoaking to a bright yellow chlorosis on bean cvs. Sprite and Bush Blue Lake (Tables 1 and 2). Two successive subclones, 3.4-kb pXV9038 and 2.4-kb pXV9039, yielded similar results on bean. To investigate the

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Nucleotide sequence data reported are available in the GenBank database under number AY842297.

host range of the chlorotic response, pXV9038 was mobilized into *X. campestris* pvs. *vesicatoria* 89-1, *glycines* 202, *vignicola* G-55, *malvacearum* G-34, and *campestris* 8004 (Table 2), and the resulting transconjugants were inoculated into their normally susceptible hosts, tomato, soybean, cowpea, cotton, and radish, respectively. All host lines responded with a chlorotic phenotype (Table 1). To ascertain whether the 2.4-kb fragment in pXV9039 contained all the sequences for full chlorosis-inducing activity on tomato, the 2.4-kb fragment was cloned into pL6 in both orientations, creating pXV9040 and pCQM20 (Table 2). The insert gave full activity in both orientations, demonstrating that the 2.4-kb fragment was capable of conferring the chlorosis-inducing activity on *X. campestris* pv. *vesicatoria* 89-1 (Fig. 1; Table 1). All fragments shorter than 2.4 kb were nonchlorotic (Table 2).

The timing of chlorosis in response to *X. campestris* pv. *vesicatoria* 89-1 transconjugants was studied (Fig. 2). In response to inoculation with 89-1 carrying pXV9038, pXV9039, and pXV9040, a strong chlorosis was evident beginning 1 day postinoculation (dpi) and increasing in strength up to 5 dpi. When 89-1 was inoculated, light chlorosis activity was observed beginning 3 dpi and increasing up to 5 dpi, suggesting two distinct chlorosis activities. Therefore, we named the locus encoded in the 2.4-kb fragment *ecf*.

To further demonstrate that the chlorosis activity resided in these fragments, *Tn5* mutagenesis was carried out. Plasmids pEC432::Tn5 and pXV9038::Tn5 lost chlorosis-inducing activity in 89-1 (Table 1). The *Tn5* mutation was exchanged into the genome of *X. campestris* pv. *vesicatoria* 75-3 and 75-3 *avrRxv*::Ω (Whalen et al. 1988), creating 75-3 *ecf*::Tn5 and 75-3 *ecf*::Tn5 *avrRxv*::Ω. *X. campestris* pv. *vesicatoria* 75-3 and 75-3 *avrRxv*::Ω induced a chlorotic response on leaves of Bonny Best, whereas 75-3 *ecf*::Tn5 and 75-3 *ecf*::Tn5 *avrRxv*::Ω induced watersoaking responses. The chlorosis-inducing phenotype was rescued by pXV9038 but not pXV9038::Tn5 (Table 1). The timing of chlorosis induction was delayed by 2 to 3 dpi in the 75-3 *ecf*::Tn5 *avrRxv*::Ω compared with the 75-3 *avrRxv*::Ω strain (data not shown).

To investigate whether an early chlorotic phenotype correlates with the presence of *ecf*, genomic DNA from *Xanthomo-*

nas strains known to be chlorotic or nonchlorotic in their hosts was hybridized with probes derived from the 2.4-kb subclone. Genomic DNA from the chlorotic *X. campestris* pv. *vesicatoria* tomato strains 75-3 and 92-14 and pepper strains 82-8, 85-10, 81-23, 68-1, and 69-1 (Table 2) hybridized at the expected size in *Eco*RI-digested DNA (data not shown). Genomic DNA from the nonchlorotic strains *X. campestris* pv. *vesicatoria* 89-1; *X. campestris* pvs. *campestris* 8004, *phaseoli* 85-6, *malvacearum*, *glycines*, *vignicola*, *alfalfae*, *holcicola*, *vitians*, *carotae*, *pruni*, and *begoniae*; and the chlorotic pathovar strain *campestris* 2D250 (Buell and Somerville 1995) did not hybridize, suggesting that *ecf* is highly specific to *X. campestris* pv. *vesicatoria*. In *X. campestris* pv. *vesicatoria* strains 75-3, 82-8, 85-10, 81-23, 68-1, and 69-1 *ecf* co-segregated with chromosomal DNA (data not shown).

Sequence determination and analysis.

The 3.6-kb clone pXV9038 was sequenced. Subclones from the inactive pXV9038::Tn5 were created and sequenced to determine that *Tn5* was inserted at base 689. Only one open reading frame (ORF), in the minus orientation, produced a long ORF (ORF1) of 2,064 nucleotides encoding a theoretical 688-amino acid protein with a molecular weight of approximately 74 kDa (base 2,239 to 176) (Fig. 3A). The results from in vitro transcription and translation reactions performed on p42 indicated a major protein product of 74.5 kDa and two minor products at 62 and 58 kDa (data not shown). The two minor products were found to correspond to internal ATGs within ORF1.

The entire sequence was analyzed for *Escherichia coli* consensus promoter sequences and none were found. A search for a PIP-box (TTCGC-N15-TTCGC) sequence found in the promoter region of several *Xanthomonas* genes revealed a perfect PIP-box between bases 3,276 and 3,251 in the minus orientation (Fig. 3A). However, the fact that *ecf* begins over 1,000 nucleotides downstream from the PIP-box, along with the fact that a subclone without the PIP-box and without external promoters is fully active (Table 1), make it unlikely that the PIP-box is part of a promoter region specific to this ORF (Fenselau and Bonas 1995). A possible ribosome-binding site, AAGGA, is centered 10 bases upstream from the ATG start codon of ORF1 (Fig. 3A) and there is no recognizable ribosome-binding site upstream of ORF2. The inverted palindrome AGCGGCGCGGCC-N23-TCGCCGCGCCGG spans bases 1,282 through 1,328 within the ORF but has unknown significance.

