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Visualisation of *hrp* gene expression in *Xanthomonas euvesicatoria* in the tomato phyllosphere

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Abstract The plasmid pUFZ75 conferred constitutive GFP expression on the bacterial pathogen *Xanthomonas euvesicatoria* (syn. *X. campestris* pv. *vesicatoria*). Colonisation of the tomato phyllosphere and invasion of tomato leaves by *X. euvesicatoria* was examined using both fluorescence and confocal laser scanning microscopy. *Xanthomonas euvesicatoria* established a limited population on the tomato leaf surface, primarily occupying the depressions between epidermal cells and around the stomata, prior to invasion of the leaf via the stomata and subsequent growth within the substomatal chamber and the leaf apoplast. Additionally, *hrp-gfp* fusions were used to report on the temporal and spatial expression of *hrp* genes during epiphytic colonisation and invasion. *Xanthomonas euvesicatoria* cells carrying *hrpG*- and *hrpX-gfp* reporter constructs were not fluorescent *in vitro* on non-*hrp*-inducing LB agar but did exhibit a low level of fluorescence on the leaf surface within 24 h of inoculation, particularly in the vicinity of stomata. Cells carrying *hrpG*- and *hrpX-gfp* fusions exhibited high levels of fluorescence 72 h after inoculation in the substomatal chamber and the leaf apoplast. Cells carrying the *hrpF-gfp* fusion were slightly fluorescent on LB agar and showed no further increase in fluorescence on the leaf surface by 24 h after inoculation, but did show a significant increase in fluorescence 72 h after inoculation in the substomatal chamber and apoplast. The apparent low-level induction of the regulators *hrpG* and *hrpX* on the tomato leaf surface may suggest that some of the genes of the *X. euvesicatoria* HrpG/HrpX regulon are up- or down-regulated prior to invasion of the stomata while still on the leaf surface.

Keywords *Xanthomonas* · Tomato · GFP · *hrp* · Confocal microscopy

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Introduction

The pathogen, *Xanthomonas euvesicatoria* (syn. *Xanthomonas campestris* pv. *vesicatoria*), causes bacterial spot of tomato. While determinants of pathogenicity and R-Avr interaction mechanisms have been studied extensively in *X. euvesicatoria* (Bonas and Lahaye 2002), the nature of the early interactions between pathogen and compatible host is much less well understood, particularly in comparison with *Pseudomonas syringae* (Preston 2000). *Xanthomonas euvesicatoria* is known to invade the host through natural openings, including stomata and hydathodes, and through wounds (Rudolph 1993); however, much less is known about the events immediately prior to invasion. *Xanthomonas euvesicatoria* has been

reported to exhibit a so-called epiphytic phase (McGuire et al. 1991), implying significant growth on the leaf surface prior to invasion. Such epiphytic growth is believed to be an important aspect of the infection process in most *P. syringae* pathovars (Wilson et al. 1999). However, studies on the early stages of growth in *X. euvesicatoria* are lacking. Improved understanding of the early events following arrival of the pathogen on the leaf surface of a susceptible plant, necessitate microscopic examination of plants inoculated under controlled conditions.

The early growth of the pathogen on the leaf surface has been examined microscopically in only a few *Xanthomonas*-host pathosystems, employing scanning electron microscopy (e.g. De Cleene 1989); immuno-fluorescence microscopy (e.g. Daniel and Boher 1981); or bioluminescence labelling with a charge-couple device camera attached to a light microscope (Dane and Marten 1994). Only in recent years has the green fluorescent protein (GFP) been used to monitor surface growth or infection by bacteria associated with aerial plant surfaces. GFP in conjunction with fluorescence microscopy (FM) has been used to study the interactions between *X. campestris* pv. *campestris* and cabbage (*Brassica oleracea*) (So et al. 2002), and *Xanthomonas oryzae* pv. *oryzae* and rice (Han et al. 2008). GFP has not, to our knowledge, been used to study epiphytic growth and colonisation of tomato leaves by *X. euvesicatoria*, particularly not in conjunction with confocal laser scanning microscopy (CLSM). The first goal of this study, therefore, was to develop a stable, constitutive GFP expression system for *X. euvesicatoria* and to use this system with both FM and CLSM to monitor epiphytic growth and colonisation, as well as invasion of stomata and growth in the mesophyll tissue.

In addition to monitoring growth and infection of bacteria, GFP also has been used as a reporter of *in planta* gene expression in leaf-associated bacteria (e.g. Leveau and Lindow 2001). Pathogenicity of the bacterial plant pathogens *Xanthomonas* and *Pseudomonas* requires expression of *hrc* and *hrp* genes, leading to the development of a type III secretion (T3S) system and secretion of effector proteins into the host cell (Buttner and Bonas 2006; Mudgett 2005). As is the case for the infection process, *hrc* and *hrp* gene regulation and *in planta* expression are best understood in the plant pathogen *P. syringae* (Preston 2000). *Pseudomonas syringae* pv. *tomato* strain DC3000 exhibited *hrp* gene induction following spray inoculation onto the leaves of tomato only once the pathogen had colonised the substomatal cavity and mesophyll tissue (Boureau et al. 2002; Preston 2000). No *hrpA-gfp* expression was detected in *P. syringae* cells on the leaf surface, however, and it was suggested that *hrp* genes probably were not required for growth on the leaf (Boureau et al. 2002; Preston 2000).

