The *Xanthomonas campestris* pv. *vesicatoria* *citH* gene is expressed early in the infection process of tomato and is positively regulated by the TctDE two-component regulatory system

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SUMMARY

Xanthomonas campestris pv. *vesicatoria* (Xcv) is the causal agent of bacterial spot disease of tomato and pepper. Previously, we have reported the adaptation of a recombinase- or resolvase-based *in vivo* expression technology (RIVET) approach to identify Xcv genes that are specifically induced during its interaction with tomato. Analysis of some of these genes revealed that a *citH* (citrate transporter) homologous gene contributes to Xcv virulence on tomato. Here, we demonstrate that the *citH* product indeed facilitates citrate uptake by showing the following: *citH* is specifically needed for Xcv growth in citrate, but not in other carbon sources; the *citH* promoter is specifically induced by citrate; and the concentration of citrate from tomato leaf apoplast is considerably reduced following growth of the wild-type and a *citH*-complemented strain, but not the *citH* mutant. We also show that, in the Xcv–tomato interaction, the promoter activity of the *citH* gene is induced as early as 2.5 h after Xcv is syringe infiltrated into tomato leaves, and continues to be active for at least 96 h after inoculation. We identified an operon containing a two-component regulatory system homologous to *tctD/tctE* influencing *citH* expression in Xcv, as well as its heterologous expression in *Escherichia coli*. The expression of *hrp* genes does not seem to be affected in the *citH* mutant, and this mutant cannot be complemented for growth *in planta* when co-inoculated with the wild-type strain, indicating that citrate uptake in the apoplast is important for the virulence of Xcv.

INTRODUCTION

The Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (Xcv) is the causal agent of bacterial spot disease of

tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum*) (Jones *et al.*, 1998). Typical disease symptoms include necrotic lesions that are often surrounded by chlorosis on all above-ground parts and, in some cases, defoliation. High temperature and relative humidity provide optimal conditions for the induction of this disease, which has a worldwide distribution. Although the reclassification of Xcv into four different species—*X. vesicatoria*, *X. euvesicatoria*, *X. gardneri* and *X. perforans*—has been proposed recently (Jones *et al.*, 2000, 2004), we refer here to the classical nomenclature, as it is still being broadly used in molecular studies on the interaction of this pathogen with its hosts.

As with many Gram-negative phytopathogenic bacteria, Xcv relies on a functional *hrp* (hypersensitive response and pathogenicity)-encoded type III secretion system (TTSS) to secrete effectors into the host cell, where they interact with host cellular processes to promote disease or to elicit defence responses in susceptible and resistant plants, respectively. In susceptible plants, type III effectors contribute collectively to the adaptation of the pathogen to the host tissue by interfering with host immune responses or mediating nutritional and virulence processes (Buttner and He, 2009; Mudgett, 2005; White *et al.*, 2009). However, the establishment of bacterial pathogens in their host and their ability to cause disease expands beyond the ability to form this secretion system and to transfer effectors through it. The infectious process is very intricate, and requires the coordinated activity of a myriad of bacterial genes, whose identity and mode of action are still largely unknown.

In a previous report, we described an adaptation of recombinase- or resolvase-based *in vivo* expression technology (RIVET), a promoter-trap method, to identify Xcv genes that are specifically induced or overexpressed during its interaction with tomato (Tamir-Ariel *et al.*, 2007). In RIVET, promoter activity drives the expression of *tnpR*, which encodes a resolvase that specifically excises a genetic marker—typically an antibiotic

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resistance gene—flanked by resolvase recognition sequences (res sites). This reaction is named 'resolution' and leads to a phenotype switch that can be easily detected and screened. The rationale behind this approach is that selectively *in vivo* induced genes have the potential to contribute to the ability of the pathogen to survive and multiply in the host tissue. Among the identified genes was a *citH* homologue, Xcv3613, according to the *Xcv* strain 85-10 genome annotation (GENBANK accession AM039952; Thieme *et al.*, 2005).

CitH possesses a CitMHS domain (citrate transporter, Pfam03600), and shows 61% and 54% identity with the characterized citrate transporters of *Bacillus subtilis* CitN (also annotated as CitH) and CitM, respectively (GENBANK accessions P42308 and P55069) (Boorsma *et al.*, 1996; Krom *et al.*, 2000). Disruption of *citH* in *Xcv* leads to reduced virulence of the bacterium on tomato when compared with the wild-type strain (Tamir-Ariel *et al.*, 2007).

The aims of this study were to further characterize *citH* and to gain an insight into its role in the *Xcv*–tomato interaction. We exploited RIVET to assess *citH* promoter activity (namely *citH* expression) in *Xcv* during the infection process. RIVET is a highly sensitive method, because a small pulse of resolvase expression is needed to mediate resolution; therefore, the method is exquisitely sensitive to low or transient gene expression (Angelichio and Camilli, 2002). This enabled us to detect *citH* expression also at the early stages of infection. We also questioned whether CitH specifically facilitates citrate uptake by assessing its contribution to growth in minimal medium with different carbon sources, as well as to citrate uptake in minimal medium and tomato apoplast extracts. We also assessed the activation of the *citH* promoter under various growth conditions, and explored how CitH could contribute to the virulence of *Xcv* by looking at several aspects of plant–microbe interactions. In this study, we also provide evidence demonstrating the involvement of a two-component regulatory system (TCS) in the regulation of *citH* expression in culture and *in planta*.

RESULTS

Functional CitH is required for growth in minimal medium with citrate, but not with other carbon sources

A *citH* mutant has been generated previously in the background of *Xcv* strain 97-2 by single recombination (Tamir-Ariel *et al.*, 2007). The vector used, pJPcit, contains an internal region of the *citH* gene (such that it does not span both the 5' and 3' ends of the transcriptional unit), and its chromosomal integration resulted in a *cis*-merodiploid strain with each of the two copies

Fig. 1 The genomic region around *citH* (citrate transporter). (A) Genomic location of *citH* according to the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strain 85–10 annotation (GENBANK accession AM039952; Thieme *et al.*, 2005). Sequence analyses revealed a similar genomic structure for this region in strain 97-2. *tctD*, *tctE* and *Xcv3609* encode a response regulator, a histidine sensor kinase and a putative periplasmic iron-binding protein, respectively, and *oprP2* encodes phosphate-selective porin P. (B) Vectors and cloned DNA fragments used in this study to generate the different strains: (1) pJPcit, containing an internal fragment of the *citH* open reading frame (ORF), used to generate the *Xcv citH* mutant (Tamir-Ariel *et al.*, 2007); (2) pHKmtctD, containing an internal fragment of the *tctD* ORF, used to generate the *Xcv tct* and *XcvPcitHtct⁻* mutants; (3) the amplified *citH* ORF and its promoter region used to generate the pMLcit complementation vector; (4) the amplified *tctDE* ORFs and their promoter region used to generate pUcittctDE and pUttctDE; (5) the entire *tct*-*Xcv3609* operon used to generate pUcitOP and pUOP.

of the *citH* gene lacking the 5' or 3' ends. As *Xcv citH* is part of a monocistronic locus, this mutation is not expected to affect downstream genes (Fig. 1).

We have shown that the *citH* mutant and the wild-type do not differ in terms of their ability to grow in rich medium (nutrient broth, NB); hence, it appears that the mutant does not have a general metabolic defect (Tamir-Ariel *et al.*, 2007). In that study, we also reported that, unlike the wild-type, the *citH* mutant is affected in its ability to grow on citrate as carbon source. Here, we show that pML122 carrying the *citH* gene with its native promoter (pMLcit; Table 1) restored the ability of the *citH* mutant to grow in M9 medium containing citrate (M9-citrate) as carbon source, although, in most experiments, this ability was not fully restored (Fig. S1, see Supporting Information). Partial complementation by pMLcit in these and other experiments (see below) could be a result, at least in part, of a certain level of plasmid loss, although, in these growth curve experiments, gentamicin (Gm) was added to the medium. The *citH* mutant did not differ from the wild-type in its ability to grow in M9 medium containing sucrose, fructose, malate or succinate as carbon source

Table 1 Strains and plasmids used in this study.