The predicted amino acid sequence of the *ecf* gene product was analyzed using several structure prediction programs. The consensus prediction indicated that the core of ECF is highly hydrophobic and includes six potential transmembrane (TM) domains (Fig. 3B). Furthermore, the tertiary structure prediction program 3D-PSSM predicted limited but significant structural similarity to domains of colicin Ia, a pore-forming TM protein present in *E. coli* (Weiner et al. 1997). The similarity includes the TonB binding box in the translocation domain (Fig. 3B) and two of the highly conserved TM sequences, C8 and C9, in the channel-forming domain (Fig. 3B and C).

Various databases were searched for similarities at both the nucleotide and amino acid levels. Comparing the *ecf* sequence within the 3.4-kb subclone at the nucleotide level, two sets of similarities were found. The first set had significant similarity to *ecf* bases 2,797 to 3,195 and comprised xanthomonad insertion elements, including a few from *X. oryzae* pv. *oryzae*, an IS4 family member (gi:23428392), IS1113 (gi:32453974), and IS1114 (gi:7453619) (Zhu et al. 2000); one from *X. campestris* pv. *vesicatoria* (gi:38564794); and one from *X. campestris* pv. *campestris* IS1478a (gi:1927196) (Fig. 3A). The sequence in these elements that is similar to the *ecf* sequence is outside the

Table 1. Responses of susceptible hosts to strains of *Xanthomonas campestris* pathovars and transconjugants upon inoculation at 5×10^8 CFU/ml

<i>X. campestris</i> pathovar	Response ^a
<i>phaseoli</i> 85-6	WS
pEC432	Chl
pXV9038	Chl
pXV9038::Tn5	WS
pXV9039	Chl
<i>vesicatoria</i> 89-1	WS
pXV9038	Chl
pXV9038::Tn5	WS
pXV9039	Chl
pCQM20	Chl
<i>glycines</i> 202	WS
pXV9038	Chl
pXV9038::Tn5	WS
<i>vignicola</i> G-55	WS
pXV9038	Chl
pXV9038::Tn5	WS
<i>malvacearum</i> G-34	WS
pXV9038	Chl
pXV9038::Tn5	WS
<i>vesicatoria</i> 75-3; <i>vesicatoria</i> 75-3 ± <i>avrRxv</i> ::Ω	Chl
<i>vesicatoria</i> 75-3 <i>ecf</i> ::Tn5; <i>vesicatoria</i> 75-3 <i>ecf</i> ::Tn5 ± <i>avrRxv</i> ::Ω	WS
pXV9038	Chl

^a Response of susceptible host to inoculation: WS = watersoaking and Chl = chlorosis.

coding sequence for the transposase but within the element at the 3' end containing one of the inverted repeats. The second set of similar sequences has significant similarity to *ecf* bases 49 to 108 and comprises the flanking inverted repeats first found in members of the widespread *avrBs3* family of avirulence genes with virulence functions, and subsequently found flanking or adjacent to other genes in *X. campestris* pv. *vesicatoria* (Noel et al. 2003). The most significant similarity was to one repeat flanking avirulence or virulence gene, *pthN* (gi:2384735), from the cotton pathogen *X. campestris* pv. *malvacearum*, followed in significance by a repeat found upstream of *hpaJ* in *X. campestris* pv. *vesicatoria* (Noel et al. 2003). In each case, the fundamental identity is to the inverted repeat sequence (approximately 95%),

with similarity dropping off completely beyond that region. Like other instances, the repeat is found downstream of the 3' end of the *ecf* ORF. There are no other significant similarities in the coding sequence to *avrBs3* genes.

Gene products with limited similarity to the *ecf* gene product were identified with PSI-BLAST (Fig. 3B). ECF is 15 to 18% identical and 48 to 52% similar to uncharacterized proteins in *X. campestris* pv. *campestris* (gi:21230004 and gi:21111523) and *X. axonopodis* pv. *citri* (gi:21241314); these three proteins are 71% identical and 86% similar. All of the xanthomonad homologues have the colicin Ia C8, C9, and TonB-box domains. In addition, ECF is 11 to 12% identical and 45 to 47% similar to a *Pseudomonas syringae* protein,

