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# Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant

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## Summary

Pathogenicity of *Xanthomonas campestris* pathovar (pv.) *vesicatoria* and most other Gram-negative bacterial plant pathogens largely depends on a type III secretion (TTS) system which is encoded by hypersensitive response and pathogenicity (*hrp*) genes. These genes are induced in the plant and are essential for the bacterium to be virulent in susceptible hosts and for the induction of the hypersensitive response (HR) in resistant host and non-host plants. The TTS machinery secretes proteins into the extracellular milieu and effector proteins into the plant cell cytosol. In the plant, the effectors presumably interfere with cellular processes to the benefit of the pathogen or have an avirulence activity that betrays the bacterium to the plant surveillance system. Type III effectors were identified by their avirulence activity, co-regulation with the TTS system and homology to known effectors. A number of effector proteins are members of families, e.g., the AvrBs3 family in *Xanthomonas*. AvrBs3 localizes to the nucleus of the plant cell where it modulates plant gene expression. Another family that is also present in *Xanthomonas* is the YopJ/AvrRxv family. The latter proteins appear to act as SUMO cysteine proteases in the host. Here, we will present an overview about the regulation of the TTS system and its substrates and discuss the function of the AvrRxv and AvrBs3 family members in more detail.


## Introduction

Plants have to cope not only with abiotic stresses but also have to defend themselves against pathogens. Among the phytopathogens are worms, fungi,

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oomycetes and bacteria which all try to acquire nutrients from the host plant for their own advantage. Although plants have basal defense mechanisms, some organisms have evolved mechanisms to suppress the basal host defense and therefore are pathogenic. Our laboratory studies *Xanthomonas campestris* pathovar (pv., indicates host range) *vesicatoria* (*Xcv*),[1] the causal agent of bacterial spot disease, and its host plants pepper and tomato. The bacteria enter the plant tissue via stomata or wounds and multiply in the intercellular spaces of the plant tissue (Stall, 1995). Bacterial pathogenicity depends on a specialized type III secretion (TTS) system, which secretes some helper and accessory proteins into the extracellular milieu that support the injection of the effector proteins, the major substrate class of the TTS system, into the host cell (Hueck, 1998; Cornelis and Van Gijsegem, 2000).

However, some host plant genotypes are able to specifically recognize a given effector protein which thus makes the corresponding pathogen strain no longer virulent on this plant. In many cases, a localized cell death response, the hypersensitive response (HR), is induced resulting in attenuation of bacterial growth (Klement, 1982; Keen, 1990). Bacterial growth inhibition is probably due to a decrease in the apoplastic water potential during the HR (Wright and Beattie, 2004). Specific recognition follows the gene-for-gene hypothesis (Flor, 1971): plants carry resistance (*R*) genes that recognize the product of corresponding effector genes in the pathogen, which were termed avirulence (*avr*) genes. To understand the molecular basis of gene-for-gene recognition and the biological function of effector proteins, both effector (or *avr*) and *R* genes are studied intensively. In this review, we will focus on the TTS apparatus and mainly discuss the effector proteins translocated by this machinery.

## The TTS system of *Xanthomonas*

Basic pathogenicity of *Xcv* in pepper and tomato is determined by the TTS system, which is highly conserved in most Gram-negative bacterial pathogens of plants and animals (Tampakaki et al., 2004). Type III-dependent protein secretion was first identified in the animal pathogen *Yersinia enterocolitica* (Heesemann et al., 1984), however, mutants in genes coding for components of the secretion machinery were first discovered in the plant pathogen *Pseudomonas syringae* by the analysis of non-pathogenic mutants (Niepold et al., 1985; Lindgren et al., 1986). In phytopathogenic bacteria, the TTS system is encoded by large gene clusters termed *hrp* (hypersensitive response and pathogenicity; Alfano and Collmer, 2004; Tampakaki et al., 2004; Fig. 1). *hrp* mutants no longer cause disease in susceptible plants, barely grow in plant tissue and are unable to induce the HR in resistant host and non-host plants (Bonas et al., 1991; Büttner and Bonas, 2002). The *hrp* gene clusters of plant pathogens (except for *Ralstonia solanacearum*) show typical features of pathogenicity islands, i.e., genes for virulence factors, mobile genetic elements, a deviant G+C content and codon usage as compared to the rest of the genome, and flanking tRNA genes (Fig. 1), all indicative for acquisition by horizontal gene transfer (Dobrindt et al., 2004). The regions flanking the *hrp* region vary in their gene content and often contain type III effector genes (Fig. 1; Alfano et al., 2000; Noël et al., 2002). The *hrp* gene cluster of *Xcv* is part of a 35.3 kb chromosomal genomic island (Bonas et al., 1991; Fenselau et al., 1992; Fenselau and Bonas, 1995; Rossier et al., 2000; Noël et al., 2002; Thieme et al., 2005), in which 18 out of the 37 putative coding sequences encode Hrp proteins. Eleven proteins are highly conserved in plant and animal pathogenic bacteria and were, therefore, termed Hrc (*hrp* conserved; Bogdanove et al., 1996; Tampakaki et al., 2004). Most Hrc proteins, probably forming the core of the TTS apparatus, also share homology to components of the flagellar assembly apparatus which is thought to be the evolutionary ancestor of the TTS system (Aizawa, 2001; Tampakaki et al., 2004).

The role of a few non-conserved Hrp proteins, which are essential for pathogenicity, could recently be clarified for *Xcv*. For example, HrpB2 and HrpE were found to be secreted and essential for secretion of other proteins by the TTS system (Rossier et al., 2000). HrpB2 is likely an extracellular part of the secretion machinery (Rossier et al., 2000). HrpE is the major subunit of the Hrp pilus, an extracellular appendix that is thought to be associated with the TTS apparatus (Koebnik, 2001; Weber et al., 2005). The Hrp pilus most likely serves as a conduit for secreted proteins. In contrast, the type III-secreted protein HrpF is essential for pathogenicity but dispensable for secretion in *Xcv* (Rossier et al., 2000). However, *hrpF* mutants in *X. oryzae* pv. *oryzae* (*Xoo*) were not completely abolished in virulence (Sugio et al., 2005). HrpF has been suggested to be the major component of the *Xcv* TTS translocon, a predicted

[1]Also designated as *Xanthomonas axonopodis* pv. *vesicatoria* (Vauterin et al., 2000) or *Xanthomonas euvesicatoria* (Jones et al., 2004).

protein channel in the plant plasma membrane that mediates the transport of effector proteins into the plant cell cytosol (Büttner et al., 2002).

The analysis of non-polar mutants in the *hrp* gene cluster of *Xcv* also identified *hpa* (*hrp* associated) genes that contribute to, but are not essential for the pathogenic interaction with the plant (Huguet et al., 1998; Noël et al., 2002; Büttner et al., 2004; U. Bonas and D. Büttner, unpublished data). For example, *hpaA* encodes a protein that is needed for the HR and full pathogenicity. HpaA is secreted by the TTS system and localizes to the plant nucleus when expressed in planta (Huguet et al., 1998). In contrast, HpaB remains in the bacterium where it plays an important role in the control of type III protein export (Büttner et al., 2004). Interestingly, the type III-secreted protein XopA (*Xanthomonas* outer protein) also shows features of an Hpa protein: The *xopA* gene is located adjacent to the left border of the *hrp* region, its expression is HrpG- and HrpX-dependent (see below). XopA shows homology to Hpa1 from *Xoo* (Zhu et al., 2000). However, XopA shows no harpin HR eliciting activity as it was shown for Hpa1 from *Xoo* and the homolog HpaG from *X. axonopodis* pv. *glycines* (Kim et al., 2003, 2004). *xopA* mutants are affected in bacterial virulence, but not in type III-mediated secretion (Fig. 1; Noël et al., 2001, 2002). As XopA is not translocated into the host cell, we speculate that it might be part of the translocon (Büttner et al., 2004).