Strains and plasmids	Characteristics	Reference or source
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
97-2	Wild-type, belongs to race T3	Astua-Monge <i>et al.</i> (2000)
110C	Wild-type, belongs to race T1	Sahin <i>et al.</i> (2003)
Xcv <i>citH</i> mutant	Km ^r ; strain 97-2 with the <i>citH</i> ORF disrupted with insertion of plasmid pJPcit	Tamir-Ariel <i>et al.</i> (2007)
Xcv <i>citH</i> comp	Km ^r , Gm ^r , Xcv <i>citH</i> -complemented strain (carrying pMLcit)	This work
XcvRes	Km ^r , reporter strain containing the res cassette inserted within <i>hpaG</i> in 97-2	Tamir-Ariel <i>et al.</i> (2007)
ResPcitH	Km ^r , Gm ^r , <i>PcitH</i> (<i>citH</i> promoter):: <i>tnpR</i> :: <i>uidA</i> in XcvRes background	Tamir-Ariel <i>et al.</i> (2007)
ResPcitHrev	Km ^r , Gm ^r , <i>PcitHrev</i> (<i>citH</i> promoter in reverse orientation):: <i>tnpR</i> :: <i>uidA</i> in XcvRes	Tamir-Ariel <i>et al.</i> (2007)
ResPhrpA	Km ^r , Cm ^r , <i>PhrpA</i> (<i>hrpA</i> promoter):: <i>tnpR</i> :: <i>uidA</i> in XcvRes	Tamir-Ariel <i>et al.</i> (2007)
ResPhrpG	Km ^r , Cm ^r , <i>PhrpG</i> (<i>hrpG</i> promoter):: <i>tnpR</i> :: <i>uidA</i> in XcvRes	Tamir-Ariel <i>et al.</i> (2007)
Xcv <i>hrpA</i> mutant	<i>hrpA</i> knock-out mutant in the background of strain 97-2	Tamir-Ariel <i>et al.</i> (2007)
XcvPcitH	Gm ^r ; <i>PcitH</i> :: <i>tnpR</i> :: <i>uidA</i> in strain 97-2	This work
XcvPcitHrev	Gm ^r ; <i>PcitHrev</i> :: <i>tnpR</i> :: <i>uidA</i> in strain 97-2	This work
XcvPcitHtct	Km ^r , Gm ^r , XcvPcitH with <i>tctDE</i> disrupted with plasmid pHKmtctD insertion	This work
Xcv <i>tct</i> mutant	Km ^r ; Xcv 97-2 with the <i>tctDE</i> disrupted with plasmid pHKmtctD insertion	This work
<i>Escherichia coli</i>		
S17-1 λ pir	λ lysogenic S17-1 derivative producing π protein for replication of plasmids carrying <i>oriR6K</i> ; <i>recA pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7 λ^- pir	Simon <i>et al.</i> (1983)
DH5 α	Cloning strain	Hanahan (1983)
DH5 α /pUcit	DH5 α carrying plasmid pUcit	This work
DH5 α /pUcittctDE	DH5 α carrying plasmid pUcittctDE	This work
DH5 α /pUcitOP	DH5 α carrying plasmid pUcitOP	This work
DH5 α /pUttctDE	DH5 α carrying plasmid pUttctDE	This work
DH5 α /pUOP	DH5 α carrying plasmid pUOP	This work
<i>Acidovorax citrulli</i> M6	Wild-type, bacterial fruit blotch pathogen, isolated from melon	Burdman <i>et al.</i> (2005)
Plasmids		
pTZ57R/T	Ap ^r ; cloning vector	Fermentas Inc.
pHP45 Ω	Ap ^r , Sp ^r ; suicide vector	Prentki and Krisch (1984)
pHPKm	Ap ^r , Km ^r ; pHP45 Ω with the Km ^r gene from pUC4K (38) replacing the Sp ^r cassette	This work
pHKmtctD	Ap ^r , Km ^r ; pHKm containing an internal fragment of the <i>tctD</i> ORF	This work
pJPGm	Gm ^r ; pJP5603 (Penfold and Pemberton, 1992) with the Km ^r gene replaced by a Gm ^r gene from pML122	Tamir-Ariel <i>et al.</i> (2007)
pJPcit	Km ^r ; pJP5603 containing an internal fragment of the <i>citH</i> ORF	This work
pJPGmntpR	Gm ^r ; pJPGm carrying a promoterless <i>tnpR</i> :: <i>uidA</i>	This work
pPcitH	Gm ^r ; pJPGmntpR with the <i>citH</i> promoter cloned upstream and in the same orientation as <i>tnpR</i> :: <i>uidA</i> to create a transcriptional fusion	This work
pPcitHrev	Gm ^r ; pBamGm1 with the <i>citH</i> promoter cloned upstream and in the opposite orientation as <i>tnpR</i> :: <i>uidA</i>	This work
pML122	Gm ^r ; broad host expression vector	Labes <i>et al.</i> (1990)
pMLcit	Gm ^r ; with the <i>citH</i> ORF including a 658-bp fragment upstream of the translation start site, cloned into the <i>SmaI</i> site	This work
pUC18	Ap ^r ; cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pUcit	Ap ^r ; pUC18 with the <i>citH</i> ORF and a 658-bp fragment upstream of <i>citH</i> cloned into <i>SmaI</i> site	This work
pUcittctDE	Ap ^r ; pUcit with <i>tctDE</i> cloned into the <i>HindIII</i> site	This work
pUcitOP	Ap ^r ; pUcit with the entire <i>tctDE</i> -Xcv3609 operon cloned into the <i>HindIII</i> site	This work
pUttctDE	pUC18 with <i>tctDE</i> cloned into the <i>HindIII</i> site	This work
pUOP	pUC18 with the entire <i>tctDE</i> -Xcv3609 operon cloned into the <i>HindIII</i> site	This work

Ap^r, Cm^r, Gm^r, Km^r, Sp^r, ampicillin, chloramphenicol, gentamicin, kanamycin and streptomycin resistance, respectively; ORF, open reading frame.

(Fig. S1). Together, these experiments strengthen the view that the *citH* product is specifically involved in citrate uptake.

In these experiments, it was also observed that growth of wild-type and *citH*-complemented strains in citrate minimal medium led to an increase in the pH of the medium from pH 7 to pH 8 (as measured after 48 h of growth). This may be indicative of citrate metabolism. In contrast, no pH change was observed in cultures of the *citH* mutant.

The wild-type, but not the *citH* mutant, imports citrate from minimal and from tomato leaf apoplast-enriched media

To further verify that CitH is involved in citrate uptake, the amounts of citrate left after the growth of the wild-type, *citH* mutant and *citH*-complemented strain were quantified in M9-citrate and in extracts of tomato leaf apoplast. The latter

Fig. 2 Citrate uptake by the wild-type, *citH* (citrate transporter) mutant and *citH*-complemented strains. (A, D) Growth of the three strains in modified M9 minimal medium with 10 mM citrate and in apoplast extract. (B, E) Citrate concentration in modified M9 medium. (C, F) Citrate concentration in apoplast medium. (A–C) 24 h after inoculation (hai); (D–F) 48 hai. This experiment was repeated twice with similar results. One batch of apoplastic fluid was used in each experiment.

medium was used as the apoplast is the site at which *Xcv* naturally populates the host. Citrate was quantified by spectrophotometric analysis of citrate lyase enzyme activity at the exponential and stationary phases of growth (after 24 and 48 h of growth, respectively). These analyses revealed that, in both media, citrate was imported by the wild-type and the *citH*-complemented strain, but not by the *citH* mutant (Fig. 2B,C,E,F). In M9-citrate, however, complementation of the *citH* mutant by pMLcit was partial, as can be seen by the larger amounts of citrate left in the medium of the complemented strain relative to the wild-type (Fig. 2B,E). In agreement with this and with the growth curves in M9-citrate (Fig. S2, see Supporting Information), in these experiments, the complemented strain also

showed intermediate growth between the *citH* mutant and the wild-type in this medium (Fig. 2A,D).