Table 2. Bacterial strains, plasmid vectors, and plasmid constructions

Designation	Relevant characteristics ^a	Source or reference
Bacterial phytopathogens		
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
75-3, 92-14	Tomato race 1, Rif ^r	Whalen et al. 1988, 1993
75-3 <i>avrRxx</i> ::Ω	75-3 with Ω insertion in <i>avrRxx</i> , Rif ^r Sp ^r	Whalen et al. 1988
75-3 <i>ecf</i> :: <i>Tn5</i> <i>avrRxx</i> ::Ω, 75-3 <i>ecf</i> :: <i>Tn5</i>	75-3 with <i>Tn5</i> insertion in <i>ecf</i> , ± <i>avrRxx</i> ::Ω Rif ^r Km ^r ± Sp ^r	This study
75-3 <i>hrp</i> :: <i>Tn5</i> mutants #38, 5, 139, 57, 132	75-3 with <i>Tn5</i> insertion in <i>hrpA</i> , <i>hrpB</i> , <i>hrpC</i> , <i>hrpD</i> , <i>hrpE</i> , and <i>hrpF</i>	Bonas et al. 1991; Ciesiolka et al. 1999
89-1	Tomato race 2, Rif ^r	Whalen et al. 1993
<i>X. campestris</i> pv. <i>vesicatoria</i> races	82-8, 85-10, 81-23, 68-1, 69-1; Rif ^r	Minsavage et al. 1990
<i>X. campestris</i> pathovars	<i>phaseoli</i> 85-6, <i>vignicola</i> G-55, <i>malvacearum</i> G-34, <i>glycines</i> 202; Rif ^r	Whalen et al. 1988
<i>Agrobacterium tumefaciens</i> C5C81 ± vir	Rif ^r Km ^r ± Tc ^r	Van Larebeke et al. 1984
<i>X. campestris</i> pv. <i>vesicatoria</i>		
85-10 <i>hrpG</i> * (85*)	85-10 <i>hrpG</i> mutant with constitutive <i>hrp</i> gene expression; Rif ^r	Wengelnik et al. 1999
85-10 <i>hrpG</i> * <i>rec:cya</i> (85*)	85-10 <i>hrpG</i> * <i>recA</i> gene fused with <i>cya</i> ; genomic integration; Rif ^r	Metz et al. in press
85-10 <i>hrpG</i> *Δ <i>hrcV</i> (85*Δ <i>hrcV</i>)	85-10 <i>hrpG</i> * with <i>hrcV</i> mutation; Rif ^r	Rossier et al. 1999
<i>Escherichia coli</i> strains		
DH5α	F- <i>recA</i> φ80- <i>dlacZ</i> ΔM15	BRL
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZDM15 Tn10</i>]; Tc ^r	Stratagene
TOP10	F- <i>mcrA</i> Δ(<i>mrrhsdRMSmcrBC</i>)φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74recA1deoRaraD139Δ(ara-leu)</i> 7697 <i>galUgalKrrpsL</i> (Str ^r) <i>endA1nupG</i>	Invitrogen
DB3.1	F- <i>gyr462endA</i> Δ(<i>sr1recA</i>) <i>mcrBmrrhsdS20</i> (<i>rBmBsupE44</i>) <i>ara14galK2lacY1proA2rpsL20</i> (Str ^r) <i>xy15λ-leumt1</i>	Invitrogen
Plasmid vectors		
pLAFR3	Broad host range cosmid, Tc ^r	Staskawicz et al. 1987
pL6	pLAFR3 deleted for <i>Plac</i> with <i>trp</i> terminators flanking the polylinker, Tc ^r	Whalen et al. 1988
pVSP61	Broad host range vector, Km ^r	DNA Plant Technology
pMD1	Binary vector plasmid with 35S promoter, NOS terminator, LB, RB, Km ^r	M. Dixon
pCRII	TA-cloning vector, Ap ^r	Invitrogen
pBluescript KS and SK	Phagemid derived from pUC19, Ap ^r	Stratagene
pRK2013, pRK2073	Helper plasmids, Km ^r , Sp ^r	Ditta et al. 1980
pENTR/SD/TOPO	Gateway entry vector	Invitrogen
Plasmid constructs		
pEC432	pLAFR3 clone from <i>X. campestris</i> pv. <i>vesicatoria</i> T 75-3 with <i>ecf</i> activity	This study
pXV9038	3.6-kb <i>Sau3A</i> I subclone from pEC432 in pLAFR3	This study
pEC432::Tn5, pXV9038::Tn5	<i>Tn5</i> insertions eliminating <i>ecf</i> activity	This study
pXV9039, pXV9040	2.4-kb <i>Bam</i> HI- <i>Hind</i> III subclone of pXV9038 in pLAFR3 and pL6	This study
pCQM20	2.4-kb subclone of pXV9038 in pL6; no external promotion	This study
pJP1	2.4-kb <i>ecf</i> subclone in pVSP61	This study
pXV9007	<i>avrRxx</i> in pLAFR3	Whalen et al. 1988
p42	2.4-kb insert from pXV9039 in pBluescript	This study
p44, p61, p64	<i>Eco</i> RI- <i>Hind</i> III subclone of p42 containing base pairs 1 to 1,114 in pBluescript, pL6, and pLAFR3	This study
p54, p62	<i>Exo</i> III subclone containing base pairs 352 to 2,358 in pBluescript and pL6	This study
p55, p63	<i>Exo</i> III subclone containing base pairs 536 to 2,358 in pBluescript and pL6	This study
pCQM30	2.4-kb <i>ecf</i> subclone in pMD1	This study
pMD1- <i>avrBsT</i>	<i>avrBsT</i> in pMD1	Orth et al. 2000
pMD1- <i>avrRpt2</i>	<i>avrRpt2</i> in pMD1	Mudgett and Staskawicz 1999
pDD62-Cya	<i>Cya</i> in pVSP61 as destination vector	Metz et al. 2005
pJM50	<i>ecf</i> Δ stop in pENTR	This study
pJMP51	<i>ecf:cya</i> fusion in pVSP61	This study

^a Rif = rifampicin, Sp = spectinomycin, *ecf* = early chlorosis factor gene, Km = kanamycin, Tc = tetracycline, and Ap = ampicillin.

HolPsyAE, in *pv. syringae* B728a (Greenberg and Vinatzer 2003). HolPsyAE is 24% identical and 55% similar to HopPmaA in *P. syringae* *pv. maculicola*, with the majority of the homology in the N-terminus (Greenberg and Vinatzer 2003). The HolPsyAE/HopPmaA N-termini do not cluster with ECF and the other ECF homologues. Analysis of ECF subcellular localization sequences uncovered a weak chloroplast thylakoid membrane targeting sequence. Moreover, the protein domain similarity search program BLOCKS uncovered some intriguing similarities to the ECF-family in proteins found in chloroplasts, including Photosystem I PsaA and PsaB, Photosystem II PsbH, Cytochrome b6-f complex subunit VI b559, NADH dehydrogenase, and Chlorophyll AB binding protein.

