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The noncanonical type III secretion system of *Xanthomonas translucens* pv. *graminis* is essential for forage grass infection

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SUMMARY

Xanthomonas translucens pv. *graminis* (*Xtg*) is a gammaproteobacterium that causes bacterial wilt on a wide range of forage grasses. To gain insight into the host–pathogen interaction and to identify the virulence factors of *Xtg*, we compared a draft genome sequence of one isolate (*Xtg*29) with other *Xanthomonas* spp. with sequenced genomes. The type III secretion system (T3SS) encoding a protein transport system for type III effector (T3E) proteins represents one of the most important virulence factors of *Xanthomonas* spp. In contrast with other *Xanthomonas* spp. assigned to clade 1 on the basis of phylogenetic analyses, we identified an *hrp* (hypersensitive response and pathogenicity) gene cluster encoding T3SS components and a representative set of 35 genes encoding putative T3Es in the genome of *Xtg*29. The T3SS was shown to be divergent from the *hrp* gene clusters of other sequenced *Xanthomonas* spp. *Xtg* mutants deficient in T3SS regulating and structural genes were constructed to clarify the role of the T3SS in forage grass colonization. Italian ryegrass infection with these mutants led to significantly reduced symptoms ($P < 0.05$) relative to plants infected with the wild-type strain. This showed that the T3SS is required for symptom evocation. *In planta* multiplication of the T3SS mutants was not impaired significantly relative to the wild-type, indicating that the T3SS is not required for survival until 14 days post-infection. This study represents the first major step to understanding the bacterial colonization strategies deployed by *Xtg* and may assist in the identification of resistance (*R*) genes in forage grasses.

INTRODUCTION

The pathogen *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a gammaproteobacterium that causes bacterial wilt of forage grasses, a disease leading to considerable forage yield losses depending on host susceptibility (Egli *et al*., 1975). Its host range comprises a broad variety of forage grasses, including *Lolium* and *Festuca* spp. (Egli and Schmidt, 1982). *Xtg* invades the plant through wounded tissue and initially colonizes the protoxylem lacuna, from where it migrates to the vascular tissue, resulting in symptoms such as wilting of leaves and necrosis of the entire plant (Masuch *et al*., 1989). Breeding for resistant cultivars based on recurrent phenotypic selection has led to cultivars with improved partial resistance to bacterial wilt. Complete resistance has not been achieved and hypersensitive response (HR) symptoms have never been observed on resistant plants. In addition, highly susceptible plants still occur after continuous recurrent selection (Michel, 2001). Breeding for resistance is also complicated by the population-based breeding schemes and the obligate cross-pollination mode of reproduction of many species, which result in highly heterogeneous populations (Brummer, 1999). A thorough understanding of this complex host–pathogen interaction will enable the development of molecular genetics tools for improved resistance breeding and the development of superior cultivars.

In plants, resistance to diseases caused by *Xanthomonas* spp. is most often based on the specific recognition of effector proteins secreted through the type III secretion system (T3SS). These type III effectors (T3Es) are recognized by plant resistance (*R*) genes, which promote an HR, limiting pathogen spread and leading to effector-triggered immunity (ETI). The T3SS is encoded by a large gene cluster on either the chromosome or a plasmid (Arnold *et al*., 2003; Zou *et al*., 2006). Expression of the *hrp* (hypersensitive response and pathogenicity) gene cluster results in the formation of a membrane-spanning secretion apparatus (Hrp-pilus), which mediates T3E translocation into the host cell (Furutani *et al*., 2009; Roden *et al*., 2004; Thieme *et al*., 2005; White *et al*., 2009). The first key component in the *hrp* regulatory cascade is HrpG, together with HrpX, part of a two-component regulatory system (Noël *et al*., 2001). Point mutations in the *hrpG* gene of *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *X. campestris* pv. *vesicatoria* (*Xcv*) result in the constitutive expression of the *hrp* gene cluster (Jiang *et al*., 2006; Wengelnik *et al*., 1999). Secreted T3Es can modulate the physiology of the plant by suppressing

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pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) of the host (Jones and Dangl, 2006) or by facilitating nutritional and virulence processes of the pathogen (Büttner and He, 2009). Mutants deficient in T3SS genes of *Xanthomonas* spp. are no longer able to trigger ETI on resistant plants, and cannot cause symptoms on susceptible plants (Cho *et al*., 2008; Darsonval *et al*., 2008; Wengelnik *et al*., 1996; Zou *et al*., 2006).

Although the genome sequences of several *Xanthomonas* spp. are available and have revealed many shared virulence factors (Bogdanove *et al*., 2011; Da Silva *et al*., 2002; Lee *et al*., 2005; Moreira *et al*., 2005, 2010; Ochiai *et al*., 2005; Pieretti *et al*., 2009; Salzberg *et al*., 2008; Studholme *et al*., 2010; Thieme *et al*., 2005; Vorhölter *et al*., 2008; reviewed in Ryan *et al*., 2011; Studholme *et al*., 2011), *Xtg* is only distantly related to the other *Xanthomonas* spp. with sequenced genomes (Hauben *et al*., 1997; Parkinson *et al*., 2009). This has hindered the identification and analysis of homologous virulence factors of *Xtg* by comparative analyses. Based on *gyrB* and 16S rRNA gene sequencing, *Xtg* seems to be most closely related to two recently sequenced *Xanthomonas* spp., which have been shown not to have an *hrp* gene cluster and, in the case of *X. albilineans*, cannot produce xanthan (Pieretti *et al*., 2009).

The objectives of this study were to use the draft genome sequence of one *Xtg* isolate and to compare the *hrp* gene cluster of *Xtg* with the *hrp* gene clusters of other *Xanthomonas* spp. Our aim was also to elucidate the importance of the T3SS for infection and *in planta* multiplication using mutants deficient in genes encoding T3SS structural components and a regulatory gene. Further, homologous genes encoding T3Es were identified in *Xtg*. An understanding of the host colonization strategies deployed by *Xtg* may provide the necessary information to predict the T3Es that potentially induce ETI on forage grasses, and therewith may assist *R* gene identification in the future.

RESULTS AND DISCUSSION

The T3SS of *Xtg* strain 29 (*Xtg*29) is distinctly different from the T3SS of other *Xanthomonas* spp.

A shotgun approach with the Genome Sequencer FLX (GS FLX) was used to sequence the *Xtg*29 genome (DDBJ/EMBL/GenBank accession numbers ANGG01000001:ANGG01000788). Using this draft sequence, we constructed a phylogenetic tree based on the core genes of selected fully sequenced genomes (Fig. 1). Although most of the strains sequenced to date fall into clade 2 of the *Xanthomonas* genus, *Xtg*29 was assigned to clade 1, together with *X. sacchari* and *X. albilineans*. Therefore, this analysis confirmed the previous phylogenetic positions assessed using *gyrB* and 16S rRNA gene sequencing (Hauben *et al*., 1997; Parkinson *et al*., 2009). As a next step, we assessed the genome sequence for the presence of genes encoding the T3SS, as the two other *Xanthomonas* spp. of clade 1, *X. albilineans* and *X. sacchari*, do not have an *hrp*-type T3SS (Pieretti *et al*., 2009; Studholme *et al*., 2011). Interestingly, we managed to identify a region covering 17 751 bp putatively encoding an *hrp* gene cluster in *Xtg*29. This region consisted of 11 *hrc* genes, eight *hrp* genes and three *hpa*