Substantial progress has been made in understanding *hrc* and *hrp* gene expression and regulation in *X. euvesicatoria* (Bonas et al. 2000; Buttner and Bonas 2006). HrpG is thought to be the response regulator of a putative two-component system in which the as yet unidentified sensor presumably detects environmental variables which induce the *hrc* and *hrp* genes (Wengelnik et al. 1996). Activation of HrpG activates expression of *hrpA* and *hrpX* (Wengelnik et al. 1996). HrpX is believed to activate expression of the remaining *hrc* and *hrp* genes (Wengelnik and Bonas 1996; Koebnik et al. 2006), the products of which are involved in T3S system structure and function or are effector proteins secreted into the plant cell (Mudgett 2005).

The *hrp* genes of *X. euvesicatoria* are known to be induced *in planta* following vacuum infiltration into leaf tissue (Schulte and Bonas 1992) and on defined *hrp*-inducing media (Wengelnik and Bonas 1996; Wengelnik et al. 1999; Wengelnik et al. 1996). However, no reporter studies have been conducted to determine where and when the *hrc/hrp* genes are first induced *in planta* following inoculation of the leaf surface. The second goal of this study was to use *hrp-gfp* reporters in *X. euvesicatoria* with FM and CLSM to examine *hrp* gene expression *in planta* following simulated natural infection of the tomato leaf surface under controlled environmental conditions. Specifically, to determine whether the *hrp* genes are expressed on the leaf surface, prior to invasion through the stomata, or only expressed on the leaf interior, in the substomatal cavity or in the apoplast of the mesophyll tissue.

Materials and methods

Media, strains, bacterial growth conditions, and chemicals

Xanthomonas euvesicatoria 75-3R is resistant to 100 μg ml^{-1} rifampicin and 75-3RS is resistant to

100 μg ml^{-1} rifampicin and 100 μg ml^{-1} streptomycin (Moss 2000). *Escherichia coli* and *X. euvesicatoria* were grown on nutrient agar (NA) (Difco, St. Louis, MO, USA.), in nutrient broth (NB) (Difco), on Luria-Bertani (LB) agar (Difco), or in LB broth (Difco), except where noted. *Escherichia coli* was grown at 37°C and *Xanthomonas* strains were grown at 28°C. When required, the following antibiotics were added to media at the concentrations specified: kanamycin (Km) 50 μg ml^{-1}; rifampicin (Rf) 100 μg ml^{-1}; streptomycin (Sm) 100 μg ml^{-1}; ampicillin (Ap) 100 μg ml^{-1}; and tetracycline (Tc) 20 μg ml^{-1}. Restriction and DNA modifiying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were synthesised by Sigma-Genosys (St. Louis, MO, USA).

Construction of a plasmid conferring constitutive gfp expression

The P_{trp}-*gfp* cassette was PCR amplified from pPROBE-NTtrp (Miller and Lindow 1997; Miller *et al.*, unpublished) using primers P1 (5'GCTCTAA GAAGCTTGGCAAATATACTGAAATAGG3'), which binds to the 5' end of P_{trp}, and P115 (5'GCTCGAATTCCTATTTGTATAGTTCATCC3'), which binds to the 3' end of *gfp*. The PCR product was cloned into pGEM-T creating pUFZ70 (Table 1). The *Xba*I-*Pst*I fragment carrying the promoter P_{trp} was excised from pUFZ70 and cloned upstream of the GFP cassette from pGreenTIR (Miller and Lindow 1997) (Table 1). This GFP cassette carries an enhanced translation initiation region (TIR) upstream of a *gfp* allele which has the S65T 'red-shift' mutation (Heim et al. 1995) and the F64L mutation that improves solubility (Cormack et al. 1996). The P_{trp}-TIR-*gfp* fusion on an *Xba*I to *Kpn*I fragment was cloned into the *Xanthomonas*-stable plasmid pUFR034 (DeFeyter et al. 1990) to create pUFZ75 (Table 1). The plasmid pUFZ75 was mobilised from *E. coli* strain JM109 into *X. euvesicatoria* strain 75-3R by triparental mating using the helper strain *E. coli* CK600(pRK2013::Tn*7*). *In vitro* fluorescence was confirmed by examining cells harvested from non-*hrp*-inducing medium using FM. Plasmid stability was assessed *in vitro* by transfer on non-selective medium followed by plating on media with and without selective antibiotics.

Construction of reporter constructs to monitor hrp gene expression in planta

To create *hrp-gfp* reporters, the upstream regions of *hrpG*, *hrpX*, or *hrpF* were cloned in front of a GFP cassette from pGreenTIR. Truncated, upstream portions of the genes were used, rather than just the promoter, because the cloning strategies employed made use of naturally-occurring restriction sites in the respective *hrp* genes. These gene fusions were cloned into *Xanthomonas*-stable vectors selected according to the availability of appropriate restriction sites, including pUFR034 (DeFeyter et al. 1990); pVSP61 (Loper and Lindow 1994); and pLAFR6 (Bonas et al. 1989). In each case, *in vitro* fluorescence and *in vitro* plasmid stability were assessed.

hrpG: The upstream region of *hrpG* (*Xba*I to *Eco*RV) (#U57625; Wengelnik et al. 1996) from pXG8 (Table 1) was fused to the GFP cassette from pGreenTIR (Table 1). The *hrpG*-TIR-*gfp* fusion on an *Xba*I to *Kpn*I fragment was cloned into pUFR034 to create pUFZG3 (Table 1). The plasmid pUFZG3 was mobilised from *E. coli* strain DH5α into *X. euvesicatoria* strain 75-3R by triparental mating using the helper strain *E. coli* CK600(pRK2013::Tn*7*).