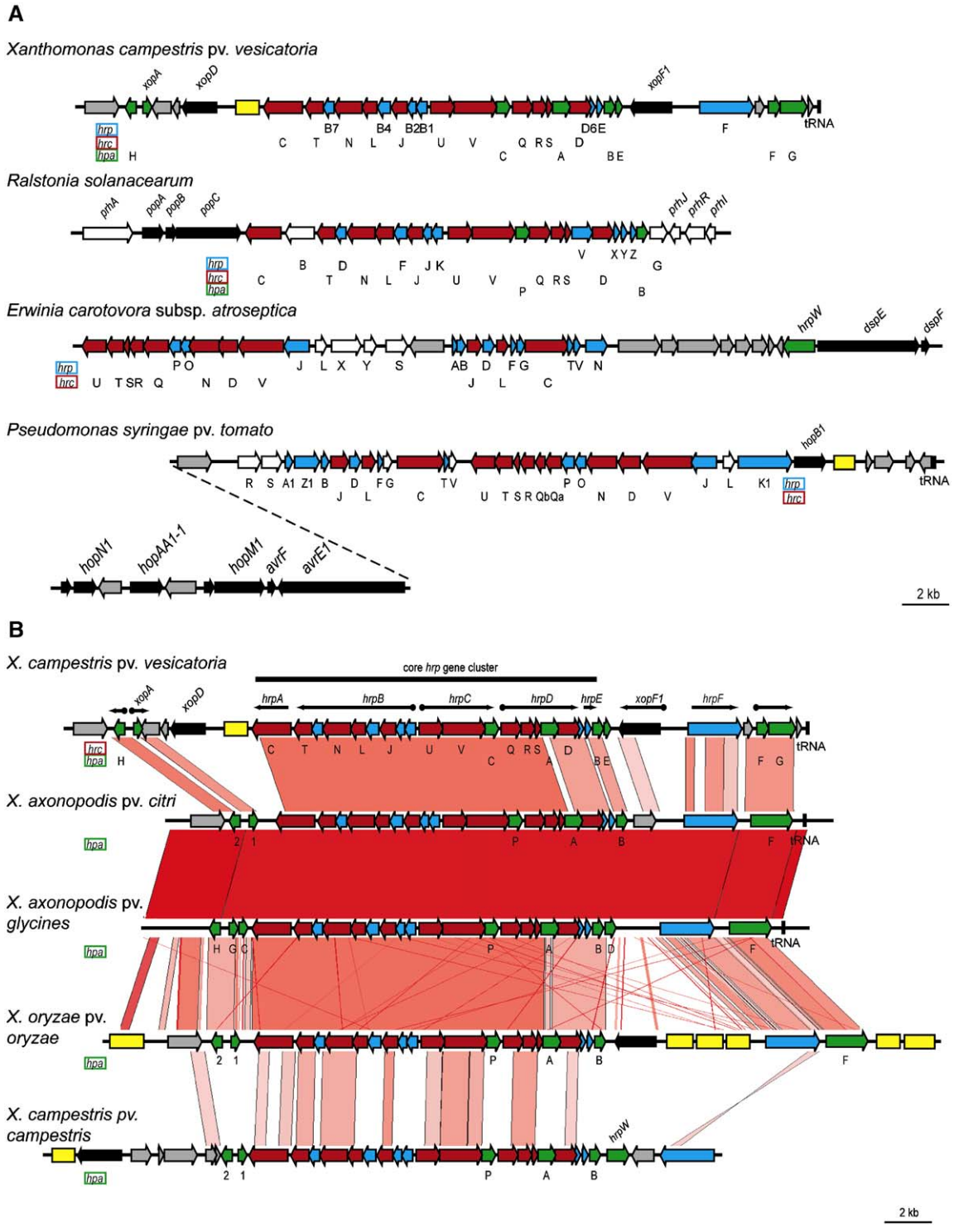
## Regulation of *hrp* gene expression in phytopathogens

Expression of the TTS system in phytopathogenic bacteria is induced in planta or in specific minimal media mimicking the plant apoplast (Schulte and Bonas, 1992a, b; Lindgren, 1997). The regulatory cascade controlling expression of *hrp* genes varies in different plant pathogens. Based on similarities in gene organization and regulation, the *hrp* regions of different pathogens were classified into two groups. Group I comprises species of *Erwinia*, *Pantoea* and *P. syringae*, whereas *R. solanacearum* and *Xanthomonas* spp. belong to group II (Fig. 1; Alfano and Collmer, 1996). Expression of group I *hrp* genes depends on the activator HrpL, a member of the extracytoplasmic function (ECF) family of alternate sigma factors, which bind to a conserved promoter sequence termed *hrp* box (Xiao and Hutcheson, 1994; Xiao et al., 1994; Wei and Beer, 1995). *hrpL* expression is controlled by HrpR and HrpS in *P. syringae* (Xiao et al., 1994), and by HrpS, HrpX and HrpY in *Erwinia* (Wei et al., 2000b; Mor et al., 2001; Chatterjee et al., 2002) and *Pantoea* (Frederick et al., 1993; Merighi et al., 2003). HrpS and HrpR of *Pseudomonas* are enhancer-binding proteins of the NtrC-family, synergistically activating the expression of *hrpL* in response to plant signals or in *hrp*-inducing minimal medium (Grimm and Panopoulos, 1989; Xiao et al., 1994; Grimm et al., 1995; Hutcheson et al., 2001). It was shown that HrpS and HrpR physically interact (Hutcheson et al., 2001). In *P. syringae* pv. *tomato* DC3000, *hrpL* expression is also regulated by the $\sigma$54 factor RpoN which is controlled by the GacA master regulator (Chatterjee et al., 2003). Intriguingly, under *hrp*-suppressing conditions, the Lon protease degrades HrpR and thus suppresses expression of the *hrp* regulon (Bretz et al., 2002). In contrast to HrpR, the negative regulator HrpV acts upstream of HrpR and HrpS and can downregulate *hrp* genes under inducing conditions (Preston et al., 1998). Recently, it was shown that the chaperone-like protein HrpG interacts with HrpV and serves as a suppressor of this negative regulator (Wei et al., 2005). Interestingly, *hrp* gene expression is also downregulated in *P. syringae* pv. *tomato* DC3000, when *hrpA*, which encodes the major subunit of the Hrp pilus, is mutated (Wei et al., 2000a). Although *hrp* gene expression in *Erwinia* strains also depends on HrpL, its activation differs from *Pseudomonas*. In case of *Erwinia*, HrpX is an $I_C$T-type sensor (Parkinson and Kofoid, 1992). In *E. amylovora*, HrpX is probably activated by phosphorylation. In turn, HrpX phosphorylates HrpY, an $RO_{III}$ response regulator (Parkinson and Kofoid, 1992). Phosphorylated HrpY, complexed with activated HrpS, binds to the *hrpL* promoter and activates *hrpL* expression in an RpoN-dependent manner (Wei et al., 2000b). In *E. herbicola* pv. *gypsophilae*, the key regulatory step is the phosphorylation of HrpY. The latter activates *hrpS* expression, and HrpS and $\sigma$54 together activate *hrpL*, whereas in this strain HrpX seems to be dispensable (Nizan-Koren et al., 2003).

Expression of group II *hrp* gene clusters is best studied in *R. solanacearum*. Here, the inducing signal is sensed by the outer membrane receptor PrhA and transduced by the putative transmembrane protein PrhR and the ECF sigma factor PrhI. Activated PrhI leads to the expression of the regulators PrhJ, HrpG and HrpB, which is homologous to HrpX from *Xcv* (Genin et al., 1992; Marenda et al., 1998; Brito et al., 2002; Fig. 1A). For HrpB-dependent activation of promoters in *R. solanacearum* the $hrp_{II}$ box (TTCG-$N_{16}$-TTCG) is required. In addition, a *cis*-acting element (YANNRT) essential for transcriptional activation,

was identified in the −10 promoter region (Cunnac et al., 2004a).

The key regulatory proteins in *Xanthomonas* are HrpG and HrpX, which are encoded outside of the *hrp* gene cluster. HrpG belongs to the OmpR-family of two-component system response regulators (Wengelnik et al., 1996) and controls the expression of the regulator HrpX (Wengelnik and Bonas, 1996; Noël et al., 2001, 2002, 2003). HrpX, an AraC-type transcriptional activator, regulates the expression of most genes of the HrpG regulon including the *hrp* operons *hrpB* to *hrpF* (Wengelnik and Bonas, 1996; Noël et al., 2001, 2002, 2003). Many HrpX-regulated genes contain a PIP box (plant-inducible promoter box; consensus TTCGC-$N_{15}$-TTCGC) in their promoter region, which was



proposed to serve as a regulatory element (Fenselau and Bonas, 1995; Wengelnik and Bonas, 1996). However, this motif is not sufficient to confer HrpX inducibility, because there are HrpX-independent PIP-box containing promoters, e.g., *avrRxv* (Ciesiolka et al., 1999). It appears that additionally, as described above for HrpB a −10 box is needed for HrpX inducibility (Tsuge et al., 2005). Remarkably, this −10 box is absent in the promoter of the constitutively expressed *avrRxv* mentioned above. Whether HrpX or a still unknown protein binds to the PIP box remains to be elucidated.