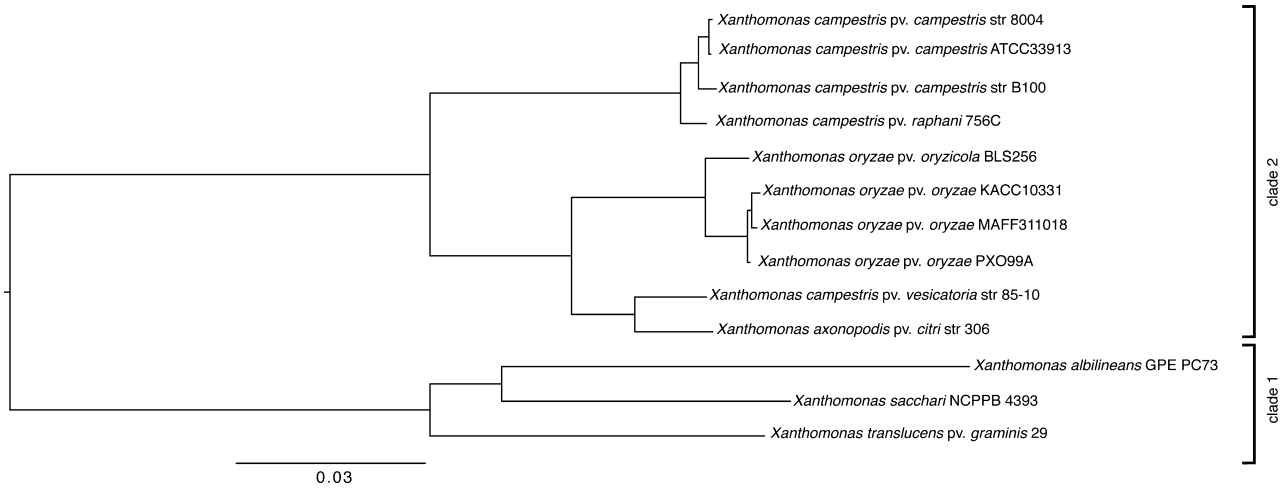

Fig. 1 Neighbour-joining tree of concatenated nucleotide sequences of all genes included in the core genome of completely sequenced *Xanthomonas* strains compared with *X. translucens* pv. *graminis* strain 29 (*Xtg*29). The strains include: *X. campestris* pv. *campestris* 8004 (CP000050; Qian *et al*., 2005), ATCC 33913 (AE008922; Da Silva *et al*., 2002) and B100 (AM920689; Vorhölter *et al*., 2008), *X. campestris* pv. *raphani* 756 (can be downloaded from: http://cmr.jcvi.org; Bogdanove *et al*., 2011), *X. oryzae* pv. *oryzae* KACC10331 (AE013598; Ochiai *et al*., 2005), MAFF311018 (AP008229; Lee *et al*., 2005) and PXO99A (CP000967; Salzberg *et al*., 2008), *X. oryzae* pv. *oryzicola* BLS256 (AAQN01000001; Bogdanove *et al*., 2011), *X. campestris* pv. *vesicatoria* strain 85-10 (AM039952; Thieme *et al*., 2005), *X. axonopodis* pv. *citri* strain 306 (AE008924; Da Silva *et al*., 2002), *Xanthomonas albilineans* str. GPE PC73 (FP565176; Pieretti *et al*., 2009) and *X. sacchari* NCPPB 4393 (AGDB01000024; Studholme *et al*., 2011).

genes (Fig. 2a). Sanger sequencing across a gap observed in the original draft sequence between the genes encoding *hrpX* and *hrcT* revealed an 815-bp sequence with high nucleotide sequence identity (88.3%) to two insertion sequence (IS) elements of *X. oryzae* pv. *oryzae* (*Xoo*) (ISXoo11 and ISXoo12). Another gap affected the 5′ region of the *hrpB4* gene between *hrcJ* and *hrcL*. Again Sanger sequencing was performed and resulted in a spanning sequence of 145 bp in length with a G + C content of almost 80%. Analysis by the EMBOSS tool palindrome (http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome) revealed two palindromic sequences within this fragment, which might have caused difficulties during amplification and sequencing of this region in the first instance.

The *hrpF* and *hpaF* genes encoding a substantial part of the type III secretion translocon present in some *Xanthomonas* spp. (Sugio *et al*., 2005) were not found in the genome of *Xtg*29 (Fig. 2a). HrpF from *Xcv* has been shown to be dispensable for type III secretion, but is presumably required for effector translocation into the host cell, as it binds to lipid bilayers and induces channel formation from the intracellular space (Büttner *et al*., 2002; Rossier *et al*., 2000). As *Xtg* makes use of breaches to gain direct access to the xylem cells in which it resides, and as these cells do not contain a cell membrane, the *hrpF* gene may be dispensable for effector translocation in *Xtg*29 and, consequently, may have been lost during evolution. However, as *Xtg*29 is so distantly related to the other *Xanthomonas* spp. with a sequenced T3SS, the *hrpF* and *hpaF* genes might nevertheless be present, but cannot be recognized on the basis of sequence identity.

Interestingly, the two genes *hrpG* and *hrpX*, encoding the response regulators of the T3SS, were found to be localized within the *hrp* gene cluster in *Xtg*29, whereas, in other *Xanthomonas* spp., they are encoded outside of the *hrp* gene cluster (Fig. 2a). A similar genetic organization with the genes encoding *hrpG* and *hrpB* (which is homologous to *hrpX* of *Xanthomonas* spp.) within the T3SS has been reported for *Ralstonia solanacearum* (*Rs*; Salanoubat *et al*., 2002). Furthermore, pairwise protein sequence identity of homologous genes revealed a lower sequence identity to *Rs*. Genealogy analysis of T3SSs based on the concatenated sequences of eight different Hrc proteins (i.e. HrcC, HrcJ, HrcN, HrcR, HrcS, HrcT, HrcU and HrcV) showed that the T3SS of *Xtg*29 clustered together with the T3SSs of other sequenced *Xanthomonas* spp. (Fig. 2b). The same topology was observed for separate phylogenetic trees of each of these proteins (Fig. S1, see Supporting Information). This analysis demonstrated that the T3SS of *Xtg*29 is clearly divergent from other T3SSs of *Xanthomonas* spp. Based on a comprehensive phylogenetic analysis of the HrcN, HrcS and HrcR proteins, the T3SS of *Xanthomonas* spp. has been hypothesized previously to have been horizontally acquired from *Rs* during evolution (Gophna *et al*., 2003). The higher degree of synteny within the T3SS, observed between *Xtg*29 and *Rs* compared with other *Xanthomonas* and *Rs*, bolsters such an evolutionary scenario and also suggests that the *hrp* gene cluster of *Xtg*29 possibly represents a more ancestral state of the T3SSs within the *Xanthomonas* genus. The T3SS of *Xtg*29 has very distinct characteristics concerning genetic organization and sequence composition, indicating potential differences in functionality during infection.