hrpX: The promoter region of *hrpX* (*Eco*RI to *Pst*I) (#U45888; Wengelnik and Bonas 1996) from pSX2 was fused with *gfp* and cloned into pVSP61 generating plasmid pUFZX1 in several steps due to the necessity of creating suitable restriction sites for cloning into pVSP61: an *Eco*RI/*Kpn*I fragment of *hrpX* in pSX2 was cloned into pUC19 creating plasmid pUFZ12 (Table 1); plasmid pUFZ13 was created by deleting the *Pst*I fragment from pUFZ12 (Table 1); the *Eco*RI fragment of pGreenTIR, including the enhanced TIR, Shine-Dalgarno sequence (SD) and *gfp*, was cloned into pBluescript II KS+ creating pUFZ19 (Table 1); the *Pst*I/*Hin*dIII fragment of pUFZ19 carrying the TIR, SD, and *gfp* was introduced into pUFZ13 upstream of *hrpX* generating pUFZ20 (Table 1); and finally, the *Sal*I/*Hin*dIII fragment of pUFZ20 carrying *hrpX*-TIR-SD-*gfp* was introduced into pVSP61 creating the *hrpX*-TIR-*gfp* fusion in plasmid pUFZX1 (Table 1). The plasmid pUFZX1 was introduced into 75-3RS by mating as described above with selection on LB agar amended with appropriate antibiotics.

hrpF: The GFP cassette from pGreenTIR (Miller and Lindow 1997) was cloned into pUC19 to create pCCGFP (Table 1). The upstream region of *hrpF*

Table 1 Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pBluescript II KS+	cloning vector, Ap^r	Stratagene, USA
pCCF3	*hrpF*-TIR-*gfp*, in pLAFR6	This study
pCCGFP	TIR-*gfp* from pGreenTIR in pUC19	This study
pCCO3	*hrpF*-TIR-*gfp* in pUC19	This study
pGreenTIR	P_{lac}-TIR-*gfp* in pUC1813, Ap^r	Miller and Lindow (1997)
pGEM-T	PCR cloning vector	Promega
pLAFR6	RK2 replicon, Mob^+, Tra^-, Tc^r; MCS flanked by terminators	Bonas et al. (1989)
pPROBE-NTtrp	P_{trp}-*gfp* in pBBR1 derivative, Km^r	Miller and Lindow unpublished
pRK2013	ColE1 replicon, $TraRK2^+$, Mob^+, Km^r	Figurski and Helinski (1979)
pRK2013::Tn7	ColE1 replicon, $TraRK2^+$, Mob^+, Tp^r, Sm^r, Sp^r	Ditta et al. (1980)
pSX2	*hrpX* in pLAFR6, Tc^r	Wengelnik et al. (1996)
pSG72	*hrpG* in pLAFR6, Tc^r	Wengelnik et al. (1996)
pUC19	cloning vector, Ap^r	New England Biolabs, USA
pUFR034	IncW, Mob^+, Par^+, *cos*, lacZα, Km^r	DeFeyter et al. (1990)
pUFZ12	*Eco*RI/*Kpn*I fragment (*hrpX*) of pSX2in pUC19, Ap^r	This study
pUFZ13	derivative of pUFZ12; a *Pst* Ifragment of pUFZ12 was deleted, Ap^r	This study
pUFZ19	TIR-*gfp* of pZGFP1 in pBlueScript II KS+	This study
pUFZ20	*hrpX*-TIR-*gfp* in pBlueScript II+ *Eco*RI digest and re-ligation of pUFZG3, *gfp* (*Eco*RI fragment) was deleted	This study
pUFZ70	P_{trp}-*gfp* in pGEM-T	This study
pUFZ75	P_{trp}-TIR-*gfp* in pUFR034, Km^r	This study
pUFZG3	*hrpG*-TIR-*gfp* in pUFR034, Km^r	This study
pUFZX1	*hrpX*-TIR-*gfp* in pVSP61	This study
pVSP61	pVS1 replicon, *lacZα*, Km^r	Loper and Lindow (1994)
pXG8	*hrpG* of pSG72 (*Hin*dIII-*Kpn*I) in pUC19	This study(1994)

(#AF056246 [#UF79116]; Huguet and Bonas 1997) containing the promoter was PCR-amplified from genomic DNA of *X. euvesicatoria* using primers hrpF2-up (5'-GCT CTA GAG TTT CTC AGC AAA TCC AGG-3') and hrpF1-down (5'-CGG GTA CCG GCG TGT TGA GCG ACA TAG-3'). The *hrpF* promoter was cloned upstream of the TIR-*gfp* cassette in pCCGFP to create pCCO3 (Table 1). The *hrpF*-TIR-*gfp* fusion was cloned into pLAFR6 to create pCCF3 (Table 1). Plasmid pCCF3 was mobilised from *E. coli* strain DH5α into 75-3RS by mating as described above with selection on LB agar amended with appropriate antibiotics.

Plant material and plant inoculation

Tomato (*Solanum lycopersicum*, syn. *Lycopersicon esculentum*) seeds were planted in commercial potting medium. Plants were grown under greenhouse conditions with standard fertilisation and pest control practices for ~6 weeks (i.e. about 7–9 true leaves).

For colonisation studies with 75-3R(pUFZ75) bacteria were grown on LB agar amended with Rf and Km for ~48 h at 28°C. Cells were scraped from the plate, suspended in 10 mM phosphate buffer (pH 7.0) and adjusted to ~10^8 CFU ml^{-1}. Tomato plants which had been incubated for 12–24 h under humid conditions were spray-inoculated with 75-3R (pUFZ75). Plants were placed in a humid chamber and maintained at 28–30°C. Leaves were sampled at 0, 24, 48, and 72 h after inoculation for FM or CLSM.