Physiological characterization of *ecf*.

We assessed the development of two other disease symptoms in *X. campestris* *pv. vesicatoria* 89-1, specifically watersoaking and tissue collapse. *X. campestris* *pv. vesicatoria* 89-1 with *ecf* appears to have a slight delay in watersoaking but no effect on final tissue collapse. Electrolyte leakage was assessed from leaves of two tomato cultivars after inoculation with strains of 89-1 with and without *ecf*. A slow rate and low level of electrolyte leakage was observed in both Bonny Best and Hawaii 7998 in response to infiltration with 89-1 carrying the *ecf* subclone pXV9040 (Fig. 4). The response to *ecf* was similar to that observed in response to infiltration of Bonny Best and Hawaii 7998 with 89-1, but different from the rapid rate and high level of electrolyte leakage in Hawaii 7998 seen in a

Fig. 1. Response of tomato cultivars to inoculation. Cv. Bonny Best after inoculation with *Xanthomonas campestris* *pv. vesicatoria* inoculated at 5×10^8 CFU/ml. *X. campestris* *pv. vesicatoria* strain 89-1 carrying pLAFR3 **A**, with early chlorosis factor gene (*ecf*) and **B**, without *ecf* at 3 days post inoculation (dpi); strain 75-3 **C**, with intact *hrp* genes and **D**, with *Tn5* insertion in *hrpA* at 3 dpi. Cv. VF36 after inoculation with *Agrobacterium tumefaciens* C5C81 *vir*⁺ carrying **E**, *ecf* in pMD1 (pCQM30) and **F**, pMD1 alone at 4 dpi.

hypersensitive response (HR) to 89-1 carrying *avrRxv* (Whalen et al. 1993). These results indicate that, although *ecf* appears to slightly delay watersoaking, it does not behave as an avirulence gene in terms of electrolyte leakage. To determine

Fig. 2. Time course of intensity of chlorosis in leaves of tomato cv. Bonny Best in response to inoculation with *Xanthomonas campestris* pv. *vesicatoria* 89-1 strains inoculated at 5×10^8 CFU/ml for up to 6 days post inoculation. The intensity of chlorosis within the inoculated area was visually scored on a scale of 0 to 4, where 0 = no chlorosis, 1 = faint chlorosis, 2 = light chlorosis, 3 = medium-bright chlorosis, and 4 = bright chlorosis. Each point represents the mean $\pm$ SE of seven independent inoculations of pL6 with early chlorosis factor gene in pXV9040 (▲) and pL6 alone (■).

To determine whether the *ecf* gene product is translocated to the host, we examined the cAMP levels in tomato leaves after infection with *Xanthomonas* strains carrying a translational fusion of the gene for the calmodulin-dependent adenylate cyclase *cya* and *ecf* (Table 2; Fig. 6). At 8 hpi, approximately 30 times more cAMP was produced in *X. campestris* pv. *vesicatoria* 85* (pJMP51) carrying the *ecf:cya* fusion than 85* alone, 85* Δ hrcV (pJMP51), or 85* carrying the nontranslocated *recA:cya* fusion. These results indicate that the gene product of *ecf* is translocated to host cells.

To test for a secreted chlorosis-inducing activity, *X. campestris* pv. *vesicatoria* 89-1 carrying *ecf* in either pXV9039 or pJP1 (Table 2) were grown in rich (nutrient yeast glycerol; NYG) and *hrp*-inductive (XVM2) medium (Feneslau and Bonas 1995) and culture filtrates were collected. No chlorosis activity on pepper or tomato was observed in culture filtrates when concentrated up to 30-fold (data not shown). This result

Fig. 5. Time course of bacterial growth in tomato cv. Bonny Best and common bean cv. Bush Blue Lake 274. Leaves were infiltrated with bacterial suspensions of 10^5 CFU/ml. Bacterial populations in leaves were sampled immediately after infiltration and up to 5 days thereafter. *X. campestris* pv. *vesicatoria* strain 75-3 in tomato ($\blacktriangle$) and in bean ($\blacklozenge$) and strain 75-3 *ecf::Tn5* in tomato ($\bullet$) and in bean ($\blacksquare$). Each point represents the mean $\pm$ SE of three replicates.

Table 3. Response of tomato and tobacco to inoculation of *Agrobacterium tumefaciens* C5C81 with transient assay vector pMD1 carrying specified genes

Plasmid ^b	Plant response ^a	
	vir ⁺	vir ⁻
VF36/Bonny Best		
pMD1- <i>ecf</i>	Chl ^c	None
pMD1	None	None
<i>Nicotiana benthamiana</i> ^d		
pMD1- <i>avrRpt2</i>	HR	None
pMD1- <i>avrBsT</i>	HR	None
pMD1	None	None

^a Response of plants to inoculation of acetosyringone-treated *A. tumefaciens* strain at an optical density at 600 nm = 0.8; vir⁺ = virulent *A. tumefaciens* containing the virulence plasmid and vir⁻ = nonvirulent *A. tumefaciens* lacking virulence plasmid.

^b Transient expression plasmid pMD1 carrying specified bacterial gene; CaMV S35 promoter driving expression of transformed DNA after delivery by *A. tumefaciens*; *ecf* = early chlorosis factor gene.