Other major virulence factors of *Xanthomonas* present in the genome of *Xtg*29

In addition to the T3SS, the type II secretion system (T2SS) is highly important for some *Xanthomonas* spp., as it facilitates host colonization by the secretion of toxins and extracellular enzymes, such as proteases, lipases and cell wall-degrading enzymes (reviewed in Büttner and Bonas, 2010). In *Xtg*29, the *xps* gene cluster encoding components of a T2SS covered a region of 11 732 bp and consisted of the genes *xpsEFGHIJKLMD*. A *gum* gene cluster was identified that included 11 genes for xanthan biosynthesis. Surprisingly, the gene *gumF*, which encodes an acetyltransferase (Becker *et al*., 1998) and is well conserved in other *Xanthomonas* genomes (Vorhölter *et al*., 2008), was missing in the otherwise complete *gum* gene cluster of *Xtg*29. Although the *gum* gene cluster is absent in *X. albilineans* (Pieretti *et al*., 2009), the other sequenced member of clade 1, i.e. *X. sacchari*, has also been shown to be capable of xanthan biosynthesis (Studholme *et al*., 2011). Flanked on one side by precursor genes for xanthan and lipopolysaccharide (LPS) biosynthesis (Vorhölter *et al*., 2008) and by the electron transfer genes *etfAB* in a generally conserved *Xanthomonas* genomic organization, and, on the other side, by *metBC* genes involved in amino acid biosynthesis (Schatschneider *et al*., 2011), an LPS biosynthesis gene cluster was identified that was made up of 19 genes (XTG29_00287 to XTG29_00305). Apart from two *wzm* and *wzt* genes coding for a polysaccharide-specific ABC transporter, also found in other *Xanthomonas* genomes (Patil *et al*., 2007; Vorhölter *et al*., 2003), other genes differed from the *Xanthomonas* LPS gene clusters characterized to date, thereby indicating a distinct LPS structure for *Xtg*29. Furthermore, an *rpf* gene cluster that regulates the synthesis of pathogenicity factors was also found in the genome of *Xtg*29 (Ryan and Dow, 2011).

A homologue to the type IV secretion system (T4SS), present in many *Xanthomonas* spp. (Alegria *et al*., 2005; Qian *et al*., 2005; Thieme *et al*., 2005), was not found in *Xtg*29. However, the function of the T4SS has not been clarified and experimental data on the extent of its contribution to disease development are still missing. In addition to the type I secretion system (T1SS), which secretes the AvrXa21 molecule in *Xoo* and is encoded by *raxABC*, all *rax* gene orthologues were identified in *Xtg*29 (Table S1, see Supporting Information). In addition, a large number of other genes with functions related to type I secretion of toxins (bacteriocins), lipases and proteins or encoding components of T1SSs were found in *Xtg*29 (data not shown).

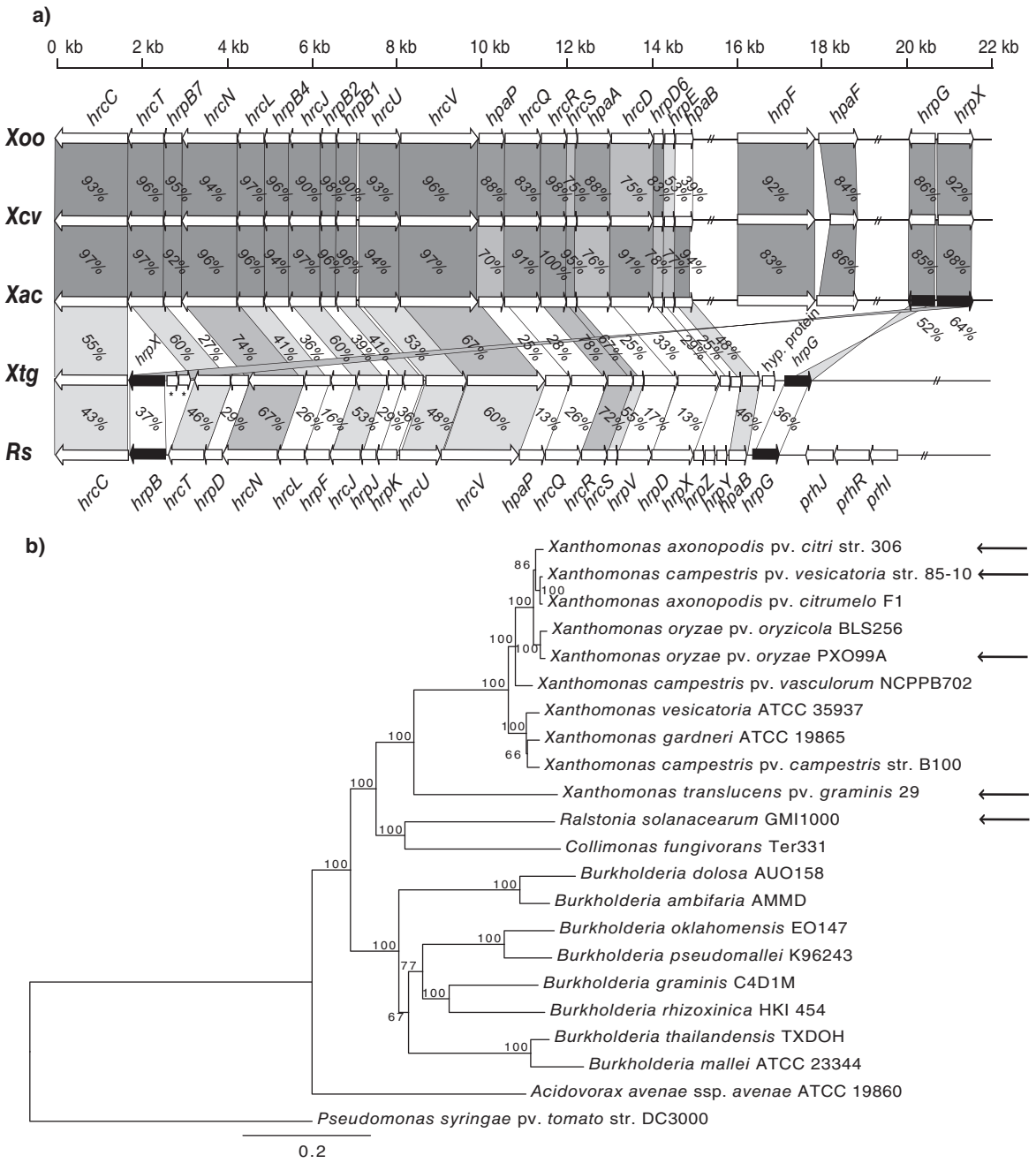

Fig. 2 (a) Genetic organization and protein sequence identity of Hrp, Hrc and Hpa proteins from different sequenced *Xanthomonas* spp. and *Ralstonia solanacearum* GMI1000 compared with *X. translucens* pv. *graminis* strain 29 (*Xtg*29) *hrp* genes. Genes encoding the type III secretion system (T3SS) of the following strains are shown: *Xoo*, *X. oryzae* pv. *oryzae* PXO99A (CP000967; Salzberg *et al*., 2008); *Xcv*, *X. campestris* pv. *vesicatoria* strain 85-10 (AM039952; Thieme *et al*., 2005); *Xac*, *X. axonopodis* pv. *citri* strain 306 (AE008922; Da Silva *et al*., 2002); *Rs*, *R. solanacearum* GMI1000 megaplasmid pGM1000MP (AL646053; Salanoubat *et al*., 2002). Arrows indicate the sizes, positions and orientations of the *hrp*, *hrc* and *hpa* genes. The two genes indicated with asterisks were shown to be similar to ISXoo11 and ISXoo12 transposases. The identity of each protein sequence with its homologue in *X. translucens* pv. *graminis* is indicated by the percentage identity and the use of a gradual black/grey colour scale. (b) Phylogenetic tree inferred with maximum likelihood for the alignment of concatenated sequences of eight different Hrc proteins (i.e. HrcC, HrcJ, HrcN, HrcR, HrcS, HrcT, HrcU and HrcV) from different *Xanthomonas* spp., *R. solanacearum* (AL646053), *Collimonas fungivorans* (NC_015856), *Burkholderia* spp. and *Acidovorax avenae* ssp. *avenae* (NC_015138). *Pseudomonas syringae* pv. *tomato* strain DC3000 (AE016853) was used as an outgroup. Numbers represent bootstrap values for 100 replications. Arrows indicate that these sequences were used in (a) for the comparison of T3SS synteny.