For reporter studies, *X. euvesicatoria* 75-3R carrying pUFZG3, pUFZX1, or pCCF3, were grown on LB agar amended with appropriate antibiotics for ~48 h at 28°C. Cells were scraped from the plate, suspended in 10 mM phosphate buffer (pH 7.0) and

adjusted to ~10^9 CFU ml^{-1}. A higher concentration of cells was employed in these experiments because the purpose was not to monitor colonisation but to detect the first induction of the *hrp* genes on the leaf surface or interior. Tomato plants incubated for 12–24 h under humid conditions were spray-inoculated with 75-3R (pUFZG3), 75-3(pUFZX1), or 75-3RS(pCCF3). Plants were placed in a humid chamber and maintained at 28–30°C. Leaves were sampled at 0, 24, 48, and 72 h after inoculation for FM or CLSM.

Fluorescence and CLSM

Leaf samples from inoculated plants were examined first by FM and subsequently by CLSM. Segments of leaf tissue (~5 mm×5 mm) cut from the leaf with a single-sided razor blade were mounted in 50% (*v/v*) glycerol on a clean glass slide and covered with a cover slip, which was held down tightly with tape. For FM, leaf samples were examined either with a Nikon Microflex UFX-IIA (Nikon, USA) or with a Zeiss Axioskop (Carl Zeiss Microscopy, Jena, Germany) using BP450-490, FT510, and LP520 filters. Following identification of leaf samples of interest, leaves were examined by CLSM using either a Leica TCS-NT confocal microscope (Leica Microsystems, Wetzlar, Germany) (Fig. 2a only) using 448 nm and 568 nm excitation filters and a 525/50 nm emission filter and other methods as described previously (Brandl and Mandrell 2002); or a Zeiss LSM510 laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany) (all other figures) using 488 nm and 543 nm excitation filters and 505–550 nm and 650 nm emission filters and other methods as described previously (Leveau and Lindow 2001).

Results

Assessment of plasmid for constitutive gfp expression

Cells of the pathogen *X. euvesicatoria* 75-3R harbouring pUFZ75 were strongly fluorescent both *in vitro* and *in planta* when examined by FM or CLSM; the plasmid was stably maintained in 75-3R both *in vitro* and *in planta*; and the transformed strain 75-3R (pUFZ75) was not significantly less virulent than the non-transformed strain 75-3R, based on the number of lesions on tomato (data not shown).

To determine the possible utility of the plasmid in other *Xanthomonas*-pathosystems, pUFZ75 was also introduced into several xanthomonads. Strong GFP expression *in vitro* was observed in *X. campestris* pv. *begoniae*, *X. campestris* pv. *dieffenbachiae, X. campestris* pv. *phaseoli*, *X. campestris* pv. *vignicola* and *X. vesicatoria* (previously *X. campestris* pv. *vesicatoria* race 2) (data not shown).

Use of pUFZ75 to monitor epiphytic growth and infection of tomato by *X. euvesicatoria*

Examined by bright-field microscopy, the abaxial leaf surface of tomato is characterised by irregularly-shaped epidermal cells and numerous stomata (Fig. 1a), as well as abundant glandular and non-glandular trichomes. Following spray-inoculation of plants with *X. euvesicatoria* 75-3R(pUFZ75), growth by the transformed pathogen was examined using both FM and CLSM. FM of tomato leaves typically revealed bacterial cells in the depressions between epidermal cells, in the depression around the stomatal guard cells, and in the stomatal opening (Fig. 1b). Some increase in density of cells of 75-3R(pUFZ75) had occurred on the leaf surface by 72 h to 96 h after inoculation. CLSM of tomato leaves confirmed these observations and revealed cells apparently entering the leaf via the stomatal opening (Fig. 2), as well as extensive growth of the pathogen in the substomatal chamber and upper layers of the mesophyll tissue (data not shown).

Reporter gene fusions for monitoring hrp gene expression in planta

hrpG: Plasmid pUFZG3 (Table 1) was stably maintained in *X. euvesicatoria* 75-3R *in vitro* and cells harbouring pUFZG3 grown on non-*hrp*-inducing media, LB agar and NA, were not visibly fluorescent when examined by FM (data not shown). The plasmid was stable *in planta* and, further, 75-3R(pUFZG3) produced a similar number of lesions to the wild-type 75-3R, though the lesions were somewhat smaller than those produced by 75-3R (data not shown).

Expression of *hrpG* was monitored after *X. euvesicatoria* 75-3R(pUFZG3) was inoculated onto tomato leaves at a high concentration (~10^9 CFU ml^{-1}). Leaves were sampled immediately after inoculation and at 24 h intervals and examined by FM or CLSM.

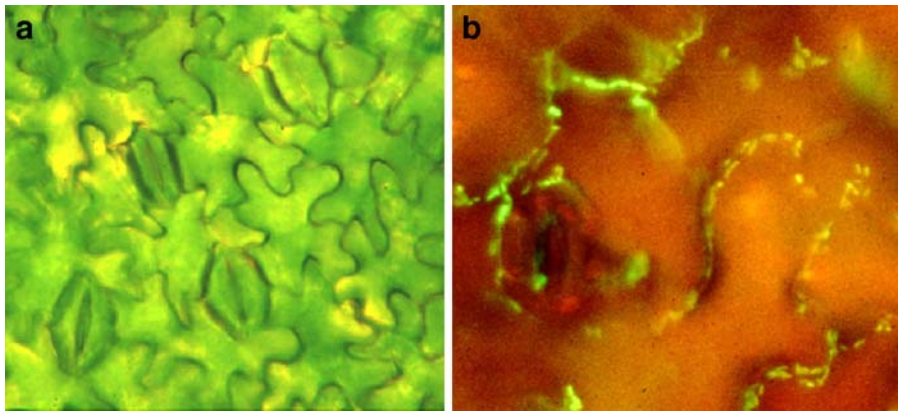

Fig. 1 Tomato leaf surface 48 h after inoculation with *Xanthomonas euvesicatoria* strain 75-3R(pUFZ75), carrying a P_{trp}-TIR-*gfp* fusion, examined by FM using a Zeiss Axioskop microscope. **a** Bright field photomicrograph (63×) showing stomata and irregularly shaped epidermal cells. **b** Fluorescence micrograph (~100×) showing bacterial cells colonising the depressions between epidermal cells, the depression around the stomatal guard cells, and the gap between the guard cells