^c VF36 routinely responded to 100% of inoculations with chlorosis; Bonny Best responded, on average, to 94% of inoculations with chlorosis.

^d *N. benthamiana* *avrRpt2* and *avrBsT* reactions included as positive control for hypersensitive response (HR).

suggests that the chlorosis-inducing factor is not secreted from *X. campestris* pv. *vesicatoria* in vitro.

Expression of *ecf*.

To understand the function of *ecf*, we analyzed the conditions under which it is expressed in *X. campestris* pv. *vesicatoria*. Northern blots confirmed a transcript size of approximately 2.1 kb in *X. campestris* pv. *vesicatoria* 89-1 strains carrying the plasmid-borne *ecf* gene that is consistent with the large ORF1 (data not shown). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of total RNA from 89-1 carrying *ecf* in pXV9039 compared with pL6 alone and normalized for the endogenous control 16S rRNA showed that *ecf* was expressed in both NYG and XVM2 to the same extent (Table 4). Our expression analyses suggest that, although translocation of ECF is dependent on the *hrp* apparatus, *ecf* expression is not dependent on nutritional conditions that induce *hrp* gene expression in *X. campestris* pv. *vesicatoria*.

DISCUSSION

Development of control strategies to prevent crop disease or lessen its negative agronomic impact requires an understanding of the factors that contribute to disease symptoms. Chlorosis is one symptom of bacterial spot disease caused by the pathogen of tomato *X. campestris* pv. *vesicatoria*. We have identified and cloned a gene from *X. campestris* pv. *vesicatoria* that induces a bright chlorosis on host and nonhost plants. The induction of chlorosis is conditioned by the single locus in *X. campestris* pv. *vesicatoria* called early chlorosis factor (*ecf*).

Fig. 6. Activity of calmodulin-dependent *cya* fusions in tomato 8 h post infection with *Xanthomonas campestris* pv. *vesicatoria* 85-10* mutant and transconjugants. Leaves were infiltrated with bacterial suspensions of 5×10^8 cells/ml and, immediately thereafter (white) and 8 h later (black), cAMP levels were determined in two leaf discs (0.5 cm² each). Each bar represents the mean $\pm$ SE of three replicates.

Table 4. Expression analysis of early chlorosis factor gene (*ecf*)^a

Growth medium	Relative expression of <i>ecf</i> ($2^{-\Delta\Delta Ct} \times 10^6$) ^b	
	Mean	Range
NYG	1.40	0.81 to 2.43
XVM2	1.26	0.75 to 2.13

^a By real-time reverse-transcriptase polymerase chain reaction on total RNA from *Xanthomonas campestris* pv. *vesicatoria* 89-1 transconjugants grown in nutrient yeast glycerol (NYG) and *hrp*-inductive (XVM2) medium.

^b Expression of *ecf* in total RNA from 89-1 carrying *ecf* in pXV9039 compared with pL6 alone. Calculations of relative expression include normalization for endogenous control gene 16S rRNA.

The chlorosis was evident beginning 2 dpi and increasing in intensity up to 5 dpi. On tomato, an additional faint chlorosis activity was apparent in a non-*ecf* strain beginning at 3 dpi and increasing up to 5 dpi, suggesting the possibility of a second chlorosis activity conditioned by at least one other locus in *X. campestris* pv. *vesicatoria*. Although DNA that hybridizes to *ecf* is found in chlorosis-inducing strains in *X. campestris* pv. *vesicatoria* and not in other pathovars of *X. campestris*, when carrying *ecf*, other pathovars are fully able to induce early chlorosis on their hosts. This suggests that the requirements for function of the protein product of *ecf* are not specific to *X. campestris* pv. *vesicatoria* or to its host, tomato.

We examined the effect of *ecf* on electrolyte leakage and growth of the pathogen in planta. Results from analysis of tomato response to infection indicate that *ecf* did not significantly affect electrolyte leakage. In both tomato and bean, we have observed that *ecf* did not affect growth at any time postinfection. The gene *ecf* delayed the onset of visible watersoaking by approximately 1 day, and it is possible that this is associated with a weak resistance-inducing activity.

Intact *hrp* genes were necessary for the *ecf* phenotype and, when directly expressed by plant cells, *ecf* induced chlorosis. Moreover, analysis of *cya* activity of *ecf:cya* in host cells indicated that the *ecf*-mediated induction of chlorosis may be a direct effect of the translocation of the *ecf* gene product to the host cell cytoplasm. Although the translocation of ECF is *hrp*-mediated, the expression of *ecf* is not dependent on *hrp*-inductive conditions. Taken together, our results suggest that ECF behaves like a virulence effector and contributes to development of symptoms. One class of virulence factors is toxins and, in fact, several pseudomonad toxins induce chlorosis (Alfano and Collmer 1996). However, ECF did not behave like a toxin, as evidenced by a lack of host response to concentrated culture filtrates from *ecf*-containing *X. campestris* pv. *vesicatoria* cells grown in rich or *hrp*-inductive medium. To the contrary, our results indicate that *ecf* encodes a translocated effector.

The coding sequence of *ecf* has been determined and analyzed for indicators of structure and function. As predicted, the 2,064-nucleotide ORF encodes a protein of molecular weight of approximately 74 kDa and produces a transcript of approximately 2.1 kb. Although a PIP-box is located upstream of *ecf*, it is unlikely to be part of the *ecf* promoter, considering the spacing and the fact that, without the PIP-box sequences, *ecf* functions normally. Moreover, *ecf* is expressed to the same extent in rich and *hrp*-inductive media. The association of PIP-boxes with effector genes does not always mean it is an essential promoter sequence, as evidenced by studies of expression of other genes including *avrRxv* (Ciesiolka et al. 1999) and *popP* (Lavie et al. 2002). Like many effectors, although translocation of ECF is dependent on induction of the *hrp* system; *ecf* gene expression is not.