Predicted genes encoding T3Es of *Xtg*29

An extensive analysis of all known *Xanthomonas* T3E protein sequences (summarized at http://www.xanthomonas.org) was performed using BLASTP and TBLASTN (Altschul *et al*., 1990) against all coding DNA sequences (CDSs) and the nucleotide sequence of *Xtg*29. In addition, the genome was searched for plant-inducible promoter (PIP) box sequences. All approaches combined revealed 35 genes homologous to genes encoding T3Es (Table 1). This number reflects the abundance and variety of T3Es typically found in other *Xanthomonas* spp. (Kay and Bonas, 2009).

In *Xtg*, the genes putatively encoding T3Es included at least three different effectors of the *xopX* family. Furthermore, two genes with predicted protein sequence similarity to AvrRxv effectors were identified in *Xtg*29. The genes that encode these two candidate effectors showed rather low G + C contents (53% and 48%), which may be indicative of acquisition by horizontal gene transfer (Dobrindt *et al*., 2004). AvrRxv has been described in *Xcv* and belongs to the AvrRxv/XopJ effector group, including members with different enzymatic activities which target multiple host physiological pathways (Mukherjee *et al*., 2007). Therefore, it is difficult to predict a function for these two effectors. Both *avrRxv*-like genes harboured an upstream PIP box, and therefore we hypothesize that these two effectors are important for the *Xtg*–forage grass interaction.

A homologous gene encoding the AvrBs2 effector has been identified in *Xtg*29. AvrBs2 encodes a putative glycerophosphoryl-diester phosphodiesterase and has been shown to be involved in both osmotic adaptation and plant host signalling (Swords *et al*., 1996). The *avrBs2* gene is highly conserved among a very broad range of *Xanthomonas* spp. (Hajri *et al*., 2009). However, experimental evidence that translocation of AvrBs2 contributes to virulence and *in planta* multiplication has only been provided for *Xcv* (Gurlebeck *et al*., 2006; Mudgett *et al*., 2000).

A number of genes presumably encoding T3Es found in *Xtg*29 are members of a 'core set' of T3Es typically found in *Xanthomonas* genomes (e.g. *xopB*, *xopF1*, *xopN*, *xopP*, *xopQ* and *xopX*), and some are less conserved. Effector composition can vary significantly between strains and pathovars, as has been demonstrated for *X. axonopodis* (Hajri *et al*., 2009). We therefore expect effector composition to vary among *Xtg* isolates. As differences in the virulence of *Xtg* isolates have consistently been observed on *Lolium multiflorum* genotypes (Kölliker *et al*., 2006), we hypothesize that this could be a result of differential effector composition.

Genes encoding effectors belonging to the highly conserved *avrBs3/pth* family, also called transcription activator-like effectors (TALEs), present in many *Xanthomonas* spp. and *Rs*, were not found in the genome of *Xtg*29. TALEs have been demonstrated to have certain amino acid residues that correspond to one nucleotide in the promoter sequence of the corresponding *R* genes (Moscou and Bogdanove, 2009; Römer *et al*., 2009a, b). Although other T3Es and resistance mechanisms that can trigger ETI are known in plant–*Xanthomonas* interactions, the absence of TALEs could be an indication for the absence of an HR, even on forage grasses with considerable resistance to *Xtg*. So far, HR symptoms have never been described in studies on bacterial wilt in forage grasses (Egli and Schmidt, 1982; Leyns, 1993; Michel, 2001). In a needle inoculation experiment using resistant *L. multiflorum* genotypes and the wild-type *Xtg*29 isolate, we failed to observe HR symptoms (data not shown). The lack of HR could be a further explanation for the absence of race specificity reported previously for the interaction of different *Xtg* isolates with Italian ryegrass genotypes (Wichmann *et al*., 2011). However, this needs to be clarified in further studies targeting directly the translocation of effector proteins into the host cell using immunochemical approaches.

The T3SS of *Xtg*29 is required for symptom evocation

In order to test whether the T3SS of *Xtg*29 is required for disease development, deletion mutants of different genes encoding T3SS components were constructed. These included two mutants of structural T3SS genes (i.e. Δ*hrpE* and Δ*hrcR*), one mutant of a response regulator gene (i.e. Δ*hrpG*) and a double mutant (i.e. Δ*hrpG/hrcR*). In addition, the Δ*hrpG* mutant was complemented with the *hrpG* gene and its promoter sequence (322 bp upstream of the start codon) in a plasmid with a broad host range. Infection of a highly susceptible Italian ryegrass (*Lolium multiflorum* Lam.) genotype with all T3SS mutants led to significantly reduced symptoms ($P < 0.05$) when compared with the wild-type *Xtg*29 isolate (Fig. 3a; Fig. S2, see Supporting Information). However, when compared with the negative control treatment, although not significant, all T3SS mutants still caused weak but detectable symptoms, particularly the Δ*hrpG* mutant. Although drastically reduced symptom development has also been observed for the T3SS mutants of other *Xanthomonas* spp. (Cho *et al*., 2008; Darsonval *et al*., 2008; Zou *et al*., 2006), it cannot be excluded that other virulence factors, such as extracellular polysaccharides (EPSs) or cell wall-degrading enzymes, secreted by the T2SS, contribute to the remaining observed symptoms.

Infection with the complemented Δ*hrpG* mutant caused 33.83% higher areas under the disease progress curve (AUDPCs) when compared with the Δ*hrpG* mutant and the wild-type strain. Significant differences ($P < 0.05$) between the complemented Δ*hrpG* mutant and other T3SS mutants were only observed for the Δ*hrcR* mutant. Previously, functional complementation of the *hrpG* gene resulted in a complete restoration of the wild-type phenotype (i.e. induction of HR on resistant plants) in *Xcv* and *X. oryzae* pv. *oryzicola* (Wengelnik *et al*., 1996; Zou *et al*., 2006). However, an HR was not observed on forage grasses after *Xtg* infection. Therefore, it was possible that the complete function of the *hrpG*

Table 1 Genes encoding predicted type III secreted effectors found in the genome of *Xanthomonas translucens* pv. *graminis* strain 29 (*Xtg*29) and putative functions or homologues from other pathogens containing a type III secretion system (e.g. *Pseudomonas syringae*, *Ralstonia solanacearum* or *Yersinia* spp.).