Cells of 75-3(pUFZG3) exhibiting GFP fluorescence were not detected on the leaf surface until 24 h after inoculation. At this time, it is important to note that a few fluorescent cells were observed, primarily in the immediate vicinity of stomata (Fig. 3). The level of fluorescence in these cells was very low compared both to 75-3R(pUFZ75), carrying the constitutive P_{trp}-*gfp* fusion, and to cells of 75-3R(pUFZG3), carrying the *hrpG-gfp* reporter construct, observed at later sample times when the pathogen had invaded the leaf tissue. It is noteworthy that at this early sampling time there were no fluorescent cells in the substomatal chambers subtending those stomata around which the fluorescent cells were observed (i.e. these fluorescent cells likely did not re-emerge from the interior of the leaf). At 48 h after inoculation, fluorescent cells of 75-3R(pUFZG3) were more numerous around the stomata, but the level of fluorescence was no greater (data not shown). At 72 h after inoculation, it is notable that there were some stomata in which the substomatal chamber and the apoplast of the mesophyll tissue

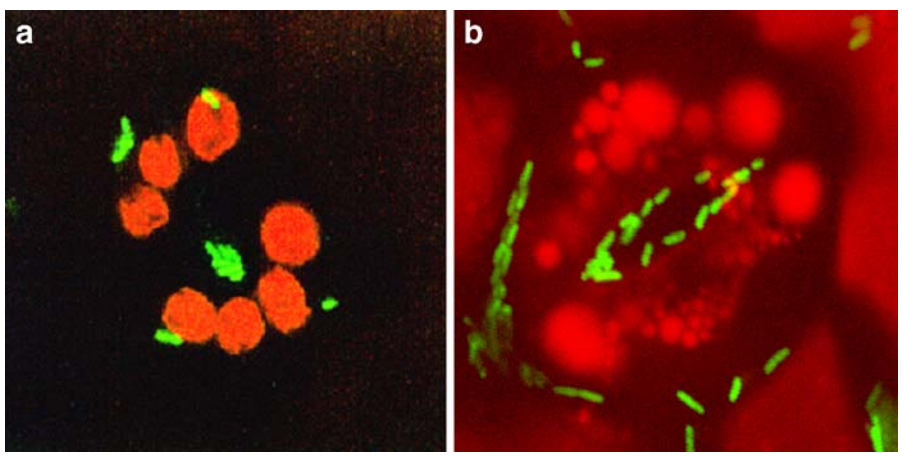

Fig. 2 CLSM micrographs of *Xanthomonas euvesicatoria* strain 75-3R(pUFZ75), carrying a P_{trp}-*gfp* fusion 48 h after inoculation of the leaf surface. **a** Single optical section through a colonised stoma showing bacterial cells in the gap between the guard cells (position indicated by red chloroplasts). GFP fluorescence of the labelled bacterial cells and autofluorescence of the plant cells were visualised through a 63×/1.2 W PL APO objective with emission filter sets BP525/50 and LP645, respectively. **b** Projection of multiple optical sections through colonised guard cells showing bacterial cells in the depression around the guard cells and in the stomatal opening