In contrast with M9-citrate, no significant differences in growth between the three strains were observed in apoplast medium (Fig. 2A,D). Therefore, under the tested conditions, the impaired ability to import citrate in the *citH* mutant in the apoplast medium cannot be attributed to the reduced growth of this strain relative to the wild-type.

The *citH* promoter is induced by citrate

So far, we have shown that, in *Xcv*, CitH functions as a transporter that specifically imports citrate into the cell. In several studies, the substrate itself has been shown to induce the expression of the corresponding transporter gene (Janausch *et al.*, 2002; Warner and Lolkema, 2002). Therefore, we aimed to assess whether transcription of the *citH* gene is specifically induced in the presence of citrate, in comparison with other carbon substrates. We used RIVET to evaluate *citH* promoter activity in different growth conditions. As mentioned, RIVET relies on the induction of a promoterless resolvase gene (*tnpR*), which captures activated promoters (Camilli and Mekalanos, 1995). As TnpR-mediated resolution is irreversible, the activation of the tested promoters at any time during growth can be detected with this assay.

A fragment containing the *citH* promoter was fused to the coding region of *tnpR* in both the correct and reverse orientations of transcription, and the transcriptional fusions were integrated into the chromosome of *Xcv*Res, a modified 97-2 strain carrying the res cassette (Tamir-Ariel *et al.*, 2007) to generate strains ResPcitH and ResPcitHrev, respectively. The res cassette confers kanamycin (Km) resistance; thus, TnpR-mediated resolution, driven in this case by activation of the *citH* promoter, results in Km-sensitive colonies. Therefore, *citH* promoter activity can be assessed with this system by evaluating the Km resistance versus sensitivity of the recovered cells. TnpR-mediated resolution was tested by plating cultures grown under the different conditions on nutrient agar (NA) and NA with Km, and the percentage resolution was scored on the basis of the fraction of colony-forming units (cfu) that were unable to grow in the presence of Km.

Under the majority of the conditions tested using modified M9 medium, resolution was shown to be affected only by the presence of citrate in the medium. Namely, *tnpR* induction via the *citH* promoter did not occur in the presence of other substrates (e.g. malate, succinate, isocitrate, fructose and glucose) as sole carbon sources. In contrast, when citrate was present in the medium as the sole carbon source or in combination with other carbon sources, full resolution of the res cassette was observed (Table 2). This was also the case when citrate and glucose were

Table 2 Measurements of TnpR-mediated resolution activated by the *citH* promoter under various conditions in culture.*

Growth conditions†	Strains		
	XcvResPcitH	XcvResPcitHrev	XcvRes
NB	No‡	No	N.A.
XVM2	Complete§	90%	No
M9 + citrate	Complete	No	No
M9 + L-malate	No	No	N.A.
M9 + L-succinate	No	No	N.A.
M9 + sucrose	No	No	No
M9 + fructose	No	No	No
M9 + glucose	No	No	N.A.
M9 + glucose + citrate	Complete	No	N.A.
M9 + isocitrate	No	No	N.A.
M9 + sucrose + fructose	90%	65%	No
M9 + sucrose + fructose + citrate	Complete	No	No
M9 + sucrose pH 5.7, 6.2 or 6.7	No	No	No
M9 + sucrose pH 5 or 8	No	No	N.A.
M9 + citrate pH 5 or 8	Complete	No	N.A.

*The TnpR resolution assay is based on the fact that *tnpR* expression, activated by a tested promoter, leads to the excision of a Km^R gene. Strains were grown in liquid medium under different conditions, and serial dilutions were plated on both nutrient agar (NA) (total cells) and NA/kanamycin (Km) (unresolved cells). The percentage resolution is determined from [(average total cfu – average unresolved cfu)/average total cfu] × 100. Tested strains were as follows: XcvResPcitH, *tnpR* under the control of the *citH* promoter; XcvResPcitHrev, the *citH* promoter in the opposite orientation relative to *tnpR*; and XcvRes, carrying the res cassette but no *tnpR*.

†Where not indicated, the pH of the medium was 7.

‡'No' indicates no resolution and means that the differences in colony-forming unit (cfu) counts between Km-lacking versus Km-containing plates were smaller than 5%.

§'Complete' indicates complete resolution (differences between counts in Km-lacking versus Km-containing plates were greater than 95%). Resolution was assessed for each strain in at least two independent experiments with similar results for each medium, with the exception of N.A. (not assessed).

both added to the medium, indicating that the presence of glucose did not repress the induction of the *citH* promoter, as shown for *citM* of *Bacillus subtilis* (Warner and Lolkema, 2002; Yamamoto *et al.*, 2000). Moreover, induction of the *citH* promoter by citrate appears to be highly specific, as the related isocitrate was not able to induce it (Table 2). No resolution was observed under the majority of the above conditions on strain ResPcitHrev (Table 2).

Surprisingly, in XVM2 medium, which contains sucrose and fructose, but does not contain citrate, resolution was observed. However, the resolution observed under these conditions does not appear to be citrate responsive, as a high level of resolution (90%) was also observed in strain ResPcitHrev. The same resolution pattern was observed when sucrose and fructose were both added to M9 medium, although no resolution was observed when sucrose and fructose were used separately in this medium (Table 2). Interestingly, when citrate was added to M9 modified medium containing sucrose and fructose, no resolution occurred in the ResPcitHrev strain, but the ResPcitH strain resolved completely. Thus, the presence of citrate seems to make this resolution event specific.

citH is expressed from the early stages of tomato infection by Xcv

We have shown that the *citH* promoter is strongly induced in *planta*, being induced by more than two orders of magnitude over the *hrpA* (*hrcC*) promoter, 48 and 72 h after inoculation (hai) at 10⁶ cfu/mL by syringe infiltration into tomato leaves (Tamir-Ariel *et al.*, 2007). As the β-glucuronidase (GUS) reporter gene (*uidA*) was used for these experiments, we could not technically detect expression prior to 48 hai, because of a lack of sensitivity of this assay at early times of infection. Here, we exploited the high sensitivity of RIVET to detect *citH* induction during the first hours after inoculation. It should be noted, however, that, unlike the GUS assay, this analysis detects the percentage of cells from the total population, which irreversibly resolved from the time of inoculation to the given point of detection. For this analysis, we used strains ResPcitH, ResPcitHrev, ResPhrpA and ResPhrpG, containing the promoter of the *citH* gene in the correct and reverse orientations, and the promoters of *hrpA* and *hrpG*, respectively, fused to promoterless *tnpR-uidA* in the background of the XcvRes strain.

Fig. 3 *In planta* resolution of the *res* (resolvase recognition) cassette induced by the *citH* (citrate transporter) promoter fused to the *tnpR-uidA* transcriptional fusion (strain ResPcitH) relative to the *hrpG*, *hrpA* and reverse *citH* promoters (ResPhrpG, ResPhrpA and ResPcitHrev, respectively). The activity of the *citH* promoter in the reverse orientation was only measured at 24 h after inoculation (hai) as this time point represents the cumulative amount of resolved bacteria, and preliminary experiments showed no or very low resolution levels for this strain. Tomato (cv. H7998) leaves were syringe infiltrated with bacterial suspensions at 10^6 cfu/mL, which were grown in nutrient broth (NB) supplemented with kanamycin (Km) to ensure that inocula contained only unresolved cells. The percentage resolution was measured several times after inoculation by extracting bacteria from leaves and plating them onto nutrient agar (NA) and NA/Km. At each time point, four plants per treatment were sampled. Data (averages and standard errors) from one experiment of two with similar results are shown.