Gene name	Sequence name	PIP box	Function/family/homology to *Pseudomonas* effectors	Best BLASTX hit	E-value	% protein identity	Length (bp)	Reference
avrBs2	XTG29_03687	–	Putative glycerophosphoryl-diester phosphodiesterase	Avirulence protein (*X. campestris* pv. *musacearum* NCPPB 4381)	0.0	62	2148	Swords *et al*. (1996)
avrRxv	XTG29_02581	+	YopJ/AvrRxv family, putative cysteine protease	Type III effector HopZ2 (*P. syringae* pv. *coronafaciens*)	3 E-14	44	246	Whalen *et al*. (1993)
avrRxv	XTG29_02457	+	YopJ/AvrRxv family, putative cysteine protease	YopP/AvrRxv family protein (*R. solanacearum* GMI1000)	8 E-90	49	1698	Whalen *et al*. (1993)
xopB	XTG29_02183	+	Homology to HopD1 (*P. syringae* pv. *tomato*)	Type III effector HopD1 (*P. syringae* pv. *oryzae* str. 1_6)	0.0	80	1887	Noël *et al*. (2001)
xopC2	XTG29_01319	–		Putative *Xanthomonas* outer protein C2 (*X. perforans* 91-118)	0.0	62	1416	Noël *et al*. (2003)
xopE1	XTG29_03655	+	Homology to *avrPphE*/HopX, predicted transglutaminase	Type III secretion system effector protein (*X. fuscans* ssp. *aurantifolii* str. ICPB 10535)	2 E-170	70	1089	Thieme *et al*. (2007)
xopF1	XTG29_00871	+	Unknown function	Outer protein F1 (*X. campestris* pv. *vasculorum* NCPPB 702)	1 E-70	33	2058	Roden *et al*. (2004)
xopF2-1	XTG29_02713	–	Unknown function	*Xanthomonas* outer protein F2 (*X. perforans* 91-118)	1 E-109	73	813	Roden *et al*. (2004)
xopF2-2	XTG29_02714	–	Unknown function	Outer protein F2 (*X. campestris* pv. *vesicatoria* str. 85-10)	2 E-148	83	855	Roden *et al*. (2004)
xopI	XTG29_02986	–	F-box protein	Type III secretion system effector protein (*X. fuscans* ssp. *aurantifolii* str. ICPB 11122)	3 E-86	83	618	Thieme *et al*. (2007)
xopK	XTG29_03500	+	Unknown function	Putative *Xanthomonas* outer protein K (*X. gardneri* ATCC 19865)	0.0	67	2610	Furutani *et al*. (2009)
xopN	XTG29_00130	–	Homology to HopAU1 (*P. syringae*), α-helical ARM/HEATS repeats	XopN effector (*X. oryzae* pv. *oryzae* PXO99A)	0.0	61	1881	Kim *et al*. (2009)
xopP	XTG29_02884	–	Unknown function	Type III effector protein XopP (*X. campestris* pv. *raphani* 756C)	0.0	56	1635	Roden *et al*. (2004)
xopP	XTG29_00851	–	Unknown function	Type III secretion system effector protein (*X. fuscans* ssp. *aurantifolii* str. ICPB 11122)	8 E-99	82	555	Roden *et al*. (2004)
xopQ	XTG29_00093	+	HopQ1-1 family protein, inosine-uridine nucleoside N-ribohydrolase	Type III effector RipB protein (*R. solanacearum* PSI07)	1 E-138	54	1407	Roden *et al*. (2004)
xopR	XTG29_00207	–	Unknown function	Putative *Xanthomonas* outer protein R (*X. gardneri* ATCC 198650	2 E-58	54	831	Furutani *et al*. (2009)
xopV	XTG29_00797	+	Unknown function	Type III effector protein (*X. arboricola* pv. *pruni*)	2 E-90	53	930	Furutani *et al*. (2009)
xopX1	XTG29_01318	–	Homology to HopAE1 (*P. syringae*)	*Xanthomonas* outer protein X (*X. vesicatoria* ATCC 35937)	0.0	74	1893	Metz *et al*. (2005)
xopX2-1	XTG29_01078	–	Unknown function	*Xanthomonas* outer protein X (*X. gardneri* ATCC 19865)	0.0	54	2124	Noël *et al*. (2002)
xopX2-2	XTG29_01080	–	Unknown function	*Xanthomonas* outer protein X2 (*X. perforans* 91-118)	0.0	55	2124	Noël *et al*. (2002)
xopY	XTG29_00726	+	Unknown function	Type III effector protein XopY (*X. oryzae* pv. *oryzicola* BLS256)	6 E-05	39	408	
xopZ1-1	XTG29_02419	–	Unknown function	Type III effector protein XopZ1 (*X. oryzae* pv. *oryzicola* BLS256)	0.0	64	3000	Song and Yang (2010)
xopZ1-2	XTG29_02420	–	Unknown function	Type III effector protein XopZ1 (*X. oryzae* pv. *oryzicola* BLS256)	2 E-60	54	681	Song and Yang (2010)
xopAD	XTG29_02463	–	Unknown function	Type III effector protein XopAD (*X. oryzae* pv. *oryzicola* BLS256)	0.0	64	7896	
xopAK	XTG29_03476	–	Homology to HopK1 (*P. syringae*)	Type III effector protein XopAK (*X. oryzae* pv. *oryzicola* BLS256)	2 E-89	56	771	Wei *et al*. (2007)
	XTG29_01559	–	Homology to HopH 1 (*P. syringae*) and *xopG*	Type III effector protein (*R. solanacearum* IPO1609)	1 E-34	50	531	Wei *et al*. (2007)
	XTG29_01817	–	Glycerophosphodiester phosphodiesterase	Hypothetical protein XsacN4_02427 (*X. sacchari* NCPPB 4393)	6 E-39	32	1017	
	XTG29_03294	–	Glycerophosphodiester phosphodiesterase	Glycerophosphodiester phosphodiesterase (*X. albilineans* GPE PC73)	0.0	86	945	

Table 1 Continued.

Gene name	Sequence name	PIP box	Function/family/homology to *Pseudomonas* effectors	Best BLASTX hit	E-value	% protein identity	Length (bp)	Reference
	XTG29_01857	+	Homology to *avrPphE*/HopX	Hypothethical protein, partial)*P. syringae* pv. *actinidiae* str. M302091)	1 E-178	90	987	
	XTG29_01339	–	Homology to HopX1	Type III effector protein (*X. arboricola* pv. *pruni*)	5 E-30	34	1131	Wei *et al*. (2007)
	XTG29_00200	+	Homology to HopR	*Xanthomonas* outer protein AM (*X. vesicatoria* ATCC 35937)	0.0	55	5166	Wei *et al*. (2007)
	XTG29_02140	+	Homology to HopAJ1 (*P. syringae*)	Outer membrane antigen precursor (*X. sacchari* NCPPB 4393)	0.0	91	2457	
	XTG29_02185	+	Homology to putative type III effector protein (*R. solanacearum*)	Hypothetical protein XGA_1070 (*X. gardneri* ATCC 19865)	3 E-62	56	798	
	XTG29_01881	–	Putative type III effector protein (*R. solanacearum*)	Type III effector protein (*R. solanacearum*)	5 E-30	38	846	
	XTG29_00627	+	Putative cysteine protease, yopT-like	HopAY1 (*P. syringae* pv. *mori* str. 301020)	2 E-68	57	1071	