ECF is a hydrophobic protein with an abundance of α -helices that potentially may localize to a membrane, as evidenced by six putative TM domains. Remarkably, the tertiary structure of ECF has limited but significant structural similarity to domains of colicin Ia, a pore-forming TM protein present in *E. coli* (Weiner et al. 1997). The colicin Ia similarity with ECF includes the TonB recognition motif (TonB binding box) in the translocation domain, and two of the highly conserved TM sequences, C8 and C9, in the channel-forming domain. In general, colicin Ia homologues do not have a high level of sequence similarity except in the C8 and C9 domains. Based on the C8 and C9 structural similarities, it is possible that, once ECF has been translocated to the host cell cytoplasm, it functions to depolarize chloroplast membranes, resulting in disruption of chloroplast function leading to chlorosis. Mutagenesis studies are essential to provide evidence for this model.

In the nucleotide sequence immediately flanking *ecf*, similarities were discovered to repeat sequences in other xanthomonads, including *X. oryzae* pv. *oryzae*, *X. campestris* pv. *vesicatoria*, and *X. campestris* pv. *campestris*. Only the region adjacent to and including an inverted repeat sequence was similar, but none of the transposon coding sequence was found. Included in the similarities were the inverted repeats found flanking members of the widespread *avrBs3* family of avirulence and virulence genes. These *avrBs3*-family flanking repeat sequences also have been found singly, adjacent to six other genes in *X. campestris* pv. *vesicatoria* involved in host-pathogen interactions (Noel et al. 2003). These two types of repeat sequences indicate that the region flanking *ecf* may have been evolutionarily active in genomic rearrangements. The *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* genomes are rich in transposable elements often found adjacent to strain-specific genes whose features suggest that they possibly were acquired by lateral transfer (da Silva et al. 2002). Recently, seven translocated effectors in *X. campestris* pv. *vesicatoria* were identified by a genetic screen in *X. campestris* pv. *vesicatoria*; four of them were novel (Roden et al. 2004).

Phytochemical gene products with limited similarity to the *ecf* gene product were found in the genomes of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *vesicatoria*, creating a loose family of putative virulence effectors. The three xanthomonad homologues have the colicin Ia C8 and C9 domains and four of six TM domains. In addition, ECF is similar to the C-terminus of a *P. syringae* effector protein, HolPsyAE (Greenberg and Vinatzer 2003). This protein has five of the six TM domains found in ECF and has a region similar to the colicin Ia sequences shared by other ECF family members. HolPsyAE is similar to HopPmaA at their N-terminal domains, where there is no similarity with ECF (Greenberg and Vinatzer 2003). HopPmaA was isolated in a translocation screen, is expressed equally well in *hrp*-inductive and rich medium, is plasmid borne, has chloroplast targeting sequences, and is similar to a prophage protein in pathogenic *E. coli* (Greenberg and Vinatzer 2003; Guttman et al. 2002). The role these latter proteins play in virulence is unknown. It will be interesting to determine whether the ECF-family factors have similar targets in plants, and whether disruption of chloroplast function is their mode of action.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria broth (LB) (Miller 1972) or SOC medium (Sambrook et al. 1989). *Xanthomonas* strains were grown at 30°C in NYG medium (Daniels et al. 1984). *A. tumefaciens* strains were grown at 28°C in LB. Bacto-agar (Difco Laboratories, Detroit) was added to media for plate cultures. The helper plasmid pRK2073 or pRK2013 was used in triparental mating (Figurski and Helinski 1979) to mobilize broad host range vectors from *E. coli* into *Xanthomonas* spp. or *A. tumefaciens*. Antibiotics (Sigma-Aldrich, St. Louis) were used for selection in both liquid and plate cultures at the following concentrations: ampicillin (Ap), 50 or 100 µg/ml; rifampicin (Rif), 100 µg/ml; tetracycline (Tc), 5 or 10 µg/ml; spectinomycin (Sp), 50 µg/ml; and kanamycin (Km), 25 or 50 µg/ml.

Molecular biology techniques.

Standard molecular biology techniques were used for manipulating plasmids, preparing DNA for sequencing and subcloning, and performing Southern blots. Exonuclease III deletion and subsequent reactions were carried out with the Erase-

a-Base System (Promega Corp., Madison, WI, U.S.A.). To assess whether the long ORF in the 2.4-kb subclone produced the predicted translation product, *in vitro* transcription or translation was performed on p42. TNT Coupled Reaction (Promega Corp.) containing 35-S methionine was used according to the manufacturer's instructions and products fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Genomic DNA was isolated according to Staskawicz and associates (1984) and restriction enzyme digested and transferred to Hybond N+ membrane (Amersham, Piscataway, NJ, U.S.A.) according to the manufacturer's protocols.

For RNA isolations, after lysing *Xanthomonas* cells from overnight liquid cultures in 0.05 M Tris, pH 9.0, 0.05 M EDTA, 0.3 M Sodium Acetate, and 0.625% SDS at 100°C, RNA in lysate was isolated with RNeasy Mini kits (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions. Total RNA (5 µg) was fractionated on a prerun 1% agarose denaturing gel (19.7 mM MOPS, pH 7.0; 6.2% formaldehyde) at 120 V. The RNA was blotted onto a nylon membrane and UV-crosslinked. Southern and Northern blot probes (25 ng) were 32P-dCTP labeled using standard protocols and purified through Sephadex G-50 spin columns. Prehybridization, hybridization, and washes were performed with the ULTRAhyb system (Ambion, Austin, TX, U.S.A.) at 42°C.