The strains were grown previously in rich medium (NA) with Km to ensure that the cells were in the unresolved state before inoculation. They were then syringe infiltrated into tomato leaves at 10^6 cfu/mL, and resolution of the *res* cassette was assessed by evaluating the Km resistance versus sensitivity of the recovered strains. The activity of the *citH* promoter was detected as early as 2.5 hai (Fig. 3). Assays performed at an earlier time (1.5 hai) showed no or low resolution levels for all treatments, with relatively high variability between replicates (not shown). The pattern of *citH* promoter activity was similar to that of *hrpG* and *hrpA*: although the level of resolution mediated by the *citH* promoter was lower during the first few hours after inoculation (2.5 and 4.5 hai), most ResPcitH cells had already been resolved at 4.5 hai. At 24 hai, no or very low resolution levels were still observed for the negative control strain ResPcitHrev (Fig. 3).

The *Xcv* TctDE TCS is sufficient to direct heterologous expression of *citH* in *Escherichia coli*

In *Xcv*, *citH* is closely linked to an operon containing two genes encoding components of a typical prokaryotic TCS, annotated as

tctD-tctE, and a third gene annotated as a putative periplasmic iron-binding protein (genes Xcv3611, Xcv3610 and Xcv3609, respectively, according to strain 85-10 annotation; GENBANK accession AM039952; Thieme *et al.*, 2005) (Fig. 1). Based on open reading frame (ORF) predictions and sequence analysis, it is likely that *tctD*, *tctE* and Xcv3609 are part of a single transcriptional unit, and their products are highly similar to putative gene products of other related xanthomonads. TctD is a predicted response regulator (RR) of the OmpR family, and TctE is a predicted histidine sensor kinase (HK). Table S2 (see Supporting Information) summarizes the similarities of the TctDE components to the putative gene products of other xanthomonads, as well as to experimentally characterized proteins.

In prokaryotes, TCSs regulate a wide variety of biological processes, including the expression of toxins and other proteins related to virulence and pathogenicity (Stock *et al.*, 2000; West and Stock, 2001). To assess whether this operon and, in particular, the TctDE system, has a role in the regulation of citrate uptake, we used *Escherichia coli* DH5 α carrying the *Xcv citH* gene with its indigenous promoter in several pUC18-modified plasmids. *Escherichia coli* was chosen as it is unable to use citrate as carbon source. Thus, this bacterium was a suitable background to assess whether *citH* may confer the ability to grow on citrate, and to examine whether this ability is regulated by the TctDE system.

First experiments revealed that DH5 α /pUcit (carrying the *Xcv citH* gene with its indigenous promoter) showed impaired growth in M9 medium with citrate as sole carbon source [Fig. 4A; note that the optical density (OD) scale is logarithmic and OD for this strain did not exceed 0.01]. This strain was also unable to alter the pH of Simmons citrate medium, where alkalization caused by citrate uptake by the cells leads to medium bluing (Fig. 4B,C). However, when either the entire *tctDE*-Xcv3609 operon, or only *tctDE*, was introduced, together with *citH* and its promoter region (in plasmids pUcitOP and pUcit-tctDE, respectively), DH5 α cells were able to grow well in both media and to change the pH in Simmons medium (Fig. 4). As controls, strains DH5 α /pUOP and DH5 α /pUtctDE, containing the entire operon or only *tctDE*, respectively, but without the *citH* gene, were created. These strains were not able to utilize citrate (Fig. 4). These results demonstrate that the *tctDE* part of *tctDE*-Xcv3609 is sufficient to direct citrate utilization in *E. coli* via the *citH* gene.

TctDE affects the ability of *Xcv* to grow on citrate

Next, we generated an *Xcv tct* mutant strain, impaired in the *tctDE*-Xcv3609 operon (Fig. 1B), to assess whether this mutant is compromised during growth in M9-citrate. Growth curve experiments revealed that the growth of the *tct* mutant on citrate was compromised relative to the wild-type. However, this mutant was

Fig. 4 Heterologous expression of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) *citH* (citrate transporter) and *tctDE* in *Escherichia coli* DH5 α . *Escherichia coli* strains were grown in M9-citrate liquid medium (A) and in Simmons citrate broth (B) and solid medium (C). In Simmons citrate medium, citrate utilization is accompanied by an increase in pH and, consequently, bluing of the medium. The strains were as follows: DH5 α /pUcittctDE containing *citH* and *tctDE* (1), DH5 α /pUcitOP containing *citH* and the entire *tctDE*-*Xcv*3609 operon (2), DH5 α /pUcit containing only *citH* (3), DH5 α /pUcittctDE containing only *tctDE* (4) and DH5 α /pUOP containing only the *tctDE*-*Xcv*3609 operon (5). *Xcv* genes were moved into *E. coli* with their indigenous promoters via pUC18-modified plasmids. All experiments were carried out twice with similar results.

Fig. 5 Growth and citrate uptake of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) *citH* (citrate transporter) and *tct* mutants, and wild-type strain, in citrate medium. (A) Growth curves of strains in M9-citrate. As growth media were not supplemented with kanamycin (Km), dilutions from cultures of the *citH* and *tct* mutants were plated at the end of the growth period on plates with and without Km to confirm that the integrated plasmids were stable during the experiments. Averages and standard errors from one experiment of three independent experiments with similar results are shown. (B) Growth of the same strains in Simmons citrate medium. Photographs of representative tubes (three for each strain per experiment of two with similar results) were taken after 48, 72, 120 and 168 h of growth.

not as impaired as the *citH* mutant in its growth ability (Fig. 5A). In agreement with these results, the *tct* mutant showed an intermediate uptake of citrate between the *citH* mutant and the wild-type in Simmons citrate medium: although bluing of the medium started to become visible after 48 h of growth in the case of the wild-type, it started to become visible only after 5 days (120 h) of growth, and became clearer after 7 days (168 h), in the case of the *tct* mutant. As expected, no bluing was observed in the medium of the *citH* mutant throughout these experiments (Fig. 5B). These findings confirm that the TctDE

system positively regulates *citH* expression and citrate uptake in *Xcv*.

The TctDE system regulates the expression of *Xcv citH* in culture and *in planta*

To verify whether the TctDE system affects *citH* expression in *Xcv*, *citH* promoter activity was tested in the background of the *tct* mutant containing the *citH* promoter fused to *tnpR-uidA* (strain *XcvPcitHtct*). GUS activity in this strain was assessed following

Fig. 6 β -Glucuronidase (GUS) expression in XcvPcItH and XcvPcItHtct⁻ in M9-citrate medium (A) and *in planta* (B). For assays with bacteria grown in culture, cells were collected at an optical density at 640 nm (OD₆₄₀) of approximately 0.2. Inoculations of tomato (cv. H7998) leaves were performed by syringe infiltration at 10⁶ cfu/mL. At each time point, three samples from three plants were taken for each treatment. GUS assays were performed using the substrate 4-methylumbelliferyl- β -D-glucuronide (MUG). Averages and standard errors from one experiment of two with similar results are shown.

growth in M9-citrate, in comparison with strain XcvPcItH, which contains the same transcriptional fusion but possesses intact *tctDE*. In XcvPcItHtct⁻, *citH* promoter activity was reduced significantly compared with that in strain XcvPcItH (Fig. 6A).

The effect of the TctDE system on *citH* expression was also assessed *in planta* following syringe infiltration of tomato leaves with strains XcvPcItHtct⁻, XcvPcItH and XcvPcItHrev. The latter served as a negative control, as it carries the transcriptional fusion of the *citH* promoter in the opposite orientation relative to the *tnpR-uidA* fusion. At all tested time points (48, 72 and 96 hai), the *citH* promoter activity in XcvPcItHtct⁻ was approximately two orders of magnitude lower than that in XcvPcItH (Fig. 6B). GUS activity in the negative control was too low to be detected reliably (not shown). Together, these results confirm that TctDE positively regulates the expression of *citH*, although its disruption does not completely abolish *citH* transcription.