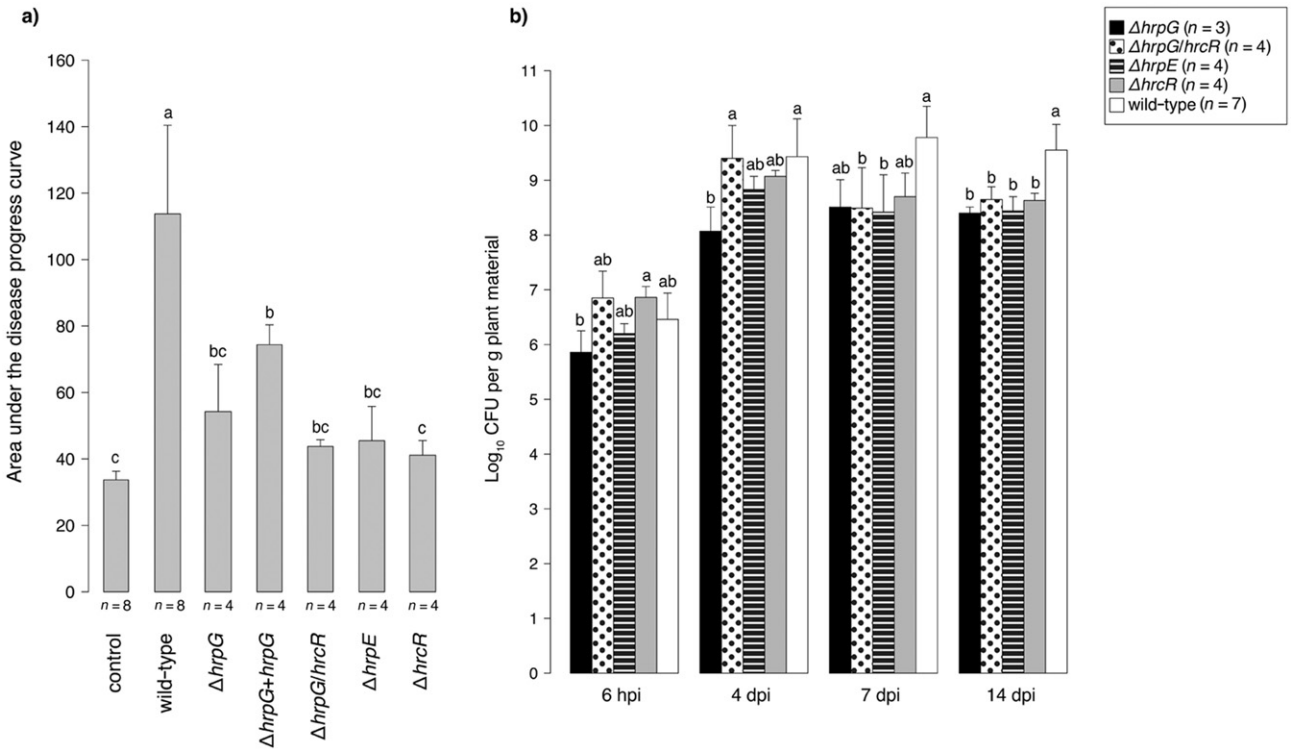

Fig. 3 (a) Mean areas under the disease progress curve (AUDPCs) from four replications for *Lolium multiflorum* genotype *Lm*K-01 infected with *Xanthomonas translucens* pv. *graminis* strain 29 (*Xtg*29) deficient in genes encoding the type III secretion system (T3SS) compared with the wild-type strain. Means with different letters are significantly different ($P < 0.05$) on the basis of a two-sided *t*-test using the Holm *P* value adjustment. The negative control treatment consisted of cutting the plants with scissors dipped in sterile physiological sodium chloride solution. (b) Colonization of *L. multiflorum* by *Xtg*29 and the *Xtg*29 strains deficient in T3SS genes. Bacterial population densities were determined for leaves of three or four different plants of *Lm*K-01 sampled at 6 h post-infection (hpi) and 4, 7 and 14 days post-infection (dpi), counting the colony-forming units (CFU) of serial dilutions. For each time point, mean population densities and standard deviations were calculated. Means indicated with different letters are significantly ($P < 0.05$) different on the basis of a two-sided *t*-test using the Holm *P* value adjustment.

gene had been restored in *Xtg*29, but the levels of expression were not as fine tuned as necessary, which resulted in less pronounced symptoms.

Mutation of T3SS components does not prevent *in planta* survival of *Xtg*29

In order to test whether the *in planta* survival of mutants deficient in T3SS genes is affected, the dynamics of bacterial population densities for the T3SS mutants and the wild-type strain (*Xtg*29) were determined. Bacteria were isolated from harvested plant material at different time points after infection [i.e. 6 h post-infection (hpi), and 4, 7 and 14 days post-infection (dpi)], and serial dilutions were plated. The population densities of all T3SS mutants quantified in leaves were not significantly different from those of the wild-type, except at 14 dpi (Fig. 3b), but also at this time point the mutants were still quantified in substantial numbers. This indicated that the T3SS of *Xtg*29 is not crucial for plant colonization. This is in contrast with most other mutant studies with *Xanthomonas* spp. deficient in T3SS genes (Büttner *et al*., 2007; Cho *et al*., 2008; Darsonval *et al*., 2008). One isolate of *Xcc* deficient in the *hrpE* gene (i.e. str. B305) could also establish populations comparable with those of the wild-type strain (Sun *et al*., 2011), but, in the other studies, *in planta* multiplication was drastically impaired. The *in planta* survival of Δ*hrpG* or Δ*hrpX* mutants of *X. fuscans* pv. *fuscans* was even more impaired relative to the T3SS mutants deficient in structural components (Darsonval *et al*., 2008). These results therefore implied that HrpG and HrpX regulate processes during early infection and the epiphytic phase (reviewed in Büttner and Bonas, 2010). Surprisingly, total *in planta* populations of the *Xtg*29 Δ*hrpG* mutant were not significantly lower than those of the other T3SS mutants or the wild-type, which demonstrates that the *hrpG* gene, despite initial significant differences at 4 dpi, establishes comparable bacterial populations later during infection.

Therefore, to characterize in more detail their role in type III secretion, gene expression of the *hrpE* and *hrcR* genes was quantified in plant material infected with the wild-type *Xtg*29 and the Δ*hrpG* mutant. Four biological replicates and three technical replicates of each sample were used to quantify *hrpE* and *hrcR* expression relative to the genomic DNA quantity obtained from the same nucleic acid extraction. We observed that, in infected plant material, the *hrpE* gene was up-regulated significantly in the wild-type isolate relative to the Δ*hrpG* mutant at all sampled time points after infection (Fig. 4). For the *hrcR* gene, significant up-regulation according to the permutation test was only observed at 4 and 14 dpi. That the *hrpG* gene activates the expression of T3SS structural genes is consistent with findings in other *Xanthomonas* spp. and *Rs* (Noël *et al*., 2001; Wengelnik *et al*., 1996; Yoshimochi *et al*., 2009). However, up-regulation was particularly pronounced at 14 dpi, where a 321-fold up-regulation of the *hrpE* gene and a 121-fold up-regulation of the *hrcR* gene were observed. The *hrpE* and *hrcR* genes remained up-regulated until 28 dpi in wild-type *Xtg*, but gene expression differences were less pronounced. Therefore, we conclude from our gene expression data and *in planta* multiplication assays that major virulence processes of *Xtg*29 occur at 14 dpi, which is the time point at which the presence of T3SS genes matters.

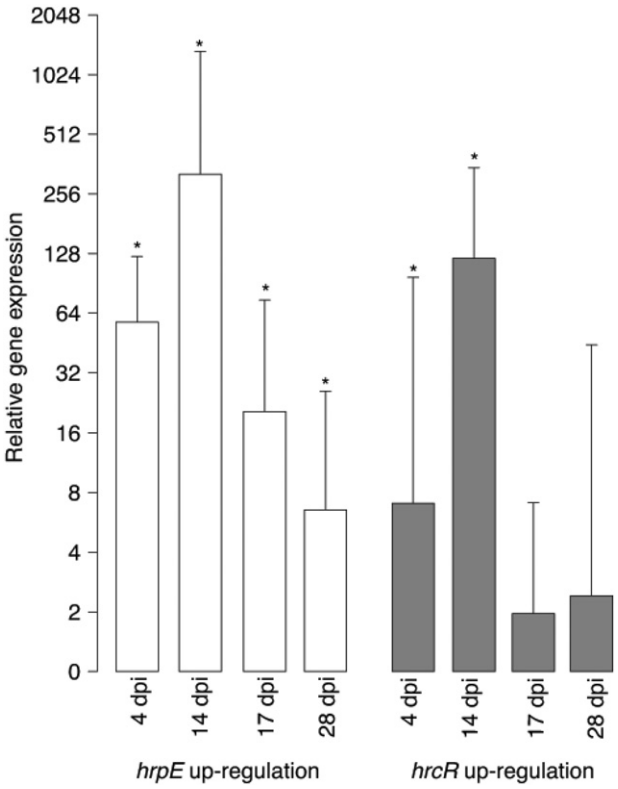

Fig. 4 Average $\log_2$ fold change of the *hrpE* and *hrcR* genes monitored in plant material infected with the *Xanthomonas translucens* pv. *graminis* strain 29 (*Xtg*29) wild-type compared with the *Xtg*29 Δ*hrpG* mutant. The average and standard errors were obtained from four biological replicates (each with three technical replicates). Genes significantly up-regulated in the wild-type ($P < 0.05$) were determined according to the pairwise fixed reallocation randomization test (using 2000 randomizations) and are indicated with asterisks. dpi, days post-infection.