## Signals and recognition of type III substrates

Functional analysis of the TTS system and the identification of secreted proteins required the establishment of an in vitro secretion assay. However, in vitro growth conditions that led to the induction of the *Xcv* TTS system were not suitable for detection of type III secretion. One breakthrough was the generation of the E44K point mutation in *hrpG* (designated *hrpG**) which resulted in constitutive expression of *hrp* genes under different growth conditions (Wengelnik et al., 1999). This mutant allowed the development of an appropriate in vitro secretion medium. Incubation of bacteria in acidic minimal medium (pH 5.2–5.4) and the addition of BSA finally led to detectable amounts of secreted proteins (Rossier et al., 1999). Interestingly, the *Xcv* TTS system also secretes heterologous proteins, e.g., PopA from *R. solanacearum*, AvrB from *P. syringae* and YopE from *Yersinia pseudotuberculosis*, indicating that the properties of the secretion signal are conserved among plant and animal pathogenic bacteria (Rossier et al., 1999). However, the nature of the TTS signal that resides in the N-terminal regions of type III-secreted proteins is still enigmatic. In contrast to *sec* signals, TTS signals show no common physicochemical properties. Furthermore, type III-secreted proteins are not processed during secretion (Holland, 2004; Tampakaki et al., 2004). Nevertheless, recent reports describe some common features of the N-terminal amino acid sequences of effectors, e.g., the presence of hydrophilic amino acids, absence of acidic residues in the first 12 amino acid residues, amphipathicity and a bias toward serine and glutamine in the first 50 residues (Lloyd et al., 2001a, 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). An alternative model, which is discussed controversially, proposes an mRNA signal in *Yersinia* outer proteins (Yops; Anderson and Schneewind, 1999; Sorg et al., 2005). However, various studies identified the signal in the first 11–17 amino acid residues of Yops (Sory et al., 1995; Schesser et al., 1996; Lloyd et al., 2001b). Similarly, the secretion signal of the effector AvrBs2 from the plant pathogen *Xcv* resides in the first 28 amino acid residues, whereas the first 58 residues are necessary for its translocation (Mudgett et al., 2000).

In addition to the secretion and translocation signal, a number of type III-secreted proteins contain a TTS-chaperone-binding site (Alfano and Collmer, 2004; Tampakaki et al., 2004). TTS chaperones are typically small, acidic and leucine-rich and share only little sequence similarity (Wattiau et al., 1994; Feldman and Cornelis, 2003; Alfano and Collmer, 2004). Usually, type III chaperones bind their substrates to stabilize them, to connect them to the TTS system and to keep them partially unfolded for their transport through the TTS pilus (Stebbins and Galán, 2001; Page and Parsot, 2002; Alfano and Collmer, 2004). TTS chaperones can be devided into classes based on their interaction with one specific effector

**Figure 1.** *hrp* regions from different plant pathogenic bacteria. (A) Schematic overview of the *hrp* regions from sequenced plant pathogens. The *hrp* clusters of *X. campestris* pv. *vesicatoria* strain 85–10 (Thieme et al., 2005), *Ralstonia solanacearum* strain GMI1000 (Salanoubat et al., 2002), *Erwinia carotovora* subsp. *atroseptica* strain SCRI1043 (Bell et al., 2004) and *Pseudomonas syringae* pv. *tomato* strain DC3000 (Buell et al., 2003) are shown. (B) Comparison of the *hrp* gene clusters of the different sequenced xanthomonads. The following strains are shown: *X. campestris* pv. *vesicatoria* strain 85–10 (Thieme et al., 2005), *X. axonopodis* pv. *citri* strain 306 (da Silva et al., 2002), *X. axonopodis* pv. *glycines* strain 8ra (Kim et al., 2003), *X. oryzae* pv. *oryzae* strain KACC10331 and *X. campestris* pv. *campestris* strain ATCC3391 (da Silva et al., 2002). Comparative analyses were carried out using the Artemis comparison tool (http://www.sanger.ac.uk/Software/Artemis/). Red areas indicate DNA regions with at least 80% identity over 400 bp and darker color indicates higher identity. Single-headed arrows represent CDS and the direction of transcription. Red arrows refer to *hrc* genes, blue arrows to *hrp* genes, green arrows to *hpa* genes, black arrows to putative/known type III effector genes, gray arrows to genes coding for proteins of unknown function, and white arrows to genes encoding regulators of the TTS system expression. Yellow boxes indicate IS elements. Arrows above the CDS indicate the operon structure and the black dots refer PIP boxes (CDS, coding sequences; *hrp*, hypersensitive response and pathogenicity; *hrc*, *hrp* conserved; *hpa*, *hrp* associated).

(class IA) or with multiple effectors (Cornelis and Van Gijsegem, 2000; Parsot et al., 2003). In *Pseudomonas*, genes encoding chaperones of class IA are often found next to the gene of the corresponding effector (Wattiau et al., 1996). In *Xcv*, so far only HpaB shows characteristic features of TTS chaperones (Büttner et al., 2004). HpaB is encoded within the *hrp* gene cluster and interacts with more than one effector and thus appears to be a class IB TTS chaperone. Interestingly, *hpaB* mutants were strongly reduced in in vitro secretion of effector proteins, but secretion of HrpF and XopA, presumably extracellular components of the TTS system, was not affected. Furthermore, the *hpaB* mutant was able to translocate the non-effector proteins HrpF and XopA into the plant cell. These results indicate that HpaB is not only a chaperone but also plays an essential role in exit control of the TTS system, i.e., translocation of effectors and inhibition of translocation of non-effectors (Büttner et al., 2004). Indeed, it was shown before in other bacteria that TTS chaperones can regulate secretion (Boyd et al., 2000; Francis et al., 2002; Wulff-Strobel et al., 2002; Thomas and Brett Finlay, 2003).

## How to identify and verify type III effectors?

### Identification of effectors based on avirulence activity

In plant pathogens, the first type III effectors have been identified as the products of avirulence genes that are recognized by corresponding plant disease resistance genes and their products, respectively. In most cases, recognition leads to the induction of the HR concomitant with arrest of bacterial growth (Klement, 1982; Staskawicz, 2001). *Xcv* strain 85–10 expresses three known avirulence proteins, AvrBs1, AvrBs2 and AvrRxv (Ronald and Staskawicz, 1988; Whalen et al., 1993; Swords et al., 1996). AvrBs1, which is encoded on the largest plasmid, and the chromosomal, highly conserved *avrBs2* gene contribute to bacterial fitness in the field and thus to the pathogen's virulence (Kearney and Staskawicz, 1990; Gassmann et al., 2000; Wichmann and Bergelson, 2004).

Based on avirulence protein translocation, in vivo screens for effectors were developed in *Xanthomonas* and *Pseudomonas* (Guttman et al., 2002; Roden et al., 2004b; Chang et al., 2005). In *Xcv*, the N-terminally deleted (amino acids 2–152) AvrBs3 derivative (see below) AvrBs3$\Delta$2 was established as a reporter. AvrBs3$\Delta$2 still induces the HR when transiently expressed in resistant plants mediated by *Agrobacterium* but cannot be secreted by *Xcv* (Szurek et al., 2001). This indicates that the N-terminal region of AvrBs3 contains a secretion and translocation signal and the C-terminus the effector domain. Translational fusions of 5′ coding regions of effector candidate genes to *avrBs3$\Delta$2* allowed the identification and verification of type III effectors (Noël et al., 2003). If a fusion protein induces the HR in AvrBs3-responsive resistant plants, the 5′ region of the candidate gene restored type III-dependent delivery of AvrBs3$\Delta$2 and thus contains a functional translocation signal (Fig. 2). Using this reporter assay, we could identify a number of type III effectors, e.g., XopC and XopJ (Noël et al., 2003). Similarly, AvrBs2 lacking its TTS signal was used as a reporter for effector translocation in a Tn*5*-based screen (Roden et al., 2004b). This screen led to the identification of a number of new effectors (Roden et al., 2004b; Table 1, see below).

### Identification of effectors based on co-regulation with the TTS system

Additional effector genes were identified by their co-regulation with the TTS system. While in *P. syringae* all known effector genes are co-regulated with the TTS system (Xiao and Hutcheson, 1994; Chang et al., 2005), this is not true for *Xanthomonas*. For example, the effector genes *avrBs1*, *avrBsT* (Escolar et al., 2001), *avrRxv* (Ciesiolka et al., 1999) and *avrBs3* (Knoop et al., 1991) are constitutively expressed. Nevertheless, a cDNA-AFLP screen for new members of the HrpG regulon in *Xcv* identified more than 20 genes, including four genes (*xopA*, *xopB*, *xopC* and *xopJ*) that were found to encode substrates of the TTS system (Noël et al., 2001, 2002, 2003).