To test whether the disruption of *tctDE* affects the virulence of Xcv on tomato, symptom development and *in planta* growth of the *tct* mutant were compared with those of the wild-type and the *citH* mutant. Under the tested conditions (dip inoculation with bacterial suspensions at 10⁵ cfu/mL and syringe infiltration at various concentrations), we were unable to detect significant

differences in these parameters between the *tct* mutant and the wild-type (not shown).

Expression of *hrp* genes is not affected by the *citH* mutation

Xcv pathogenicity depends on a functional Hrp TTSS. In *Pseudomonas syringae* pv. *phaseolicola*, the addition of citrate to the medium resulted in a reduction in expression of several *hrp* genes (Rahme *et al.*, 1992). Therefore, we set out to assess whether the reduced virulence of the *citH* mutant could be associated with a negative effect on *hrp* gene transcription.

The expression of *hrpA*, *hrpX* and *hrpG* genes in the *citH* mutant was compared with that in the wild-type in XVM2, an *hrp*-inducing medium (Wengelnik *et al.*, 1996a), by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). *hrpA* encodes an outer membrane structural component of the Hrp TTSS that belongs to the PulD superfamily of proteins (Wengelnik *et al.*, 1996a). *hrpX* encodes an AraC-type regulator of several *hrp* genes (Wengelnik and Bonas, 1996), and *hrpG* encodes a global regulator, which belongs to the OmpR family of RRs of typical TCSs (Wengelnik *et al.*, 1996b). Under the tested conditions, the levels of transcription of these *hrp* genes were probably unaffected by the *citH* mutation (Fig. S2A).

As Hrp TTSS is essential for hypersensitive response (HR) induction in incompatible interactions, we assessed the ability of the *citH* mutant to induce HR in resistant pepper and H7981 tomato plants. These experiments were performed as a means to assess whether the *citH* mutation affects *hrp* expression *in planta*. The tested strains were infiltrated at concentrations of 10⁵ and 10⁸ cfu/mL. In pepper, wild-type 97-2 and *Acidovorax citrulli* M6 served as positive controls for HR induction, whereas the nonpathogenic Xcv 97-2 *hrpA* mutant, defective in its ability to form the Hrp TTSS, served as a negative control. At the lowest inoculation dose, visible HR symptoms could not be detected (not shown). At the highest inoculation dose, faint HR symptoms started to appear at 24 hai for the positive controls, as well as for the *citH* mutant, with HR becoming clearer at 48 hai, and with no differences between the *citH* mutant and the wild-type (Fig. S2B).

In H7981 tomato plants, *A. citrulli* M6 and Xcv 97-2 served as positive controls for HR induction, whereas the 97-2 *hrpA* mutant served as a negative control. The Xcv T1 race strain 110C is virulent on this tomato cultivar, and thus enabled the comparison between compatible and incompatible interactions. Again, the lowest inoculation dose resulted in no visible HR, but, at the highest inoculum concentration, HR induced by *A. citrulli* M6, Xcv 97-2 and the Xcv *citH* mutant was clearly visible at 48 hai, whereas no symptoms were detected following inoculation with the *hrpA* mutant and strain 110C at this early time

Fig. 7 Individual and mixed inoculation of the *citH* (citrate transporter) mutant and wild-type 97-2 by syringe infiltration of tomato (cv. H7998) leaves at 10^7 cfu/mL. Three plants were inoculated with an equal mixture of wild-type and mutant strains (or with each strain alone). Five leaf discs were removed at each time point from each plant and macerated for serial dilutions and plating. Averages and standard errors from one experiment of three with similar results are shown. (A) *In planta* growth of the *citH* mutant and wild-type strains, individually inoculated (left panel) or co-inoculated (right panel). t_0 , t_2 and t_3 represent 0, 2 and 3 days after inoculation (dai), respectively. (B) Competitive index (CI) and relative increase ratio (RIR) values, calculated as described by Macho *et al.* (2007). CI is defined for co-inoculations as the mutant-to-wild-type output ratio divided by the mutant-to-wild-type input ratio. RIR is calculated with the same formula but for individual inoculations. No significant differences ($P = 0.05$) were observed between individual and mixed inoculations at t_2 and t_3 as determined by Student's *t*-test.

point (Fig. S2B). Together, these results indicate that disruption of *citH* probably does not affect *hrp* gene expression in *Xcv*.

The *citH* mutant is not complemented for growth *in planta* by the wild-type

The *citH* mutant is compromised in its ability to cause disease symptoms and to grow *in planta* relative to the wild-type (Tamir-Ariel *et al.*, 2007). Here, several experiments were carried out in which the wild-type and the *citH* mutant were co-inoculated using different inoculum concentrations to assess whether there was an interaction when both strains coexisted in the same niche. The occurrence of such an interaction could point to a possible mechanism by which *citH* influences *Xcv* virulence.

In co-inoculation experiments using relatively low bacterial concentrations (10^3 cfu/mL of each strain), the mutant grew to significantly lower levels than the wild-type, and the growth patterns did not differ from those observed in individual inoculations (not shown), suggesting that the presence of the wild-type does not complement the mutant under these conditions. Macho *et al.* (2007) have reported that the inoculation dose influences the complementation of growth *in planta* of a mixed population of phytopathogenic bacteria. Their findings indicate that the complementation of a mutant affected in growth *in planta* may occur when highly concentrated inocula are used. Therefore, we also co-inoculated the *citH* mutant and the wild-type using a mixture of 10^7 cfu/mL from each strain. These experiments also indicated that, when co-inoculated, the growth of the *citH* mutant could not be complemented by the wild-type (Fig. 7).

DISCUSSION

We have previously identified *citH* as a novel virulence gene of *Xcv* (Tamir-Ariel *et al.*, 2007). Here, several experiments were performed that demonstrate that *citH* encodes a specific citrate transporter. The *citH* mutant was considerably impaired in growth compared with the wild-type and the *citH*-complemented strains when citrate was the carbon source. In contrast, the *citH* mutant was able to grow as well as the wild-type on other tested carbon sources, including other tricarboxylic acid (TCA) cycle intermediates, confirming that this mutant is not impaired in general metabolism, and that the *citH* mutation specifically affects its ability to grow on citrate.

As many of the characterized di- and tricarboxylate transporter genes are induced by the presence of their substrates (Janausch *et al.*, 2002; Warner and Lolkema, 2002), we aimed to determine the conditions under which the *citH* promoter is activated. In particular, we asked whether *citH* transcription is specifically induced by citrate. Indeed, using RIVET, we showed that the induction of the *citH* promoter occurred almost exclusively in the presence of citrate. Nevertheless, a surprising finding from these experiments was that, although *citH* induction was not observed in the presence of sucrose or fructose as sole carbon source, it did occur with a combination of both compounds. Interestingly, in the presence of combined sucrose and fructose, resolution also occurred in the ResPcitHrev strain, carrying the *citH* promoter in the opposite direction relative to *citH* transcription. This finding suggests the possible occurrence of a cryptic promoter towards the opposite direction of the *citH* neighbour

gene, *oprP2* (Fig. 1); however, this hypothesis should be verified further. The resolution pattern observed in the presence of sucrose and fructose remains unclear at this point.

We also report novel regulatory elements of the citrate transporter belonging to a TCS. TCSs have been shown to regulate the transporters of organic acids in bacteria (Janausch *et al.*, 2002; Yamamoto *et al.*, 2000). Thus, we looked at an operon containing a predicted TCS, annotated as *tctD-tctE*, which is closely linked to *citH* in the *Xcv* chromosome. *tctD* and *tctE* encode putative RR and HK, respectively, showing homology to TctD and TctE of *Salmonella enterica* serovar *typhimurium*. In this bacterium, the TctDE system regulates the *tctI* operon, encoding the structural components of a tricarboxylate transporter (Valdivia *et al.*, 2000; Widenhorn *et al.*, 1989). Here, characterization of a *tct* mutant of *Xcv*, as well as heterologous expression of the *Xcv citH* and *tctDE* genes in *E. coli*, demonstrated that the TctDE system positively regulates the expression of *citH*. Importantly, disruption of *tctDE* did not completely abolish *citH* expression in *Xcv*; thus, it appears that, in the absence of functional TctDE, there is a basal level of *citH* expression, which could be mediated by other, yet unidentified, regulatory elements.