CONCLUSIONS

Using the whole genome sequence of *Xtg*29, we showed that the T3SS of *Xtg*29 is present and required for symptom development, but is divergent from that of other sequenced *Xanthomonas* spp. In addition, our genealogy analyses propose that *Xtg*29 harbours a more ancestral state of the T3SS, and is important to infect a broad host range. Overall, our results show that the *hrpG* gene activates the expression of the T3SS, and that the T3SS is not primarily responsible for *in planta* survival. From our results and data published by others, we hypothesize that the T3SS may not be

required for xylem-colonizing pathogens, as some vascular pathogens, such as *Xylella fastidiosa*, *Pectobacterium carotovorum* and *Xanthomonas albilineans* (Kim *et al*., 2009; Pieretti *et al*., 2009; Van Sluys *et al*., 2002), do not even have an *hrp* gene cluster. Furthermore, *Xtg* gains direct access to the protoxylem lacuna, which is the area of primary multiplication (Masuch *et al*., 1989), and from where it infects the xylem. Therefore, it is thought that, initially, the T2SS, which secretes necessary cell wall-degrading enzymes to gain access to the xylem vessels, is more important for initial *in planta* growth. Nonetheless, the T3SS of *Xtg* must provide some advantage to bacterial fitness, especially during later infection stages, otherwise we would not have observed significant differences at any time point after infection.

EXPERIMENTAL PROCEDURES

Bacterial isolates and cultivation conditions

The bacterial isolates and plasmids used in this study are listed in Table S2 (see Supporting Information). *Xtg*29 has been characterized previously for virulence on different genotypes and cultivars (Kölliker *et al*., 2006; Wichmann *et al*., 2011). *Xtg* was grown either in Circle Grow (CG) broth (Molecular Probes, Eugene, OR, USA) or on CG and GYC (glucose, yeast extract, $CaCO_3$) plates containing 1.5% agar at 28 °C. *Escherichia coli* cells were cultivated in Luria–Bertani (LB) broth or on LB plates at 37 °C. Antibiotics were used in *E. coli* cultures at the following concentrations: 50 μg/mL ampicillin, 50 μg/mL kanamycin and 25 μg/mL streptomycin. We prepared electrocompetent *Xtg*29 cells and conducted electroporation using the protocol described in Oshiro *et al*. (2006). Sucrose (5%) was added to the medium when selecting for the second crossing-over event.

Sequencing, genome assembly and gap closure

Genomic DNA of isolate *Xtg*29 (Kölliker *et al*., 2006) was extracted from 30 mL of bacterial culture grown at 28 °C in CG broth (Molecular Probes) using the cetyltrimethylammonium bromide (CTAB) method (Ausubel *et al*., 1987). The DNA was nebulized to obtain random fragments with an average size of 3 kb. The GS Titanium Library Paired End Adaptors Kit (Roche, Mannheim, Germany) was employed to generate a 3-K Paired End fragment library. After titration with a GS Titanium SV emPCR Kit (Lib-L) v2 (Roche), an emulsion polymerase chain reaction (PCR) was carried out with the GS Titanium LV emPCR Kit (Lib-L) v2 (Roche). Samples were analysed using a GS Titanium Sequencing Kit XLR70t and the GS Titanium PicoTiterPlate Kit 70x75 (both from Roche) by means of a 454 Genome Sequencer FLX System (Roche). Sequencing reads were assembled using the GS *de novo* Assembler software (Newbler; release 2.5.3, 454 Life Sciences, Roche Corporation). Relative coverages of individual contigs were calculated as described previously for another bacterium with high G + C content (Schwientek *et al*., 2012).

Gap closure was performed using the BigDye® Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA). A sequencing premix of 12 μL containing 3 μg genomic DNA, 833 mM betaine and 1 pmol of primer was prepared and denatured at 98 °C for 5 min (Kieleczawa, 2006); 8 μL of BigDye® Terminator v3.1 ready reaction mix (Applied Biosystems) was added and cycle sequencing was performed using an initial step of 95 °C for 5 min, followed by 99 cycles of 95 °C for 5 min, 50–55 °C for 20 s and 60 °C for 4 min. The samples were purified using ethanol/ethylenediaminetetraacetic acid (EDTA) precipitation, and analysed with an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems).

Genome analysis and annotation

Functional annotation of the genome was achieved comparing several software tools employed for previous *Xanthomonas* genome projects. The gene predictor Prodigal (Hyatt *et al*., 2010) outperformed Glimmer 3.02 (Delcher *et al*., 2007), Gismo (Krause *et al*., 2007) and Reganor (Linke *et al*., 2006), the latter implementing combined activity of Glimmer and Critica in terms of identifying translational start sites (data not shown). Hence, Prodigal was used to predict CDSs that received locus tags with the prefix XTG29. Functional information was obtained for the CDSs by means of the GenDB Metanor pipeline (Meyer *et al*., 2003) for Gram-negative bacteria. Functional annotation was copied from orthologous genes of the strain *Xcc* B100 for which the annotation of several CDSs with metabolic functions has been updated recently (Schatschneider *et al*., 2011). Genome comparisons were performed using EDGAR (Blom *et al*., 2009) and are made available by means of the public EDGAR project '*Xanthomonas translucens graminis* 29' at http://edgar.cebitec.uni-bielefeld.de/. DNA and protein sequences were compared using BLASTN, BLASTX or BLASTP (Altschul *et al*., 1990). Predicted *Xtg*29 amino acid sequences of T3SS genes were compared after CLUSTALW alignment using the resulting identity matrices (Hall, 1999). T3Es were found using EDGAR software, BLASTP and TBLASTN programs against all proteins and the nucleotide sequence of *Xtg*29, and searching the whole genome sequence for PIP box sequences, i.e. TTCGB-N_{15}-TTCGB, where B is any base other than adenine (Fenselau and Bonas, 1995).

Cloning of plasmids, plasmid isolations and PCR conditions

Plasmid isolations were performed with the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA). Restriction enzymes were used according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). Cloning reactions were performed using the Dephos and Ligation kit (Roche, Penzberg, Germany). PCRs were conducted in 20-μL volumes using Hotstar DNA Polymerase (Qiagen, Hilden, Germany) or Phusion (Qiagen), depending on the required proofreading activity. The PCR conditions were as follows: initial denaturation at 94 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 50–60 °C annealing for 40 s, 72 °C extension for 1 min/kb and a final extension of 7 min at 72 °C. Constructs were verified by sequencing plasmid DNA using gene-specific or M13 primers. Sequencing reactions were performed using 20 ng of DNA.