### Identification of effectors by in silico prediction

In the era of high-throughput sequencing of bacterial genomes, effector candidates can also be uncovered based on homology to known effectors from other pathogens. In addition, the presence of eukaryotic motifs in the predicted gene products can indicate a function inside the host cell (Büttner and Bonas, 2003; Fig. 2). Furthermore, conserved sequence motifs such as PIP boxes in the promoter regions of *Xanthomonas* genes can be used to identify genes which might be co-regulated

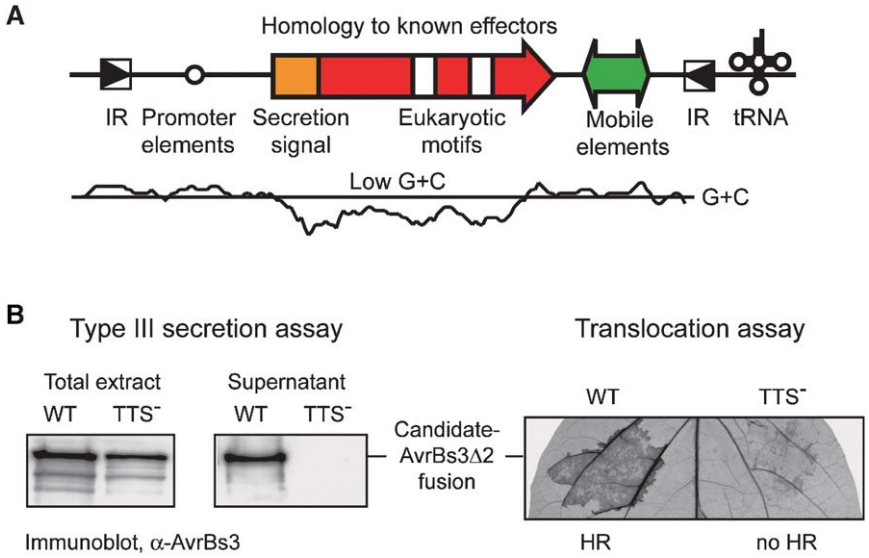



**Figure 2.** Strategies for the prediction and verification of type III effector proteins. (A) Schematic overview of an effector locus. Effector genes can be predicted by the presence of promoter motifs and signs of acquisition by horizontal gene transfer (red). On the amino acid level, there might be homology to known type III effectors, presence of eukaryotic features and putative TTS signals. (B) In vitro and in vivo assays to confirm type III effectors. Effector candidate fusions to the AvrBs3Δ2 reporter expressed in wild-type (WT) and TTS mutant (TTS$^-$) *Xcv* strains can be visualized by immunoblotting using an AvrBs3-specific antibody. Using these strains, in vitro type III secretion can be tested in secretion medium (left panel). The right panel shows a translocation assay in which the *Xcv* strains were inoculated into an AvrBs3-responsive resistant plant. The wild-type strain used is normally virulent on this plant (see text for details).

with the TTS system (Büttner et al., 2003; see above). In *P. syringae*, prediction algorithms for effectors have been developed based on a characteristic N-terminal amino acid composition (see above), which presumably serves as export signal (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). A number of effector genes show signs of horizontal gene transfer, e.g., low G+C content of the DNA, compared to the average of the genome, and flanking mobile elements or tRNA genes (Büttner et al., 2003; Noël et al., 2003; Thieme et al., 2005; Fig. 2 and Table 1). Based on the genome sequence, *Xcv* strain 85–10 contains 14 known effector genes (Table 1). Remarkably, *xopC* is the only specific effector gene unique for *Xcv*, because there is no homolog in the database and similarity to *R. solanacearum* proteins is only partial (Table 1). Interestingly, the *xopC* region shows signs of horizontal gene transfer and is associated with a putative effector integron consisting of genes encoding a transposase and cointegrate resolution proteins, flanked by inverted repeats (Noël et al., 2003). Similar integron elements are also present in *X. axonopodis* pv. *citri* (*Xac*). These elements are always associated with effector genes or candidates and could be involved in the horizontal transfer of virulence factors (Noël et al., 2003). In contrast to the unique XopC protein, XopQ homologs are present in different pathogens. The effector belongs to the HopQ1-1 (formerly HopPtoQ) family (Rohmer et al., 2004) and is a putative inosine-uridine nucleoside *N*-ribohydrolase (Roden et al., 2004b; Table 1). Unique effectors like XopC could be important for a specific host interaction, whereas the wide distribution of an effector gene in different bacteria like XopQ suggests a more general role in virulence.

## What do we know about the function of type III effectors?

The first type III effector from plant pathogens was isolated as an avirulence protein from *P. syringae* pv. *phaseolicola* in 1984 (*avrA*; Staskawicz et al., 1984). Since then, more than 40 Avr proteins and many type III effectors, respectively, were identified in plant pathogens possessing a TTS system, e.g., *Ralstonia*, *Xanthomonas*, *Pantoea* and *Erwinia*. Although the first type III effectors were identified because of their activity in restricting the host range of the pathogen by inducing the HR (avirulence activity), it was assumed that there has to be a function of TTSS effector proteins for the producer's advantage

**Table 1.** Known type III-effector proteins of *Xcv* strain 85-10

| Gene name | Predicted function/ homology* | HrpG-dep.† | G+C‡ | Homologs§ in | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | (%) | *Xac* | *Xcc* | *Xoo* | *Pst* | *Rs* | *Eca* | |
| *avrBs1* | Unknown function | – | 42.23 | – | + | – | – | – | – | Ronald and Staskawicz (1988) and Escolar et al. (2001) |
| *avrBs2* | Putative glycerophosphoryl-diester phosphodiesterase | nd | 63.59 | + | + | + | – | – | – | Swords et al. (1996) |
| *avrRxv* | YopJ/AvrRxv-family, putative cysteine protease | – | 52.32 | – | (+) | – | – | + | – | Whalen et al. (1993) and Ciesiolka et al. (1999) |
| *xopB* | Homology to HopD1 (*P. syringae* pv. *tomato*) | + | 55.54 | – | – | – | + | – | – | Noël et al. (2001) |
| *xopC* | Unknown function | + | 47.50 | – | – | – | – | (+) | – | Noël et al. (2003) |
| *xopD* | SUMO cysteine protease; C48 family | + | 54.76 | – | + | – | – | – | – | Noël et al. (2002) and Hotson et al. (2003) |
| *xopF1* | Unknown function | nd | 65.47 | (+) | (+) | + | – | – | – | Roden et al. (2004b) |
| *xopF2* | Unknown function | nd | 64.72 | (+) | (+) | + | – | – | – | Roden et al. (2004b) |
| *xopJ* | AvrRxv/YopJ-family, putative cysteine protease | + | 56.86 | – | (+) | – | – | + | – | Roden et al. (2004b) |
| *xopN* | Unknown function | nd | 63.44 | + | + | + | – | – | – | Roden et al. (2004b) |
| *xopO* | Homology to HopK1 and AvrRps4 (*P. syringae* | nd | 52.04 | – | – | – | + | – | – | Roden et al. (2004b) |
| *xopP* | Unknown function | nd | 61.66 | + | + | + | – | + | – | Roden et al. (2004b) |
| *xopQ* | HopQ1-1 family protein, putative inosine-uridine nucleoside *N*-ribohydrolase | nd | 68.88 | + | + | + | + | + | – | Roden et al. (2004b) |
| *xopX* | Unknown function | nd | 65.95 | + | + | + | – | – | – | Metz et al. (2005) |
| *ecf* | Early chlorosis factor, homology to HopAE1 (*P. syringae* pv. *syringae*) | – | 64.88 | – | – | + | – | – | – | Morales et al. (2005) |

*Predicted function and homology to known type III-effector proteins from *Pseudomonas syringae* or other *Xanthomonas* spp. For *Pseudomonas* effectors the unified nomenclature was used (Lindeberg et al., 2005).

†HrpG- and HrpX-dependent co-regulation with the TTS system (nd, not determined).

‡G+C content of the DNA in the coding region.

§Homologs were determined using BLAST algorithms. (+) indicates partial homology or a disrupted homolog in the corresponding genome. (*Xac*: *X. axonopodis* pv. *citri*; *Xcc*: *X. campestris* pv. *campestris*; *Xoo*: *X. oryzae* pv. *oryzae*; *Pst*: *P. syringae* pv. *tomato*; *Rs*: *R. solanacearum*; *Eca*: *E. carotovora* subsp. *atroseptica*).