Under the tested conditions, the *tct* mutant did not show reduced virulence compared with the wild-type strain. It is well known that many virulence determinants in plant pathogenic bacteria possess a subtle phenotype. This is often a result of a lack of sensitivity of the available virulence assays, combined with functional redundancy and/or a relatively slight contribution to virulence of each determinant (Alfano and Collmer, 1996).

To explore how *citH* contributes to the virulence of *Xcv*, we looked at several aspects of the plant–pathogen interaction. In *P. syringae* pv. *phaseolicola*, the expression of *hrp* genes is affected by carbohydrates, amino acids and organic acids. In this bacterium, when citrate was added to minimal medium as the sole carbon source, the expression of *hrpC* was reduced; when it was added in tandem with fructose or sucrose, the expression of *hrpAB*, *hrpC* and *hrpD* was reduced (Rahme *et al.*, 1992). In *Xcv*, under the tested conditions in culture and *in planta*, *hrp* gene expression was not affected by the *citH* mutation, indicating that the effects of citrate transport on *Xcv* virulence are probably independent of the secretion of virulence effectors through Hrp TTSS.

Our data indicate that the *citH* mutation does not affect the HR-inducing ability of *Xcv*. These findings are similar to those recently reported by Mellgren *et al.* (2009) for *P. syringae* pv. *tomato*. The authors found that *mgo*, encoding the TCA enzyme malate:quinone oxidoreductase, and *dctA1*, probably encoding a dicarboxylate transporter, were required for normal growth *in planta* of this bacterium on *Arabidopsis thaliana*. HR assays revealed that the *mgo* mutation did not affect *hrp* expression in *P. syringae* pv. *tomato*, and the defective phenotypes of both

mgo and *dctA1* mutants were attributed to a nutritional deficiency (Mellgren *et al.*, 2009). In our study, the lack of differences in growth ability between the *citH* mutant and the wild-type in apoplast extracts could suggest that the *citH* mutation does not affect the virulence of *Xcv* through a nutritional defect. However, the possibility that CitH confers a nutritional advantage to *Xcv* cannot be discarded. First, these were *in vitro* assays, which may not reflect the complexity of a dynamic plant–pathogen interaction. Second, both mutant and wild-type grew relatively poorly in apoplast extracts under the tested conditions. Third, we also showed that the *citH* mutant could not be complemented for growth by the wild-type in co-inoculation experiments, a fact that may point to a nutritional deficiency of the former.

As the pH of the citrate medium increases during the growth of *Xcv*, it can also be hypothesized that citrate uptake by *Xcv in planta* could contribute to create more favourable conditions for infection. This stems from the fact that alkalization of the host apoplast is required for the successful establishment of many phytopathogenic bacteria (Alfano and Collmer, 1996, 2004). However, if this was the case, one would expect that the *citH* mutant would benefit from the presence of the wild-type in the plant apoplast, which did not occur in co-inoculation experiments. Therefore, it is not likely that the role of CitH in virulence is associated with apoplast alkanization.

As the timing of gene induction during the infection process may provide clues with regard to the role played by a specific gene product in pathogenicity, we attempted to determine when the *citH* promoter is activated *in planta*. Induction of the *citH* promoter was compared with that of the promoters of two previously characterized *hrp* genes, *hrpG* and *hrpA* (Wengelnik *et al.*, 1996a, b). *hrpG* and *hrpA* must be activated early on in the disease process to allow the pathogen to go undetected by the host defence mechanisms and to gain access to host intracellular reservoirs. Our analyses showed that, under the conditions tested, the *citH* promoter was also induced early during the first hours of the infection process. Previously, we have shown that *citH* expression continues for at least 96 hai (Tamir-Ariel *et al.*, 2007). Taken together, it appears that CitH might be needed from early on in the infection process until later stages of the interaction with the host tissue.

This report expands our knowledge on a novel virulence factor of *Xcv*, and sheds some light on its regulation. Although, from co-inoculation experiments, it appears that CitH-mediated citrate uptake may confer a nutritional advantage to *Xcv* during infection, the literature provides some clues about other possible roles for citrate that can be studied in the future. Citrate is known to be a good chelating agent. It forms complexes with metal ions, thereby allowing a more efficient transport of these micronutrients (Lopez-Bucio *et al.*, 2000). In addition, citrate can potentially participate in the maintenance of osmotic and ionic (in addition of pH) balances (Lopez-Bucio *et al.*, 2000). Another

possibility is that citrate is needed for the functionality of as yet unidentified molecules in *Xcv* that contribute to its virulence. Finally, recent studies have implicated the TCA cycle as playing a role in the maintenance of the balance of reactive oxygen species (ROS) (Mailloux *et al.*, 2007). Thus, if *Xcv* competes for the citrate reservoirs, the plant may be less efficient in producing ROS, and the pathogen may be better equipped to deal with the oxidative stress imposed by the plant.

Support for the importance of citrate in plant–microbial interactions comes from several studies. Genes encoding TCA cycle enzymes have been identified in several other IVET-based studies of bacteria in diverse plant niches, such as *Ralstonia solanacearum* in the xylem, *Erwinia amylovora* in fruit tissue, *P. syringae* pv. *syringae* in the phyllosphere and *P. fluorescens* in the rhizosphere (Brown and Allen, 2004; Marco *et al.*, 2003; Rainey, 1999; Zhao *et al.*, 2005). In contrast, these genes were not detected in IVET studies of bacterial interactions with animal hosts, possibly pointing to their specificity for plant–microbe interactions (Brown and Allen, 2004). Importantly, two recent studies point to the importance of citrate in plant–microbe interactions. In one study, Rico and Preston (2008) demonstrated that the metabolic pathway that enables citrate utilization by *P. syringae* pv. *tomato* is specifically induced in tomato leaf apoplast. A second study showed that the interruption of a citrate transporter in *Pectobacterium atrosepticum* results in reduced virulence of this pathogen on potato tubers (Urbany and Neuhaus, 2008).

Finally, it is important to mention that the requirements of functional *citH* to achieve both wild-type levels of virulence *in planta* and to grow on citrate as sole carbon source were observed in our experiments despite the fact that, according to the *Xcv* genome annotation, this bacterium contains another gene encoding a putative citrate transporter (Xcv3602, encoding a protein that is 72% homologous to a citrate:H⁺ symporter from *Klebsiella pneumoniae*). If this gene indeed encodes an additional citrate transporter, whether it possesses a role in the *Xcv*–host interaction, and under what conditions it is expressed, are questions we intend to study in the future.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *Xcv* 97-2 (race T3; *X. perforans*, according to the new nomenclature; Jones *et al.*, 2004) and mutant strains were grown on NA/NB (Becton, Dickinson and Co., Sparks, MD, USA), XVM2 minimal medium (Wengelnik *et al.*, 1996a) or modified M9 minimal medium at 28 °C. M9 medium contained 2.5 mg/mL NaCl, 5 mg/mL NH₄Cl, 0.3% casamino acid, 5 mM MgSO₄, 0.1 mM CaCl and different carbon sources that were

added to a final concentration of 10 mM. M9 medium was buffered with appropriate buffers according to the desired pH as follows. For *in vitro* resolution experiments, phosphate buffers were used for media in the pH range 5.7–8, and potassium hydrogen phthalate buffer (0.01 M potassium hydrogen phthalate and 0.0045 M NaOH) was used when the desired pH of the medium was pH 5. For growth curves of *Xcv* strains in M9 medium with different carbon sources (including citrate), and for the quantification of citrate remaining in M9-citrate following growth, media were buffered to pH 7 with phosphate buffer (0.02 M monobasic sodium phosphate, 0.03 M dibasic sodium phosphate; pH 7). *Escherichia coli* strains were routinely grown on Luria–Bertani (LB) medium at 37 °C. Liquid and solid Simmons citrate media (Simmons, 1926) were used to qualitatively assess the pH change in the medium following citrate uptake by *E. coli* and *Xcv* strains. Antibiotics were added at the indicated concentrations: Km, 25 µg/mL; cephalixin (Cp), 20 µg/mL; Gm, 30 µg/mL in solid medium and 10–15 µg/mL in broth; chloramphenicol (Cm), 25 µg/mL; ampicillin (Ap), 100 µg/mL.