The Δ*hrpG*, Δ*hrcR* and Δ*hrpE* fragments used for site-directed mutation were generated using Soeing PCR (Horton, 1995) by connecting the PCR fragments amplified from the corresponding flanking regions with primers containing the sequence of a *Sal*I or *Bam*HI restriction site at the 5′ end of each primer. PCR fragments were subcloned into the pCR®4Blunt-TOPO® vector (Invitrogen, Carlsbad, CA, USA). The Δ*hrpG*, Δ*hrcR* and Δ*hrpE* fragments were cut from the pCR®4Blunt-TOPO® vector and ligated into

the suicide vector pKNG101 (Kaniga *et al*., 1991). Control and selection of single and double crossing-over events were performed using PCR on single colonies and the primers listed in Table S3 (see Supporting Information). For the complementation of the Δ*hrpG* mutant, the *GFPuv* gene of the plasmid pDSK-GFPuv (Wang *et al*., 2007) was excised using *Eco*RI and *Pst*I, and replaced with the open reading frame (ORF) and the promoter region (321 bp upstream) of *hrpG*.

Generation of rifampicin-resistant *Xtg* strains

To selectively re-isolate *Xtg*29 and *Xtg*29 Δ*hrpG*, *Xtg*29 Δ*hrpE* and *Xtg*29 Δ*hrcR* mutants from plant material, rifampicin resistance was induced. The rifampicin concentration was increased continuously in CG broth from an initial value of 10 μg/mL over intervals of 30, 50, 100 and 150 μg/mL, to a final concentration of 300 μg/mL, in a rotary shaker at 28 °C and 150 rpm. One millilitre of bacterial culture was used to inoculate 4 mL of CG medium supplemented with the required amount of rifampicin. Cells were grown for 24 h until the rifampicin concentration had increased.

Screening for *Xtg* symptoms and *in planta* growth

To monitor *Xtg* symptoms and *in planta* multiplication, the highly susceptible genotype *Lm*K-01 (Wichmann *et al*., 2011) was used. Inoculation was performed by cutting plants with scissors dipped into a bacterial suspension [optical density at 600 nm (OD_{600}) = 0.6]. Plants were kept at 16 h light per day at 19 °C/23 °C (average day/night temperature). The assessment of bacterial wilt symptoms was performed using four replications per genotype × treatment combination. Scoring for bacterial wilt symptoms was performed at 7, 14, 21 and 28 dpi according to a scale ranging from completely healthy (1) to dead (9), with intervals as described in Wichmann *et al*. (2011). A negative control treatment consisted of cutting the plants without inoculum.

In planta growth was determined by counting the colony-forming units (CFU) per gram of fresh plant material, including leaves and tillers (2 cm above the soil). To ensure exhaustive sampling, two preliminary experiments using the Δ*hrpG* mutant and the *Xtg*29 wild-type were carried out (data not shown). As highly similar results were obtained in these two preliminary experiments, the four most meaningful time points after infection were monitored for all resulting mutants: 6 hpi and 4, 7 and 14 dpi. Experiments were performed using three or four biological replicates. Plant surface sterilization was performed using a 1% Chloramine-T solution (Honeywell Riedel de-Haën, Seelze, Germany). Serial dilutions were prepared on CG (Molecular Probes) plates supplemented with 300 μg/mL of rifampicin (AppliChem, Darmstadt, Germany). Bacterial cell counts per gram of fresh weight plant material were determined after incubation of the plates at 28 °C for 7 days. In order to verify the identity of the isolated bacteria, PCR using primers targeting the flanking regions of the corresponding mutation (Table S3) was conducted on 10 different colonies per treatment and time point.

Gene expression analyses of T3SS genes in *Xtg*29 and *Xtg*29 Δ*hrpG*

To quantify *hrpE* and *hrcR* expression *in planta*, plant material was sampled at 4, 14, 17 and 28 dpi with *Xtg*29 and the Δ*hrpG* mutant, and four biological and three technical replicates were performed per treatment and time point. The harvested plant leaves were ground in liquid nitrogen and 200 mg of plant material were used for total nucleic acid extraction. This was performed using a modified hot phenol extraction: 1 mL of neutral phenol and 500 μL of RNase-free water were added to the plant material and incubated at 65 °C for 6 min. After cooling and centrifugation, the aqueous phase was transferred to a new tube and an equal volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1) mix was added. After two extractions using an equal volume of chloroform, the nucleic acids were precipitated at −70 °C overnight using 100% ethanol. After washing once with 70% ethanol, pellets were resuspended in 200 μL of RNase-free water. After this step, RNA and DNA were treated separately. RNA purification was performed using the RNeasy MinElute Cleanup Kit (Qiagen) including DNase treatment. For DNA purification, RNase A was added to the samples and incubated for 30 min at 37 °C. After RNase inactivation at 70 °C for 10 min, the sample was purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany).

Reverse transcription was performed with 5 μL of purified RNA (1 μg/μL) using gene-specific primers of the *hrpE* and *hrcR* genes (Table S3) and Superscript II (Invitrogen), according to the manufacturer's recommendations. PCR amplifications of DNA and cDNA were performed using *hrpE* and *hrcR* primers in 20-μL volumes using 2 μL of cDNA or DNA. Quantitative real-time PCR was performed using SsoFast EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions were as follows: initial denaturation step at 98 °C for 3 min, 45 cycles of 5 s at 98 °C and 10 s at 60 °C. The specificity of the primer pairs was verified by melting curve analysis. The analysis of relative gene expression was performed using REST® software (Qiagen).

Data analyses and statistics

Geneoius software version 5.5.3 was used for protein sequence comparison and genealogy analyses. We used ClustalW (Larkin *et al*., 2007) for sequence alignments, and the phylogenetic trees were inferred using maximum likelihood. Bootstrap values were calculated on the basis of 100 replications. Disease symptoms were described using the AUDPC values (Wichmann *et al*., 2011). In order to compare AUDPC values resulting from infection with the T3SS mutants or the wild-type *Xtg* strain, multiple *t*-tests using Bonferroni family wise error rate correction were performed. The same procedure was used when testing for significant differences in CFU values *in planta* after infection with the T3SS mutants or the wild-type *Xtg* strain. $P < 0.05$ was considered to be significant. These statistical analyses were all performed in R (The R Development Core Team, 2008) using the packages: stats, graphics and coin (Hothorn *et al*., 2006).

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Nucleotide sequences are accessible through DDBJ/EMBL/GenBank under the accession numbers ANGG01000001: ANGG01000788

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Phylogenetic trees inferred with maximum likelihood for the separate alignment of eight different Hrc proteins (HrcC, HrcJ, HrcN, HrcR, HrcS, HrcT, HrcU and HrcV) from different *Xanthomonas* spp., *Ralstonia solanacearum*, *Collimonas fungivorans*, *Burkholderia* spp. and *Acidovorax avenae* ssp. *avenae*. *Pseudomonas syringae* pv. *tomato* strain DC3000 was used as an outgroup. Numbers represent bootstrap values for 100 replications.

Fig. S2 Symptoms on *Lolium multiflorum* seedlings at 7 days post-infection with different *Xanthomonas translucens* pv. *graminis* type III secretion system mutants (i.e. Δ*hrcR*, Δ*hrpE*, Δ*hrpG*, Δ*hrpG*/Δ*hrcR*, Δ*hrpG* + *hrpG*) and the wild-type or negative control treatment, i.e. cutting the plants with scissors dipped in sterile physiological sodium chloride solution. Symptoms are primarily characterized by increased wilting of the entire seedling when compared with the control treatment.

Table S1 *rax* gene homologues from *Xanthomonas oryzae* pv. *oryzae* found in the genome of *Xanthomonas translucens* pv. *graminis* 29.

Table S2 Strains and plasmids used in this study.

Table S3 Primer sequences and their application used in this study. Underlined sequences indicate recognition sites for restriction enzymes used for cloning.