(virulence activity). A contribution to virulence could indeed be observed for several Avr proteins and effectors, respectively (Kearney and Staskawicz, 1990; Swarup et al., 1991, 1992; Lorang et al., 1994; Yang et al., 1994, 2000; Ritter and Dangl, 1995; Bogdanove et al., 1998; Huguet et al., 1998; Jackson et al., 1999; Alfano et al., 2000; Chang et al., 2000; Chen et al., 2000; Gassmann et al., 2000; Shan et al., 2000; Tsiamis et al., 2000; Vera Cruz et al., 2000; Guttman and Greenberg, 2001; Badel et al., 2002; Guo et al., 2002; Lavie et al., 2002; Abramovitch et al., 2003; Deng et al., 2003; Holeva et al., 2004; Lopez-Solanilla et al., 2004; Losada et al., 2004; Yang and White, 2004; Kay et al., 2005; Lim and Kunkel, 2005; Lin and Martin, 2005; Metz et al., 2005). However, in many cases, a virulence function of type III effectors is difficult to assess, because the pathogens have several effectors which can be functionally redundant and often show no homologies to proteins with known function. It is interesting that effector proteins are quite diverse whereas the analysis of their counterparts, the known resistance gene products, revealed a large number of related proteins in different plant species (Dangl and Jones, 2001; Lahaye, 2002; Martin et al., 2003). This suggests that plants use similar mechanisms to detect a wide range of diverse effector proteins to defend themselves and avoid pathogen's growth. However, although effector proteins generally vary in their amino acid sequence, some of them are homologous and were therefore grouped into families. Does this mean that pathogens also pursue common mechanisms to manipulate the host, to defend themselves against plant's defense and to grow? Here, we will focus our discussion on two major families of type III effectors from *Xanthomonas* characterized by remarkable features (Fig. 3), which indicated a function for the proteins in virulence.

## A family of cysteine proteases

A growing number of type III effectors appear to act as cysteine proteases in plants. Based on primary sequence analysis, they were classified into four groups: the AvrRxv/YopJ (C55) family (Orth et al., 2000), the XopD (C48) family (Hotson et al., 2003), the HopAR1/YopT (C58) family (Shao et al., 2002) and the AvrRpt2 (C70) family (Mudgett and Staskawicz, 1999). Members of the AvrRxv/YopJ family have been identified in mammalian and plant pathogens suggesting that they play an important role in the interaction with the host. Examples include YopJ and YopP from *Y. pseudotuberculosis* and *Y. enterocolitica*, respectively, AvrRxv, AvrXv4, AvrBsT and XopJ from *Xcv*; PopP1 and PopP2 from *R. solanacearum*; ORFB from *Erwinia*, HopZ1 (formerly known as HopPmaD), HopZ2 (formerly known as AvrPpiG1) and HopZ3 (formerly known as HopPsyV) from *P. syringae* (Whalen et al., 1993; Galyov et al., 1994; Ciesiolka et al., 1999; Alfano et al., 2000; Astua-Monge et al., 2000; Arnold et al., 2001; Noël et al., 2001, 2003; Lavie et al., 2002; Deng et al., 2003; Deslandes et al., 2003). Comparison of predicted secondary structures of these effectors revealed similarities to the cysteine protease from adenovirus (AVP; Orth et al., 2000; Orth, 2002), which shows similarity to yeast Ulp1, a SUMO protease of the C48 family (Orth, 2002). All members of this family contain a conserved catalytic triad necessary for the HR induction (Fig. 3A; Orth et al., 2000; Shao et al., 2002; Hotson et al., 2003; Roden et al., 2004a). The substrate of YopJ appears to be the small ubiquitin-like modifier protein SUMO-1. SUMO is post-translationally ligated to lysin residues of target proteins (Hochstrasser, 2000a, b) which, e.g., blocks ubiquitination and subsequent proteolysis and thus stabilizes the target proteins. Removal of SUMO from host proteins by a SUMO protease would allow binding of ubiquitin leading to degradation of the protein. Neither YopJ nor AvrXv4 showed protease activity in vitro (Orth et al., 2000; Roden et al., 2004a). This is in good agreement with the results for AVP, which requires an activator for catalytic activity (Orth, 2002). Overexpression of YopJ resulted in a decrease of SUMO bound to proteins (Orth et al., 2000, 2002). Furthermore, similar results were shown for AvrXv4 transiently expressed in planta (Roden et al., 2004a). For XopD, the ability to remove SUMO from plant proteins in plant extracts was demonstrated (Hotson et al., 2003). XopD, isolated from *Xcv* based on its position in the left *hrp* flanking region and homology to PsvA from *P. syringae* pv. *eriobotryae* (Noël et al., 2002), belongs to the C48 cysteine proteases, whereas YopJ, AvrBsT, AvrRxv, AvrXv4 and XopJ are C55 cysteine proteases (Fig. 3A, for review see Hotson and Mudgett, 2004). Interestingly, proteins of both cysteine protease families share another characteristic: XopD, PopP2, AvrRxv, AvrBsT and AvrXv4 contain putative nuclear localization signals (NLSs; Ciesiolka et al., 1999; Noël et al., 2002; Deslandes et al., 2003; Roden et al., 2004a). Indeed, PopP2 from *R. solanacearum* and XopD from *Xcv* localize to the plant cell nucleus where the latter accumulates in subnuclear foci (Deslandes et al., 2003; Hotson et al., 2003; L. Noël and U. Bonas, unpublished). In contrast, AvrXv4 and AvrRxv localize in the plant cell cytoplasm (Roden et al., 2004a; Bonshtien et al., 2005) suggesting

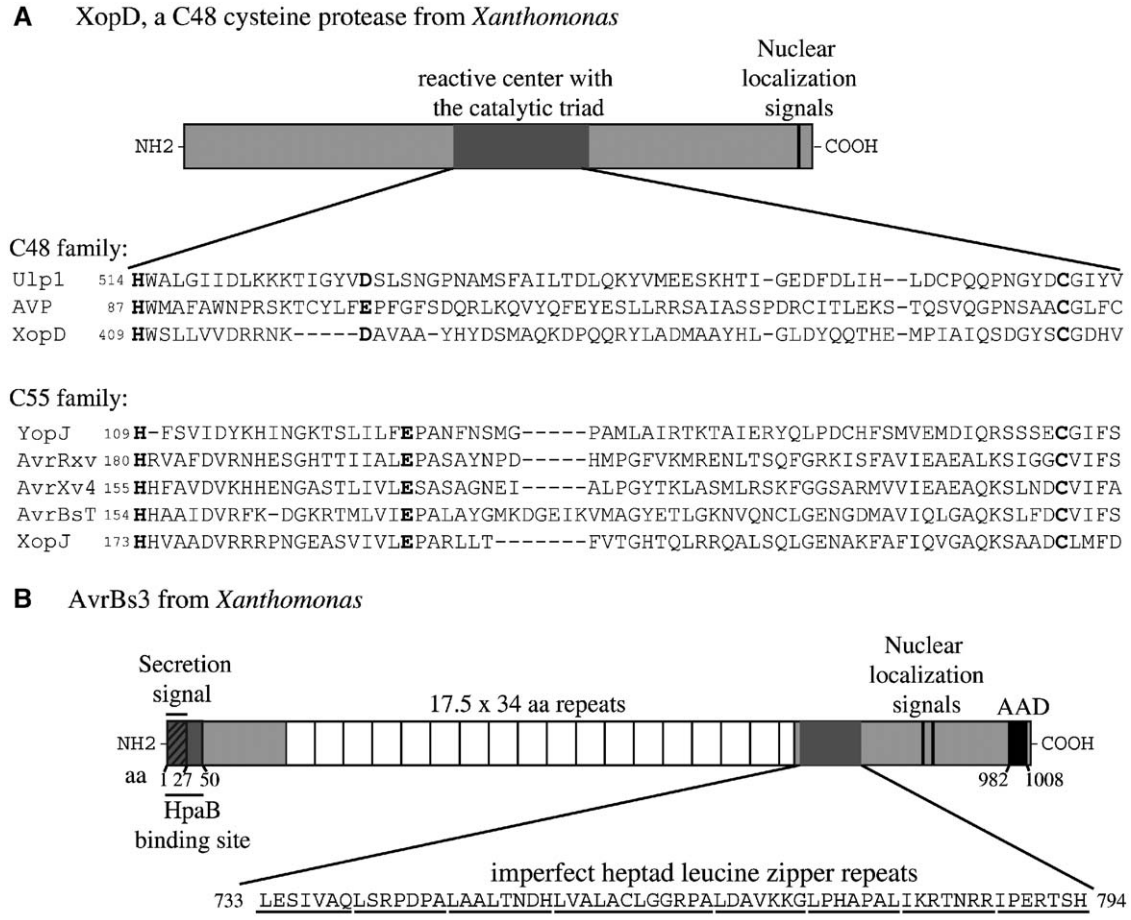



**Figure 3.** Structural features of representatives of two type III effector families from *Xanthomonas*. (A) XopD as an example for a C48 cysteine protease. The characteristic features of cysteine protease effectors are presented: The reactive centre containing the catalytic triad (black box) is essential for the function of the family members and is shown for different members of the C48 and C55 cysteine protease families from *Xanthomonas* (XopD, AvrRxv, Avrxv4, AvrBsT, XopJ), *Yersinia* (YopJ), yeast (Ulp1) and adenovirus (AVP). The residues forming the catalytic triad are shown in bold. The nuclear localization signal is indicated (black bar). (B) Structural features of AvrBs3 from *Xcv*. The central repeat region consists of 17.5 nearly identical tandem repeats of 34 amino acids. The C-terminal region contains different eukaryotic motifs: imperfect heptad leucine zipper repeats (black box; Gabriel, 1999); two functional nuclear localization signals (NLS; black bars; Van den Ackerveken et al., 1996; Szurek et al., 2001); and an acidic activation domain (AAD; Szurek et al., 2001). The secretion signal and the HpaB binding site are indicated (gray hatched and gray, respectively; Szurek et al., 2002; Büttner et al., 2004).

that the plant target proteins of these structurally related effectors differ. For example, XopD might manipulate the transcriptome by deSUMOylating transcriptional regulators, which then will be degraded. The fact that a pathogen delivers a set of proteins with similar functions into the plant cell where they localize to different compartments is probably a very efficient way to attack a wide range of plant proteins by mimicking different plant SUMO proteases.