Sequence of *ecf* and flanking DNA was obtained by sequencing several subclones of the 3.5-kb *ecf* subclone pXV9038 (Table 1). The 3.5-kb clone containing *ecf* had sixfold coverage and flanking DNA had onefold coverage. Sequencing reactions were performed on ABI 377 DNA sequencers (Perkin-Elmer, Norwalk, CT, U.S.A.) using standard protocols and vector sequences for dye-labeled primers. Other internal sequencing primers were designed as necessary using Oligo 5.1 (National Biosciences, Plymouth, MN, U.S.A.). Sequence data was manipulated using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI, U.S.A.), MacVector 5.0.1 (Kodak, New Haven, CT, U.S.A.), and Geneworks version 2.5N (Oxford Molecular Group-Accelrys San Diego, CA, U.S.A.). The nucleotide and amino acid sequences were analyzed by programs available on the internet.

Plant growth, inoculations, and physiological analyses of host responses.

All plants were grown from seed in plastic pots with standard potting mix in a greenhouse. Plant inoculations of *Xanthomonas* strains at 5×10^8 CFU/ml in H₂O were performed on four different plants and experiments were done in triplicate. Inoculated plants were observed at 24-h intervals from 1 to 15 dpi and disease symptoms (chlorosis, watersoaking, and tissue collapse) were noted and scored for each strain. To assess the effect of gene expression in planta, *A. tumefaciens*-mediated gene transfer was performed on leaf tissue (Tai et al 1999). *A. tumefaciens* strains were suspended in 1 ml of induction medium (10 mM 2-[N-Morpholino] ethanesulfonic acid [Sigma-Aldrich], 10 mM MgCl₂, and 200 µM acetosyringone [3',5'-dimethoxy-4'-hydroxyacetophenone] [Aldrich, Milwaukee, WI]) and incubated at 28°C for 2 h. After induction, *A. tumefaciens* strains were diluted to an optical density at 600 nm = 0.2, 0.4, 0.6, and 0.8, and tomato and tobacco were inoculated with the dilution series and leaves observed as described above for 5 dpi. Three independent experiments were performed each, with several independent transconjugants.

To assess levels of electrolyte leakage, *Xanthomonas* strains were grown overnight in NYG liquid medium, brought to a cell density of 5×10^8 CFU/ml in distilled water, and vacuum infiltrated into leaves of tomato. During a time course postinfiltration, six leaf disks of 0.5 cm² were taken from each plant using a cork borer and were suspended in 3 ml of distilled H₂O

in 10-ml beakers for 1 min with vigorous shaking. A conductivity meter was used to read the solution in microOhms every 6 h for up to 64 h postinfiltration. Three plants were used for each *Xanthomonas* strain and the experiment was repeated three times.

To determine the level of bacterial growth in planta, plant leaves were infiltrated with a 10^5 CFU/ml in 10 mM MgCl₂ bacterial solution by vacuum infiltration, and bacteria were isolated from leaf disks during the course of 5 dpi (Whalen et al. 1988). In all, seven independent growth curves were performed in bean and tomato.

X. campestris pv. *vesicatoria* 89-1 (p39) and 89-1 (p40) (Table 1) were tested for the presence of a secreted toxin. Overnight cultures were grown in 5 ml of NYG or XVM2 (Wengelnik et al. 1996). Some of the cell suspension was inoculated directly after assessing cell density. Cell suspensions were pelleted twice and supernatants were filtered with a 0.2-micron filter to remove bacteria. Culture filtrates were concentrated 5- to 30-fold with centricon concentrators (Amicon/Millipore, Bedford, MA, U.S.A.) with a molecular weight cutoff of 10 kDa. Tomato and pepper plants were inoculated and plant responses were observed for 1 to 4 dpi.

Expression analysis.

Real-time RT-PCR was used to analyze expression of *ecf*. The *ecf* and *Xanthomonas* rRNA expression primers were created using default settings in Primer Express 2.0 (Applied Biosystems, Foster City, CA, U.S.A.). The *ecf* expression forward primer (5'-GTTGCTCATGCGTGATTGCT-3') and *ecf* expression reverse primer (5'-TTACGCAGCTTCTCCACGAA-3') amplicon is 111 bp in length. The *ecf* TaqMan probe consists of an oligonucleotide (5'-CTGTCATCGAAGTCCGAAGCTCCTG-3') covalently attached to the 5' reporter dye FAM (6-carboxyfluorescein) and the 3' quencher dye TAMRA (6-carboxytetramethylrhodamine) (Applied Biosystems).

To design an endogenous control for real-time RT-PCR of RNA from *X. campestris* pv. *vesicatoria* 89-1, Clustal W was used to align *xanthomonas* 16S rRNA sequences (GenBank identifiers: 4544327, 3153161, 4544328, 4544326, 1657412, 1922934, 1657411, 2266886, 3153162, 4544329, 4544325, and 28170767). The 16S rRNA forward primer (5'-TGACGGTACCCAAAGAATAAGCA-3') and 16S rRNA reverse primer (5'-ACGCTTGCACCCTTCGTATTA-3') amplicon is 72 bp in length. The 16S rRNA TaqMan probe consists of an oligonucleotide (5'-ACTTCGTGCCAGCAGCCGCG-3') covalently attached to dyes as above.