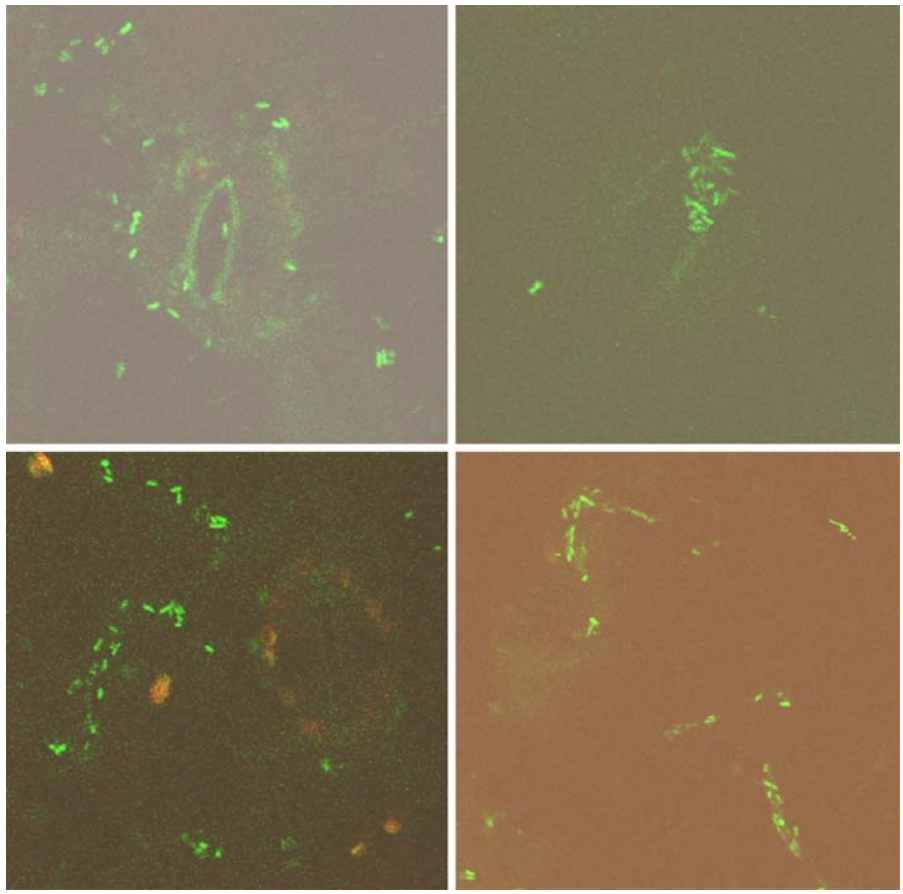

Fig. 3 CLSM micrographs of *Xanthomonas euvesicatoria* strain 75-3R(pUFZG3) carrying the *hrpG-gfp* fusion examined 24 h after inoculation onto the leaf surface. Each image represents a single optical 'section' of a different part of the leaf surface

were heavily colonised with highly fluorescent cells (Fig. 4). Even at this time, however, there were few fluorescent cells on the epidermis around the stomata through which entry apparently occurred, though occasionally bright fluorescent cells re-emerged from the substomatal chamber into the mounting medium used in the CLSM. Large numbers of bacteria in substomatal chambers and colonised mesophyll tissue preceded the appearance of water-soaking symptoms by several days.

hrpX: Cells of 75-3R carrying pUFZX1 (Table 1) grown on non-*hrp*-inducing LB agar and NA were not visibly fluorescent when examined by FM (data not shown). Most importantly, similar observations were made with the pathogen carrying the *hrpX* reporter (data not shown) to those made with the *hrpG* reporter *in planta*, indicating that a low level of *hrp* expression occurred on the leaf surface.

hrpF: Cells of 75-3RS harbouring pCCF3 (Table 1) grown on non-*hrp*-inducing media LB agar and NA were slightly fluorescent when examined by FM (data not shown). The fluorescence intensity of the cells on the leaf surface at 24 h after inoculation was not detectably higher than that of the cells harvested from LB or NA; hence, it remains unclear whether the *hrpF* fusion was induced on the surface of the leaf. However, strong expression of *gfp* did occur in cells of 75-3RS(pCCF3) occupying the sub-stomatal chamber and colonising the apoplast of the mesophyll tissue at 72 h after inoculation (Fig. 5).

Discussion

Relatively little is known about the initial stages of interaction between *X. euvesicatoria* and the susceptible host tomato following arrival of the pathogen on the leaf surface. In this study, a plasmid-borne, constitutively expressed *gfp* fusion in the pathogen was employed in conjunction with FM and CLSM to examine spatial aspects of epiphytic growth and

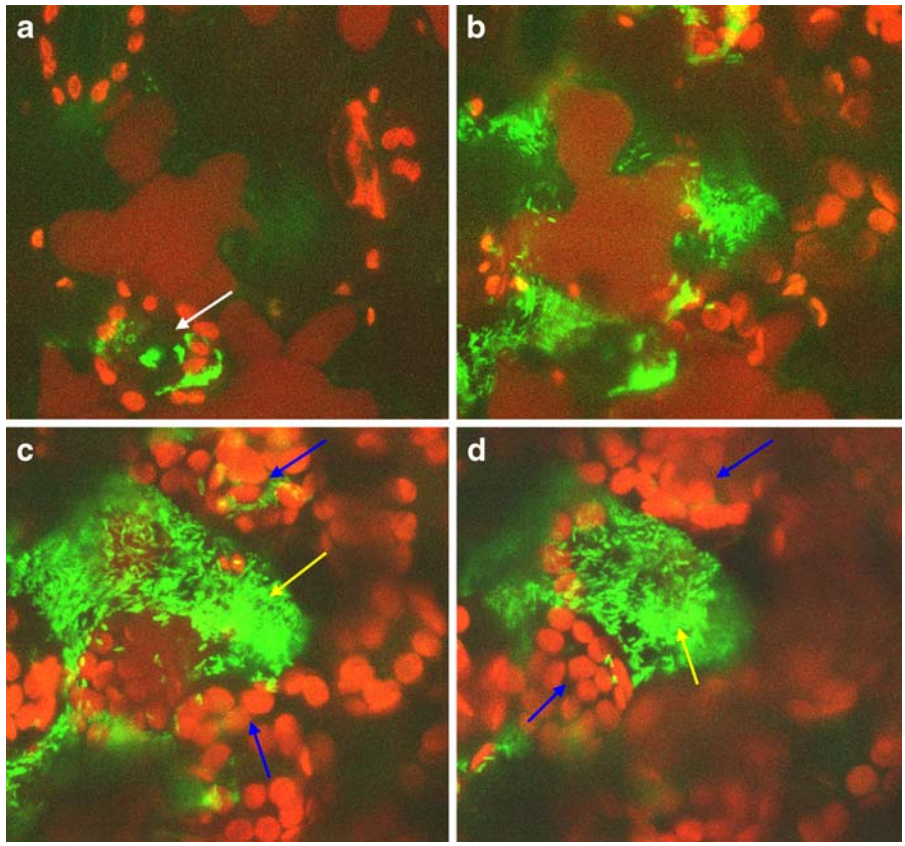

Fig. 4 CLSM micrographs of *Xanthomonas euvesicatoria* strain 75-3R(pUFZG3) carrying the *hrpG-gfp* fusion 72 h after inoculation of the leaf surface. Scan from leaf surface down through a stoma (*white arrow*), into the sub-stomatal chamber (*yellow arrows*) and into the mesophyll tissue (*blue arrows*). Each image represents a projection of multiple optical sections: **a** sections 1–7; **b** sections 8–14; **c** sections 15–21; and **d** 22–26

invasion of the stomata and mesophyll tissue. Subsequently, inducible *hrp-gfp* fusions were used to examine spatial and temporal aspects of *hrp* gene expression. In particular, we wished to determine whether the *hrpG* or *hrpX* regulatory genes were expressed on the leaf surface or not until the pathogen entered the substomatal chamber or mesophyll tissue.