One major question concerns the proteins targeted by type III effectors. Using YopJ as a bait in a yeast two-hybrid approach, several MAPK kinases were isolated (Orth et al., 1999). A second example is PopP2 from *R. solanacearum*, which was shown to interact with the corresponding R protein RRS1-R in *Arabidopsis* (Deslandes et al., 2003). Since RRS1-R recognizes PopP2, it is unlikely that RRS1-R is the virulence target of PopP2 (for review see; Alfano and Collmer, 2004). HopAR1 (formerly known as AvrPphB) from *P. syringae* coimmunoprecipitates the PBS1 kinase from *Arabidopsis*, indicating a physical interaction of both proteins (Shao et al., 2003). AvrRpt2, another cysteine protease from *P. syringae*, cleaves RIN4, an interactor of the matching R protein, RPS2. A physical interaction between AvrRpt2 and RIN4 is likely, but involves a plant protein, recently identified as cyclophilin (Coaker et al., 2005; Kim et al., 2005b). The isolation of additional target proteins including targets of the *Xcv* SUMO-proteases will shed

more light on the way of action of these families of type III effectors.

## A family of transcriptional activators

The effectors discussed above most likely interfere with host cellular processes by their enzymatic function as proteases. In contrast, effector proteins of a second large family, AvrBs3 family members presumably manipulate the host cell transcriptome more directly. Interestingly, members of the AvrBs3 family were only found in *Xanthomonas* sp. with the exception of Brg11 from *R. solanacearum* (Cunnac et al., 2004b), which is, however, more distantly related. Members of the *Xanthomonas* AvrBs3 family share 90–97% amino acid sequence identity and are characterized by striking features (Fig. 3B): all members contain a repetitive region in the central part of the polypeptide, imperfect heptad leucine zipper (LZ) repeats, NLSs and an acidic activation domain (AAD) (Herbers et al., 1992; Yang et al., 1994, 2000; Yang and Gabriel, 1995a, b; Van den Ackerveken et al., 1996; Zhu et al., 1998; Gabriel, 1999; Lahaye and Bonas, 2001; Szurek et al., 2001; van 't Slot and Knogge, 2002; Yang and White, 2004). Genetic analyses of AvrBs3-like effectors revealed that in most cases the known virulence and avirulence activities in the plant cell depend on the NLSs and the AAD (see below). The repeat region consists of 5.5 (Avrxa5) to 28.5 (PthXo3) nearly identical direct repeats of 34 amino acid residues and confers the avirulence specificities of these effectors (accession AAQ79773; Herbers et al., 1992; Yang et al., 1994, 2000; Yang and Gabriel, 1995b; Zhu et al., 1998; Lahaye and Bonas, 2001; Yang and White, 2004). The presence of nearly identical tandem repeats in all family members suggests that this family is naturally growing by recombination events, which indeed could be shown (Yang and Gabriel, 1995a; Yang and White, 2004). Interestingly, Hax2 from *X. campestris* pv. *armoraciae* (*Xca*; Kay et al., 2005) and Brg11 from *R. solanacearum* are distinct from all other family members, because their repeats consist of 35 amino acid residues. Strikingly, both genes were isolated from bacteria which are pathogenic on *Arabidopsis* (Deslandes et al., 1998; Kay et al., 2005) suggesting a correlation between repeat structure and host range of the bacteria.

AvrBs3, the founder of this family, was isolated from *Xcv* based on its avirulence activity on resistant pepper plants (Bonas et al., 1989). However, it could be shown for an increasing number of family members that their primary function is in virulence. Diseases caused by *Xanthomonas* spp., the observed symptoms on plants and specific plant reactions are shown in Fig. 4. For instance, AvrBs3 specifically induces a hypertrophy in susceptible pepper plants and other solanaceous species (Fig. 4A4). The hypertrophy, which is the enlargement of single mesophyll cells, might play a role in bacterial dispersal (Marois et al., 2002). In field studies, it has indeed been shown that AvrBs3 promotes bacterial spreading (Wichmann and Bergelson, 2004). Also PthA, the first effector that was isolated based on its virulence activity from the citrus canker pathogen *Xac*, causes pustles visible on the surface of leaves, stems and fruits (Swarup et al., 1991). This hyperplasia caused by PthA is, in contrast to the hypertrophy, due to multiplication of plant cells (compare Figs. 4A4 and 4B4). Apl1 from *Xac* also induces citrus canker-like symptoms (Ishihara et al., 2003). The recently isolated family members *hax2* to *hax 4* from *Xca* contribute additively to the aggressiveness of the pathogen on *Raphanus sativus* (Kay et al., 2005). Also for other members of the AvrBs3 family a virulence function could be observed: Avrb6 from *X. campestris* pv. *malvacearum* increases the release of bacteria to the surface of infected cotton plants (Yang et al., 1994). The rice pathogen *Xoo* encodes several AvrBs3 homologs that contribute to bacterial blight disease (Fig. 4C), e.g., AvrXa7 and, to a lesser extent, Avrxa5 enhance bacterial growth and lesion formation in rice (Bai et al., 2000). Bai et al. (2000), Yang et al. (2000) and Yang and White (2004) described even more family members from *Xoo* contributing to virulence (*aB* and *pthXo* genes). It should be emphasized that studies of *Xoo* strains revealed a large number of AvrBs3-like effectors compared to *Xcv* strains, which bear 1, 2 or no homologs of AvrBs3 (Lee et al., 2005; Ochiai et al., 2005; Thieme et al., 2005). It would be interesting to understand why the rice pathogen has evolved so many *avrBs3* family members to conquer its host. The central questions are how AvrBs3 and its homologs induce the observed plant reactions and which host proteins or genes are targeted.

## Following a type III effector from the pathogenic bacterium into the plant cell

### The destination: visualization of type III effectors at their site of action

Although AvrBs3 has been studied for quite some time its biochemical function is not entirely clear yet. The presence of NLSs and an AAD in the