Genetic manipulations

Routine molecular manipulations and cloning procedures were carried out as described previously (Sambrook *et al.*, 1989). Unless stated otherwise, enzymes were purchased from Fermentas (Burlington, ON, Canada). Kits for plasmid and PCR product extraction and purification were purchased from Real Biotech Corporation (Taipei, Taiwan). Genomic DNA from *Xcv* was prepared with the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Southern blot hybridization was performed using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Oligonucleotide primers used in this study were purchased from Hy Laboratories (Rehovot, Israel) and are listed in Table S1 (see Supporting Information).

Generation of a *citH*-complemented strain

This strain was created by amplification of the *citH* region from *Xcv* 97-2 by PCR using FBamPcitH and RCitcompNewSma as primers, followed by TA cloning into pTZ57R/T (Fermentas). The cloned fragment was then verified, excised with *Sma*I and cloned into pML122, previously digested with the same enzyme and dephosphorylated. This vector, named pMLcit, was verified by restriction digests and electroporated into the *citH* mutant to create a *citH*-complemented strain (*Xcv citH* comp; Fig. 1).

Extraction of tomato leaf apoplast and bacterial growth in apoplast medium

Tomato leaf apoplast was extracted by submerging detached leaflets in water under light and alternately applying and releas-

ing vacuum until the leaflets were completely infiltrated. The leaflets were then blotted dry, rolled into 5-mL syringes and placed in 15-mL Falcon tubes. The tubes were centrifuged at 4 °C for 6 min at 1000 *g*. A fraction of the liquid obtained was immediately used for analysis of the activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PD), as an indicator for cytoplasmic contamination of the samples. The rest of the extract was stored at –80 °C until use as growth medium, at which point it was filtered through a 0.4-µm-pore-size nitrocellulose membrane, and the appropriate antibiotic was added. G6PD was assayed spectrophotometrically (340 nm) at 25 °C. To 0.05 mL of extract, 0.05% β-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and a protease inhibitor tablet (Roche, Indianapolis, IN, USA) were added. This mix was then added to 0.89 mL of 50 mM glycylglycine buffer (pH 7.5) containing 0.05 mL of nicotinamide adenine dinucleotide phosphate (NADP) (6 mM). The reaction was started by the addition of 0.05 mL of glucose-6-phosphate (40 mM). Activity was measured as the rate of increase in absorbance at 340 nm (ΔA_{340}). The activity of the apoplast extract measured in multiple trials was determined to be approximately 1% of that of whole leaf extract. Cultures were grown in 0.6 mL of apoplast medium in 15-mL tubes. Samples of 0.11 mL were removed during the exponential and stationary phases of growth, and placed into 96-well plates for spectrophotometric measurement of growth at 640 nm. The same samples were used for the quantification of citrate as described below.

Quantification of citrate in medium after bacterial growth

The quantification of citrate in growth medium was determined enzymatically using the ENZYTEC™ Citric Acid Kit (Scil Diagnostics GmbH, Viernheim, Germany). The kit was adapted for use in 96-well microplates, such that, to 0.1 mL of kit solution 1, 0.1 mL of apoplast extract, or 0.02 mL of minimal medium (diluted 10-fold), was added. Double-distilled water was then added to a final volume of 0.3 mL. The mixture was mixed and, after 5 min at 25 °C, A_{1340} (the absorbance at 340 nm) was read. Then, 0.002 mL of kit solution 2 was added and mixed and, after 10 min at 25 °C, A_{2340} was read. Each microplate had a blank (medium without bacteria) and standard solutions. $\Delta A_{\text{citrate}}$ was determined as follows: $\Delta A_{\text{citrate}} = (A_{1340} - A_{2340})_{\text{sample}} / \text{standard} - (A_{1340} - A_{2340})_{\text{blank}}$. The concentration of citrate in µg/µL was determined based on the standard curve.

Generation of *Xcv* mutants for the assessment of *tnpR*-mediated resolution driven by the *citH* promoter

A 658-bp DNA fragment from strain 97-2, upstream of the *citH* ORF (thus containing the *citH* promoter), was amplified using

primers FBamPcitH and RBamXhoPcitH, and cloned upstream of a promoterless *tnpR-uidA*, to create a transcriptional fusion, as follows: the amplified PCR product was digested with *Bam*HI and cloned into pJPGmntpR, which was cut with the same enzyme and dephosphorylated. The orientation of the cloned fragment was determined by restriction analysis, as well as by PCR and sequencing of the cloned fragment. In pPcitH, the *citH* promoter was cloned in the same orientation as the transcription of *tnpR-uidA*, whereas, in pPcitHrev, the promoter was cloned in the opposite orientation to *tnpR-uidA*. The two vectors were conjugated into *Xcv* 97-2 to obtain *XcvPcitH* and *XcvPcitHrev*, respectively, and into *XcvRes*, to obtain *ResPcitH*, and *ResPcitHrev*, respectively.

Resolution of the *res* cassette for assessment of promoter activity in culture

Cells were grown in 5 mL of NB supplemented with Km for 48 h at 28 °C with shaking. Then, a 1:500 dilution was made in 5 mL of fresh medium to be tested, and cells were grown at similar conditions to the stationary growth phase. Three replicates were made for each growth medium. Serial dilutions were plated from each replicate onto NA (to obtain the total number of cfu) and onto NA supplemented with Km (to obtain the number of unresolved cfu). To obtain the number of resolved cfu, the average number of unresolved cfu was subtracted from the average of the total number of cfu. The percentage resolution was determined based on the ratio of resolved cfu to the total number of cfu: % resolution = [(average total cfu – average unresolved cfu)/average total cfu] × 100.

Plant material and inoculation procedures

Tomato (*S. lycopersicum* L.) cultivar Hawaii 7998 (H7998; Yu *et al.*, 1995) was grown from seeds in a glasshouse (25–28 °C). Inoculation of plants for the assessment of growth *in planta* was carried out by dipping 4–5-week-old plants into bacterial suspensions of 10⁵ cfu/mL containing 0.02% of the surfactant Silwet L-77 and 10 mM MgCl₂, as described previously (Tamir-Ariel *et al.*, 2007). Serial dilutions of plant extracts were plated on plates supplemented with appropriate antibiotics. Some of the dilutions were also plated on medium with no antibiotics, which confirmed that there was no loss of the integrated plasmids in the mutants (similar tests were also performed for *in vitro* experiments). In co-inoculation experiments, growth *in planta* was determined following syringe infiltration of leaves of 4–5-week-old plants with bacterial suspensions of 10³ or 10⁷ cfu/mL in 1 mM MgCl₂. Leaf discs were extracted and treated as described previously (Tamir-Ariel *et al.*, 2007). The assessed strains were discriminated on the basis of antibiotic resistance (the *citH* mutant is resistant to Km, whereas the

wild-type is not). Competitive index (CI) and relative increase ratio (RIR) values were obtained as described by Macho *et al.* (2007). Briefly, CI and RIR were calculated from mixed and individual inoculations, respectively. CI was calculated as the mutant-to-wild-type output ratio divided by the mutant-to-wild-type input ratio. RIR was calculated with the same formula using the data from individual inoculations. CI and RIR values were analysed by Student's *t*-test. To determine promoter induction *in planta* by resolution or GUS assays, plants were syringe infiltrated with bacterial suspensions of 10^6 cfu/mL in 1 mM MgCl₂. HR assays were performed on 4–5-week-old plant leaves of pepper (*C. annuum* var. *annuum* California type) and tomato cv. Hawaii 7981 (H7981; Yu *et al.*, 1995) by syringe infiltration with bacterial suspensions of 10^5 or 10^8 cfu/mL in 1 mM MgCl₂.