Plasmid pUFZ75, based-upon pUFR034 (DeFeyter et al. 1990) provided strong, stably-maintained fluorescence in *X. euvesicatoria*, as well as in *X. vesicatoria* and several *X. campestris* pathovars (data not shown), including *X. campestris* pv. *citri* (Cubero et al. 2005). How the strength of fluorescence compares to other recently developed plasmids used

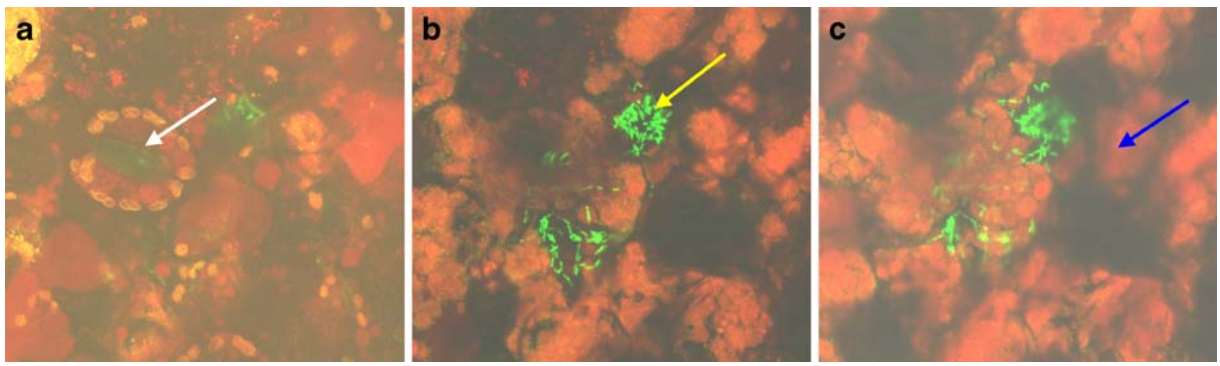

Fig. 5 CLSM micrographs of *Xanthomonas euvesicatoria* strain 75-3RS(pCCF3) carrying the *hrpF-gfp* fusion 72 h after inoculation of the leaf surface. Scan from leaf surface down through a stoma (*white arrow*), into the sub-stomatal chamber (*yellow arrow*) and into the mesophyll tissue (*blue arrows*). Each image represents a projection of multiple optical sections: **a** sections 1–5; **b** sections 6–10; and **c** sections 11–16

in xanthomonads, including pPneo-*gfp*, developed for use in *Xanthomonas oryzae* pv. oryzae (Han et al. 2008), and pDSK-GFPuv, developed for *Agrobacterium* but tested in *X. euvesicatoria* and *X. campestris* pv. *campestris* (Wang et al. 2007) is not known. Before settling on a constitutive plasmid for a study, researchers are encouraged to compare the three vectors in their pathosystems for plasmid stability, strength of fluorescence, and impacts on fitness and pathogenicity.

FM and CLSM of tomato leaves 48 h or 72 h after inoculation with 75-3R(pUFZ75) showed cells occupying the depressions between epidermal cells, the depressions around the guard cells of the stomata, and in the stomatal opening, possibly in the process of entering the stomata. From 72 h onwards, the substomatal chambers contained numerous cells of the pathogen and the mesophyll tissue subtending those substomatal chambers was heavily colonised. Even at 72 h or 96 h after inoculation, however, growth on the leaf surface was not extensive. This contrasts with the behaviour of non-pathogenic strains and pathovars of *P. syringae* which typically establish a substantial population on the leaf surface (Wilson et al. 1999).

The *hrc* genes of *X. euvesicatoria* are necessary for the formation of the T3S system. In this study, GFP reporters were constructed for two regulatory genes, *hrpG* and *hrpX*, and the structural gene, *hrpF*. While the use of reporters for two genes, *hrpG* and *hrpX*, adjacent to each other in the regulatory cascade might seem somewhat redundant, both were included because while we wished to focus on the regulator at the top of the system, *hrpG*, it was unclear how much up-regulation in expression would be observed in this gene (Wengelnik et al. 1999). Further, because activated HrpG turns on expression of *hrpX*, we would expect similar spatial and temporal patterns of *hrp-gfp* expression *in planta* for the two constructs; hence, the two constructs acted as internal controls for unrecognised problems.