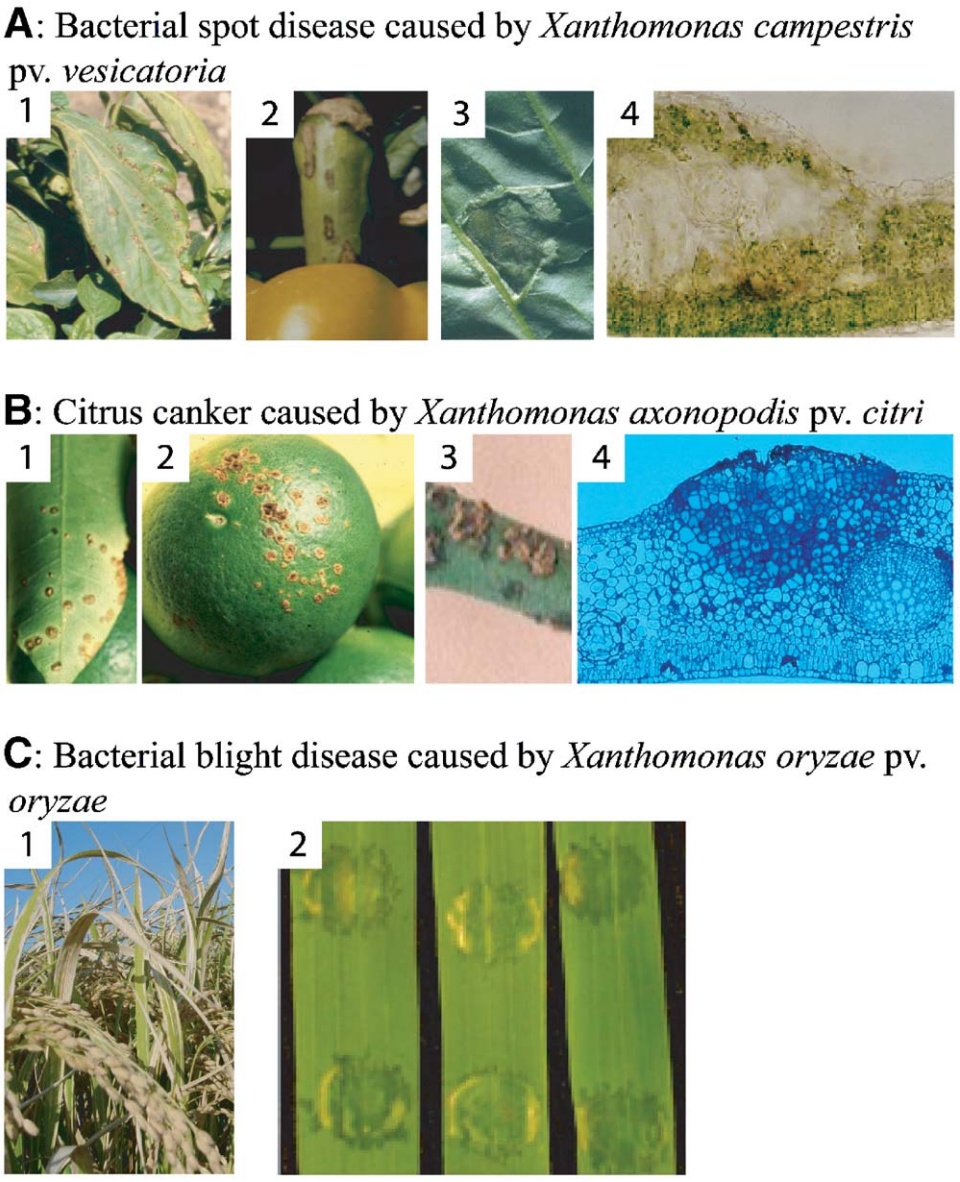



**Figure 4.** Plant diseases caused by different *Xanthomonas* species. Xanthomonads are phytopathogenic bacteria which cause various disease symptoms on important crop plants: (A) *Xanthomonas campestris* pv. *vesicatoria* causes bacterial spot disease on pepper and tomato. Water-soaked lesions are induced on leaves and fruits which later become necrotic (A1 and A2). AvrBs3 of *Xcv* induces hypertrophy in susceptible plant leaves visible as pustules on the lower epidermis (A3). Light microscopy analysis of a pepper leaf section infected with *Xcv* strain I74A expressing *avrBs3* visualizes the hypertrophy, the enlargement of single mesophyll cells (A4). This photograph is reprinted with permission from Marois et al. (2002). (B) *Xanthomonas axonopodis* pv. *citri* is the causal agent of citrus canker. The symptoms are raised circular lesions, which later becoming pustules which then darken and thicken into brown corky canker visible on leaves (B1), fruits (B2) and stems (B3). Photographs are reprinted with permission from Dean W. Gabriel: http://www.biotech.ufl.edu/PlantContainment/canker.htm. The pustule formation is due to tissue hyperplasia, an excessive mitotic cell division (B4). This photograph is reprinted with permission from Swarup et al. (1991). (C) *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight disease in rice. Typical symptoms are water-soaked to yellowish stripes on leaf blades which start at leaf tips and increase in length extending to the sheath (C1). The photograph of rice was provided by Dr. Jan Leach, Bioagricultural Sciences and Pest Management Department at Colorado State University. (C2) Infections of IR24 rice leaves with *Xoo* PXO99. On the left, the wild-type strain was inoculated. The middle leaf is inoculated with a PXO99ME2 mutant missing *pthXo1* and the right leaf with PXO99ME2 with *pthXo1* on a plasmid. Disease symptoms are stronger if *pthXo1* is present. The leaves were photographed 3 d after inoculation. The photographs were provided by Dr. Bing Yang and Dr. Frank White, Department of Plant Pathology, Kansas State University.

C-terminus suggested that AvrBs3 is transported into the plant cell and localizes to the nucleus (Van den Ackerveken et al., 1996; Szurek et al., 2001) at that time providing a first indication that bacterial effectors act inside the host plant cell. Using *Agrobacterium*-mediated transient expression, AvrBs3 was expressed inside the cells of resistant pepper plants and induced a specific HR (Van den Ackerveken et al., 1996). This result strongly suggested that AvrBs3 is recognized inside the plant cell upon delivery by *Xcv*. Since then, the transient expression assays were crucial to demonstrate the activity of a growing number of effectors inside the plant cell.

While the injection of type III effectors from *Yersinia* into the host cell cytosol was first shown in 1994 (YopE; Sory and Cornelis, 1994), a direct proof of translocation by phytopathogenic bacteria was demonstrated only much later. Two approaches using biochemistry and immunocytochemistry were developed to demonstrate type III-dependent translocation into the plant cell. For the biochemical approach (Casper-Lindley et al., 2002) the effector AvrBs2 was fused to the calmodulin-dependent adenylate cyclase domain (Cya) of *Bordetella pertussis* cyclolysin, which is only active in eukaryotic cells, but not in bacteria. The detection of Cya activity in the plant cell strongly indicated that AvrBs2 is delivered into the plant cell (Casper-Lindley et al., 2002). The ''Cya-assay'' has been used to show the translocation of a number of effectors into the plant cell (Roden et al., 2004b; Schechter et al., 2004). Also for an AvrBs3::Cya fusion protein, a Cya activity in the plant was measured after delivery by *Xcv* (S. Köthke, F. Thieme and U. Bonas, unpublished). Using immunocytochemistry, a more direct proof for the translocation of a type III effector was possible and allowed us to directly visualize the effectors AvrBs3 and AvrBs4 in the plant cell. AvrBs3-specific antibodies detected both proteins in plant nuclei of susceptible and resistant pepper plants after infection with *Xanthomonas* (Szurek et al., 2002; B. Szurek and U. Bonas, unpublished). Furthermore, these experiments clearly demonstrated that the N-terminal amino acids 1–27 of AvrBs3 are essential for the type III-dependent translocation of AvrBs3 by *Xcv* into the plant cell and that localization to the nucleus depends on the NLSs (Szurek et al., 2002). Recently, the bacterial protein HpaB was identified, which is probably necessary for the translocation of AvrBs3 at the right moment. HpaB shows typical features of type III chaperones and physically interacts with the first 50 amino acids of AvrBs3 (see above; Büttner et al., 2004).

## Upon arrival: AvrBs3 dimerizes in the plant cell cytoplasm

As mentioned above, AvrBs3 family members possess a putative LZ and an AAD suggesting a role in transcriptional activation in the eukaryotic host cell. Transcription factors are often post-translationally modified and form oligomers. Indeed, AvrBs3 dimerizes in the cytoplasm prior to nuclear import (Gürlebeck et al., 2005). Surprisingly, the dimerization is not conferred by the LZ-like motif, which is known to be involved in homo- and heterodimerization of plant bZip factors (Landschulz et al., 1988; Meshi and Iwabuchi, 1995), but by the repeat region in AvrBs3. Interaction studies in yeast revealed that a certain number, but not defined repeats of AvrBs3 are necessary for dimerization indicating that the leucine-rich backbone of the central region confers the dimerization rather than the variable residues of the repeats (positions 12 and 13; Gürlebeck et al., 2005). Although the heptad leucine repeats are not essential for homodimerization of AvrBs3, they might be involved in heterodimerization, e.g., with a plant bZip factor. Interestingly, it was shown that the region of Apl1 containing the LZ motif can contribute to recognition specificity in addition to the central repeat region (Ishihara et al., 2004). Although the C-termini of AvrBs3 and AvrBs4 containing the LZ are almost identical and interchangeable, they differ in their recognition specificity (Bonas et al., 1989, 1993; Ballvora et al., 2001). However, the avirulence activities of effector proteins are determined rather by the host than by the pathogen and the LZ motifs of the effectors plays probably a major role in virulence. Interestingly, ATR13, an effector recently cloned from the downy mildew *Hyaloperonospora* (formerly *Peronospora*) and recognized by the resistance protein RPP13 from *Arabidopsis* also contains a heptad repeat region, next to four direct repeats (Allen et al., 2004). Although ATR13 is clearly distinct from the AvrBs3 family, the presence of a putative LZ and direct repeats in an oomycete pathogen suggests a more widespread usage and importance of these structural motifs for phytopathogens. The interesting question remains if these motifs indeed confer interaction with plant proteins.