Two-step real time RT-PCR was used to study *ecf* expression. The RT reaction was performed in three steps for the two targets independently on 1 µg of total RNA following Invitrogen's (Carlsbad, CA, U.S.A.) instructions (2 pmol of each primer, 1 mM dNTPs, 1× RT buffer, 10 mM DTT, 40 units RNaseout [Invitrogen], and 200 U of RT [Invitrogen]). The Geneamp 5700 sequence detector (Applied Biosystems) was used to perform real-time PCR on 2 µl of cDNA. PCR reactions (50 µl) contained 100 nmol of each primer/probe, 1 mM dNTPs, 1× TaqMan Gold buffer, 10 mM MgCl₂, and 2.5 U of TaqMan Gold polymerase. After heating samples to 95°C for 10 min, 40 cycles were performed, consisting of 95°C for 15 s and 62°C for 90 s, followed by 72°C for 10 min. rRNA cDNAs were diluted 100-fold compared with *ecf* samples before real-time PCR. The sizes of the PCR products from the experimental and control reactions were confirmed with agarose gel electrophoresis (data not shown). Calculations for relative expression were performed according to the comparative threshold cycle (Ct) method as described by the real-time PCR system manufacturer (Applied Biosystems). The average Ct values used in the calculation represent the mean and standard deviation.

tion of three independent RNA samples, with each sample analyzed in triplicate with real-time RT-PCR.

Translocation analysis.

The Gateway cloning system (Invitrogen) was used to construct the *ecf:cya* fusion (Table 2). Oligo 5.1 (Molecular Biology Insights Inc., Cascade, CO, U.S.A.) was used to create *ecf* gateway-specific primers. The *ecf*-gateway forward primer (5'-CACCATGCAGATCAAAAC-3') and *ecf*-gateway reverse primer 2 (5'-TTCCGACTGAGGCACTGG-3') product is 2,068 bp in length. Full-length *ecf* Δ stop codon was PCR amplified using Pfu Turbo polymerase (Stratagene, La Jolla, CA, U.S.A.). PCR reactions contained 100 ng of each primer, 200 μ M dNTPs, 1 $\times$ Pfu Turbo polymerase buffer, 100 ng of p42 template DNA (Table 1), and 2 U of Pfu Turbo polymerase. Reactions were heated to 95°C for 3 min, and then 30 amplification cycles were performed at 95°C for 30 s and 55°C for 30 s, followed by 72°C for 2.5 min.

After confirming the size of the PCR product by agarose gel electrophoresis, amplified DNA was cloned directly into pENTR/SD/TOPO (Invitrogen) to produce the entry vector, pJMP50 (Table 2). The TOPO cloning reaction was performed using 120 ng of fresh PCR product, 1 μ l of salt solution, and 1 μ l of TOPO vector as supplied by the manufacturer and according to their instructions (Invitrogen), and transformed into TOP10 *E. coli* (Invitrogen). Confirmation of the entry clone pJMP50 was verified by restriction enzyme digestion using *Bsr*GI and by sequencing with M13 forward and M13 reverse primers. pJMP50 DNA was linearized with *Eco*RV and a 4.7-kb band was gel purified. The LR recombination reaction was used to transfer it into the destination vector, pDD62-Cya (Table 2). The LR reaction (16 μ l) contained 150 ng of attL1-*ecfA* stop-attL2 and 150 ng of pDD62-Cya, 1 $\times$ LR Clonase Reaction Buffer, and 4 μ l of LR Clonase enzyme mix and was performed and stopped according to manufacturer's instructions (Invitrogen). Library Efficiency DH5 α competent cells (Invitrogen) were transformed. The sequence of the resulting pJMP51 (*ecf:cya* fusion) was verified by sequencing. For expression in *Xanthomonas* spp., pJMP51 and pDD62-cya were conjugated into *X. campestris* pv. *vesicatoria* 85* and 85* Δ hrcV (Table 2).

To assess the activity of *ecf:cya* in vitro, *Xanthomonas* cells were grown overnight and sonicated and, after addition of bovine calmodulin, cAMP levels were measured according to Casper-Lindley and associates (2002) with a cAMP enzyme immunoassay according to the manufacturer's instructions kit (Cayman Chemicals, Ann Arbor, MI, U.S.A.). To determine whether *ecf:cya* is translocated into tomato cells, *Xanthomonas* strains at 5×10^8 cells/ml in 10 mM MgCl₂ were hand infiltrated into tomato leaves as described earlier. For each sample, two leaf discs (0.5 cm² each) were collected and stored frozen at -80°C until assayed for cAMP as described by Casper-Lindley and associates (2002). Triplicate samples were analyzed and the experiment was repeated three times.

ACKNOWLEDGMENTS

We gratefully acknowledge M. Huber for performing the in vitro transcription/translation reaction, members of the SFSU spring 1995 Biology 744 class for beginning to sequence and analyze pXV9038, E. Moe for tomato seed production, C. Boucher for help with the sequence analysis, B. Staskawicz for greenhouse facilities, and G. Ponciano for critical reading of the manuscript. All the research and M. C. Whalen were supported by NIH AREA R15 GM59022 and NIH MBRS SCORE grant SO6 GM52588; C. Q. Morales and J. Posada were supported by NIH MBRS RISE R25 GM59298; D. Franklin and J. Posada were supported by NIH MARC T34 GM08574; I. Rivas and M. Bravo were supported by NIH Bridges to the Baccalaureate R25 GM50078; D. Franklin was supported

by ASPB 2003 Summer Undergraduate Research Fellowship; and the work was supported by NIH RIMI P20 RR11805.

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