Generation of *E. coli* strains for heterologous expression of *citH*

The *Xcv citH* gene with its indigenous promoter was excised from plasmid pMLcit using *Sma*I, and cloned into pUC18, previously digested with *Sma*I and dephosphorylated. The resulting vector, named pUcit, was electroporated into DH5 α to create DH5 α /pUcit. For the generation of strain DH5 α /pUcitctDE, *tctDE* with its indigenous promoter was PCR amplified with the proofreading enzyme Phusion™ Hot Start (Finnzymes, Espoo, Finland) using primers FHindtctDE and RHindtctDE. The resulting fragment was treated with *Hind*III and cloned into pUcit previously treated with the same enzyme. Strain DH5 α /pUcitOP was created in a similar way but, in this case, the entire *tctDE*-*Xcv3609* operon was PCR amplified using primers FHindtctDEop-er-on and RHindtctDEop-er-on, and cloned into pUcit, following treatment with *Hind*III. As controls, *tctDE* and *tctDE*-*Xcv3609* were similarly cloned into pUC18 (without *citH*) to generate plasmids pUcttDE and pUOP, and strains DH5 α /pUcttDE and DH5 α /pUOP, respectively (Fig. 1).

Generation of *tct* and *XcvPcitHtct*[−] mutants

tctDE mutant strains were generated in the background of wild-type 97-2 and *XcvPcitH* containing the *PcitH::tnpR::uidA* transcriptional fusion. Primers FtctDSac and RtctDSac were designed to amplify an internal region within the *tctD* ORF. The PCR product was sequenced and cloned into pHPKm, a pHP45 Ω derivative containing a Km resistance gene instead of the original streptomycin resistance cassette (Tamir-Ariel *et al.*, 2007). The resulting suicide vector, designated pHKmtctD, was transformed into strains 97-2 and *XcvPcitH* to generate *Xcv tct* and *XcvPcitHtct*[−] mutants, respectively (Fig. 1), which were verified by Southern blot.

Growth curves in M9 medium

Growth curve experiments with *Xcv* strains were performed in modified M9 medium with different carbon sources (at 10 mM, as described above). *Xcv* strains were previously grown for 24 h in NB, and then washed twice by centrifugation (3000 *g*, 4 °C, 10 min) and resuspended in the corresponding M9 medium. Cells were then transferred to 30 mL of fresh medium in 100-mL Erlenmeyer flasks (three per treatment) to an initial OD₆₄₀ of about 0.005, with the exception of growth curves in M9-citrate with Gm (see below), where the initial OD₆₄₀ was ~0.05. Cultures were grown at 28 °C with shaking (200 r.p.m.), and growth was assessed using a Helios Gamma (Thermo Spectronic, Cambridge, UK) spectrophotometer. For growth curves on M9-citrate that included the *citH*-complemented strain (*citH* mutant carrying pMLcit), wild-type and *citH* mutant strains, both carrying pML122, were used, and Gm was added at 10 μ g/mL to both NB and M9-citrate. Growth curves of *E. coli* strains carrying pUC18-derived plasmids in M9-citrate were performed similarly, with the following modifications: *E. coli* strains were previously grown in LB, Ap (100 μ g/mL) was added to the medium and the strains were grown at 37 °C.

Assessment of *citH* promoter activity by GUS assays

The effect of the *tct* mutation on *citH* promoter activity was assessed *in planta* and in culture by GUS (*uidA*) assays with the substrate 4-methylumbelliferyl- β -D-glucuronide (MUG). For assays with bacteria grown in culture, cells were grown in M9-citrate and collected at OD₆₄₀ ~ 0.2. Assays were performed as described previously (Tamir-Ariel *et al.*, 2007). For *in planta* assays, bacteria at 10^6 cfu/mL in 1 mM MgCl₂ were inoculated into leaves of 5-week-old plants as described above. Each mutant was inoculated into three plants, and samples from each plant were collected at 48, 72 and 96 hai, with a 0.8-cm-diameter corkscrew, and macerated. Macerated tissues were centrifuged at low speed (1000 *g*) for 30 s to pellet the plant material. The supernatants were transferred to a clean tube and centrifuged again at 12 000 *g* for 2 min to pellet bacterial cells. The pellets were then resuspended in double-distilled water, and samples from each treatment were diluted and plated for cfu counts. The remaining cells were stored at −80 °C until use for GUS activity measurements, as described previously (Jefferson *et al.*, 1987).

Semiquantitative RT-PCR to assess the expression of *hrp* genes

The *citH* mutant and the wild-type were grown in NB, and then diluted 1:1000 into XVM2, from which cultures were collected during exponential growth. XVM2 was chosen as it is known to induce the expression of *hrp* genes (Wengelnik *et al.*, 1996a), and has also been shown to induce the expression of *citH*

(Tamir-Ariel *et al.*, 2007; this study). RNA extraction was carried out using the MasterPure™ RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). A further DNA degradation treatment was conducted using TURBO DNase (MO BIO Laboratories Inc., Carlsbad, CA, USA). cDNA was prepared with random hexamers using an ImProm-II(tm) Reverse Transcription System (Promega, Madison, WI, USA). PCRs were carried out in an Eppendorf (Hamburg, Germany) thermal cycler using REDTaq PCR ReadyMix (Sigma-Aldrich). Specific primer sets, F16sRNA-RT/R16sRNA-RT, FhrpA-RT/RhrpA-RT, FhrpXv-RT/RhrpXv-RT and FhrpG-RT/RhrpG-RT, were used for amplification of 16sRNA, *hrpA*, *hrpX* and *hrpG* cDNA, respectively. The PCR proceeded for 30 cycles of denaturation at 95 °C for 4 min, annealing at 57 °C for 45 s and elongation at 72 °C for 50 s. Samples were collected after 10, 15, 20, 25 and 30 cycles, and separated by electrophoresis in 1% agarose gels. The gels were stained in an ethidium bromide solution (0.5 µg/mL) for 20 min and photographed with transmitted UV light at 295 nm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Growth curves of *citH* mutant and wild-type strain in M9 minimal medium supplemented with different substrates as carbon sources (at 10 mM). Growth curves in M9-citrate medium also included the *citH*-complemented strain (*citH* mutant carrying pMLcit). In M9-citrate, wild-type and *citH* mutant carrying pML122 were used, and gentamicin was added to the medium at 10 µg/mL. Representative data (averages and standard errors) from one experiment (of at least two per carbon source with similar results) are shown.

Fig. S2 Effects of the *citH* disruption in *hrp* gene expression. (A) Effect on mRNA expression levels of *hrpA*, *hrpX* and *hrpG* in culture. Semiquantitative reverse transcription-polymerase chain reactions (RT-PCRs) were performed with total RNA prepared from cultures of the *citH* mutant (c) and wild-type 97-2 (wt) strains, grown to an optical density at 640 nm (OD₆₄₀) of 0.2 in XVM2. Numbers indicate the amplification cycles from which samples were run in the gel. (B) Hypersensitive response (HR) on California-type pepper (left) and tomato H7981 (right) following syringe infiltration with bacteria at 10⁸ cfu/mL. Photographs were taken at 48 h after inoculation (hai). Treatments: *Acidovorax citrulli* M6 (positive control for HR), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) 97-2 and the 97-2 *citH* and *hrpA* mutants (*citH*⁻ and *hrpA*⁻, respectively). On tomato, an additional treatment was Xcv 110C. In contrast with strain 97-2, which is avirulent on this cultivar (thus inducing HR), strain 110C is virulent on this cultivar; therefore no symptoms are observed at 48 hai. All experiments were performed twice with similar results.

Table S1 Primers used in this study.

Table S2 Similarities of the *Xanthomonas campestris* pv. *vesicatoria* (Xcv) TctDE components to the proteins of other two-component systems (TCSs).

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