Moderate induction of *hrpG- gfp* and *hrpX-gfp* was observed on the leaf surface as early as 24 h after inoculation. At this time, there were no fluorescent cells in the substomatal chamber of the stoma around which the fluorescent cells were located, suggesting that cells exhibiting *hrpG-gfp* and *hrpX-gfp* expression had not emerged from the leaf interior. These data suggest that *hrpG* and *hrpX* induction and low-level *hrpG* and *hrpX* expression occurred in some cells within 24 h of arrival on the leaf surface. HrpG, which is proposed to be at the top of the regulatory cascade (Wengelnik et al. 1999), activates expression of *hrpA* and *hrpX* (Wengelnik and Bonas 1996), but the HrpG regulon also appears to include numerous genes other than the *hrp* genes (Noel et al. 2001). It may be, therefore, that some genes of the HrpG regulon are necessary for growth on the leaf surface and/or invasion of the stomata. This contrasts with observations made with *P. syringae* pv. *tomato* DC3000 carrying a *hrpA-gfp* reporter in which no evidence was found for *hrp* gene activation on the leaf surface (Boureau et al. 2002). This could be because no *hrp* genes in *P. syringae* pv. *tomato* DC3000 were induced on the leaf surface, because the level of *hrpA-gfp* expression was below the detection limit of the instrument in that study, or because the *hrpA* gene is not part of the leaf surface-induced *hrp* regulon in *P. syringae*.

By 72 h after inoculation, numerous strongly fluorescent cells of *X. euvesicatoria* 75-3R(pUFZG3) were observed in the substomatal chambers and mesophyll tissue. Presumably this was due to a further induction of *hrpG* and a higher level of expression of *hrpG-gfp*. The higher level of expression could be the result of a different chemical composition of the apoplast (Rico and Preston 2008) compared to the leaf surface, or there could be cell-density triggers involved in *hrp* regulation, since cell densities in the apoplast of the mesophyll tissue were much higher than on the leaf surface. The fact that Wengelnik et al. (1999) did not observe significant up-regulation of *hrpG* on *hrp*-inducing medium, may just reflect the very different chemical composition of the tomato leaf apoplast and their *hrp*-inducing medium. *Pseudomonas syringae* pv. *tomato* DC3000 carrying the *hrpA-gfp* reporter similarly was observed to fluoresce strongly in the substomatal chamber 72 h after spray-inoculation (Boureau et al. 2002). In both pathogens, growth in the substomatal chamber and *hrp* gene induction occurred several days before the appearance of water-soaking or other visible symptoms of infection.

Expression of the *hrpX-gfp* fusion was observed at similar times, in similar locations, and at similar levels to that of the *hrpG-gfp* fusion. This is not surprising, since expression of the *hrpX* gene is believed to be activated by HrpG (Wengelnik and Bonas 1996; Wengelnik et al. 1996). Thus, the

observations with the *hrpX-gfp* reporter support the observations with the *hrpG* reporter.

It was hoped that *in planta* monitoring of *hrpF-gfp* expression would provide information on the expression of *hrp* structural genes, to contrast with the data on the *hrp* regulatory genes *hrpG* and *hrpX*, since *hrpF* is a structural gene which encodes a translocon protein (Huguet and Bonas 1997; Rossier et al. 2000). The goal was to determine whether the *hrp* structural genes were induced on the leaf surface or, by deduction, only the non-*hrp* genes of the HrpG/HrpX regulon. The gene *hrpF* was selected over other *hrp* genes, because it exhibited the highest level of expression *in vitro* on *hrp*-inducing medium (Wengelnik and Bonas 1996; Wengelnik et al. 1996) and *in planta* (Schulte and Bonas 1992) when examined with a *gusA* reporter. Cells of *X. euvesicatoria* 75-3RS(pCCF3) carrying the *hrpF-gfp* fusion grown on non-*hrp*-inducing media were already slightly fluorescent when examined by FM, suggesting that the basal level of expression of *hrpF* was higher than that of either *hrpG* or *hrpX*. Cells harbouring pCCF3 on the leaf surface were not detectably more fluorescent than those grown on non-*hrp*-inducing medium, indicating either that GFP was not sensitive enough to detect a difference or that there was no *hrpF* induction on the leaf surface. Cells carrying the *hrpF* reporter did, however, exhibit a high level of fluorescence when colonising the substomatal chamber and mesophyll tissue at 72 h after inoculation, as was observed with both the *hrpG* and *hrpX* reporters.

To summarise the observations made with the *hrp-gfp* reporters, low levels of *hrpG* and *hrpX* expression were detected on the leaf surface 24 h after spray-inoculation, though high-level expression did not occur until much later in the substomatal chamber and leaf apoplast. The HrpG/HrpX regulon includes several genes in addition to *hrc*, *hrp*, *hpa* and *avr* (Koebnik et al. 2006; Noel et al. 2001); hence, it seems possible that these non-*hrc*/*hrp* genes are activated on the leaf surface to enhance survival in the phyllosphere environment, to facilitate opening/invasion of the stomata (Melotto et al. 2008), to avoid induction of host defence responses, the innate immune response, once the bacterium is inside the substomatal chamber or mesophyll (Wilson et al. 2006), or to be primed to suppress innate immunity. With regard to survival in the tomato phyllosphere under field conditions, it is known that *hrpG* and *hrpX* mutants are significantly reduced in survival ability compared to the wild-type, but also compared to a *hrpE* mutant (Moss 2000), suggesting that factors other than the T3S system and pathogenicity are important for phyllosphere survival.

Future studies should determine how soon after arrival on the leaf surface *hrpG* is expressed, possibly using a quantitative reporter such as *inaZ* (Miller et al. 2000) and specifically which genes in the HrpG/HrpX regulon are up- or down-regulated on the leaf surface prior to invasion of the stomata. The overriding problem in such studies, however, will be determining where the cells are expressing the genes in more quantitative, but non-microscopic techniques. For example, whether a low overall level of ice-nucleation activity derives from many cells located on the surface expressing a low level of *hrpG-inaZ* activity versus one cell located in the substomatal chamber expressing a high level of *hrpG-inaZ*.

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