## Searching for proteins targeted by AvrBs3

Potential interaction partners of AvrBs3 were identified by yeast two-hybrid based analysis of pepper and tomato cDNA libraries (Szurek et al., 2001; B. Szurek, D. Gürlebeck, J. Stuttmann and U.

Bonas, unpublished). One of the proteins interacting with AvrBs3 is importin $\alpha$, which specifically interacts with the NLSs of AvrBs3 in yeast and in vitro (Szurek et al., 2001; Schornack et al., 2004). Importin $\alpha$ is the only interactor of an AvrBs3 family member reported so far. Since the NLSs of AvrBs3 family members are conserved, importin $\alpha$ is supposed to interact with all AvrBs3-like effectors. In agreement with this hypothesis, an interaction between importin $\alpha$ and AvrBs4 was demonstrated (Szurek et al., 2001; Schornack et al., 2004). Importin $\alpha$ is part of the nuclear import machinery in eukaryotes and binds to the NLSs of a target protein via its armadillo repeats. Then, importin $\beta$ interacts with importin $\alpha$ and mediates the import of the protein complex into the nucleus (Görlich et al., 1994, 1995; Mattaj and Englmeier, 1998; Komeili and O'Shea, 2000). AvrBs3 localizes in both susceptible and resistant plants to the nucleus but induces different plant reactions (hypertrophy or the HR, respectively). Importin $\alpha$ is most likely expressed in resistant and susceptible plants and therefore it is presumably not the product of the so far unknown resistance gene *Bs3* which recognizes AvrBs3. Indeed, the most simple model to explain gene-for-gene interactions was the receptor-ligand model in which the products of the plant *R* gene and the *avr* gene of the pathogen physically interact with each other (Gabriel and Rolfe, 1990). However, such direct interactions were only observed for AvrPto from *P. syringae* and Pto from tomato, AvrPita from *Magnaporthe grisea* and Pi-ta from rice and for PopP2 from *R. solanacearum* and RRS1-R from *Arabidopsis* (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000; Deslandes et al., 2003). Interestingly, not only the kinase Pto but also Prf, a typical NB-LRR resistance protein was found to be necessary for the recognition of AvrPto in tomato. This is consistent with the "guard hypothesis" (Van der Biezen and Jones, 1998; also see Schornack et al., this issue), which proposes the interaction of the effector with a plant protein, its virulence target. In resistant plants, the R protein monitors the modification of this virulence target which leads to the induction of defense reactions. With regard to the guard model, Pto could be a virulence target of AvrPto, guarded by Prf. However, yeast three-hybrid screens using Pto and AvrPto as baits did not isolate Prf (Bogdanove and Martin, 2000). Also for other type III effectors, guarded virulence targets in *Arabidopsis* were reported: PBS1 interacts with the *Pseudomonas* effector HopAR1, and RIN4 is targeted by AvrRpt2, AvrRpm1 and AvrB1 from *Pseudomonas* (Mackey et al., 2002; Shao et al., 2003; Kim et al., 2005a). While the known guarding R protein for PBS1 is RPS5, RIN4 is protected by at least two R proteins: RPM1 and RPS2 (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Mackey, 2003).

Could the AvrBs3-interactor importin $\alpha$ also be a virulence target that is guarded by different R proteins? Since importin $\alpha$ is necessary for a large number of plant proteins to be imported in the nucleus, it presents an attractive target for bacterial effectors to modify plant reactions. Therefore, importin $\alpha$ should be well guarded by the plant. Indeed, the corresponding resistance gene of AvrBs3, *Bs3*, only confers recognition of nuclear localized AvrBs3. However, deletion derivatives of AvrBs3 which are still nuclear imported but lack the AAD or the repeat region do not induce the HR in *Bs3* pepper plants. Thus, Bs3 cannot be the guardian of importin $\alpha$· Bs4, a typical NB-LRR R protein recognizes not only wild-type AvrBs4 (97% sequence identity with AvrBs3) but also derivatives which cannot be imported into the nucleus (e.g., AvrBs4ΔNLS; S. Schornack, T. Lahaye and U. Bonas, unpublished data; Ballvora et al., 2001; Schornack et al., 2004). Thus, Bs4 is active even if importin $\alpha$ is not targeted by AvrBs4. Based on these data it is likely that importin $\alpha$ is recruited by the AvrBs3 family members for nuclear localization and not a virulence target guarded by R proteins. Besides *Bs4*, several *R* genes that correspond to *avrBs3*-like genes in the pathogen have been isolated. Interestingly, although they recognize very homologous type III effectors, the *R* genes identified so far are quite diverse. For example, *xa5* from rice is a recessive allele which encodes a homolog of the $\gamma$-subunit of transcription factor IIA and confers recognition of Avrxa5 from *Xoo* (Blair et al., 2003; Iyer and McCouch, 2004). The recently isolated *Xa27* gene from rice is induced upon infection with *Xoo* strains expressing the AvrBs3 homolog AvrXa27. Strikingly, the only differences between the resistant and susceptible alleles lie within the promoter region (Gu et al., 2004, 2005).

## Searching for genes targeted by AvrBs3

AvrBs3 family members were postulated to act as transcriptional activators. As mentioned above, AvrXa27 induces the expression of the corresponding resistance gene *Xa27*. Our group has investigated the AvrBs3-dependent transcript profile of susceptible pepper plants after *Xcv* infection. More than 20 genes *up*regulated by *A*vrBs3 (*upa*) were identified (Marois et al., 2002; Kay and Bonas, unpublished). The homologies of the predicted *upa* gene products to a pectate lyase, $\alpha$-expansins and auxin-induced proteins (Marois et al., 2002) suggest

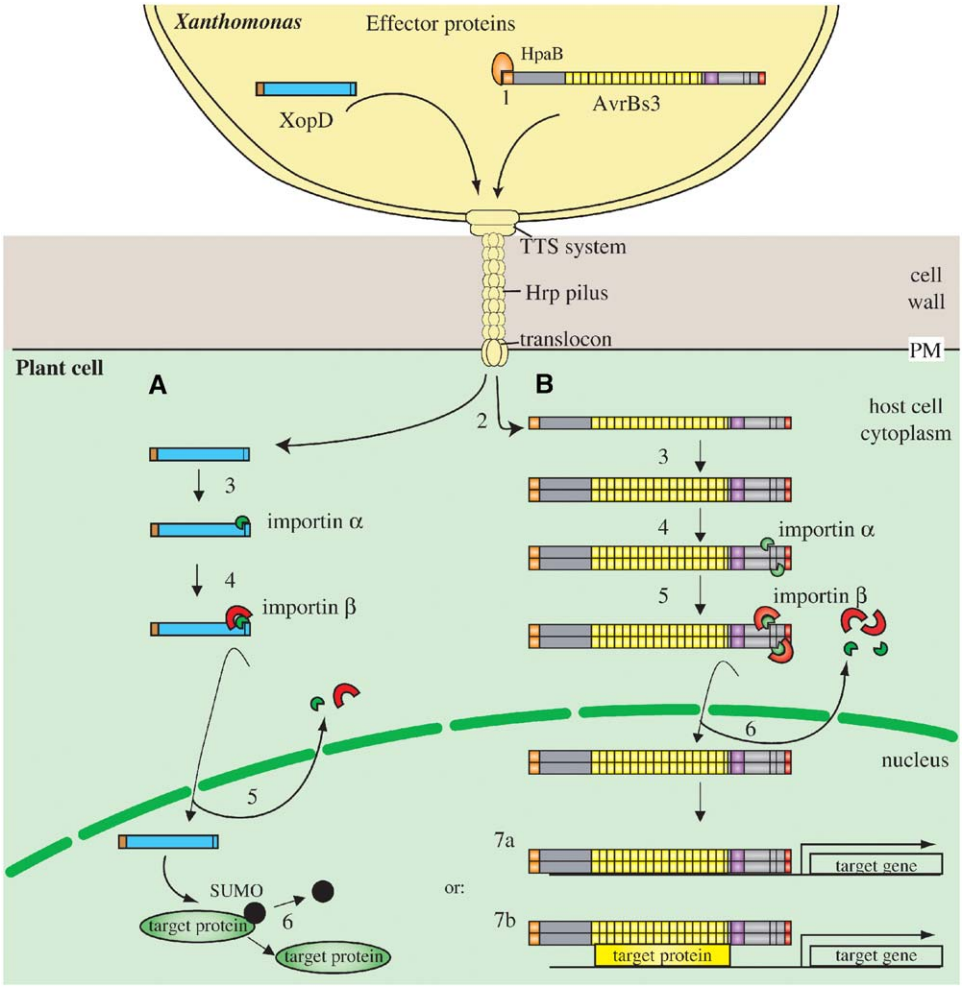



**Figure 5.** Model for the activities of the *Xanthomonas* type III effectors XopD and AvrBs3. (A) Proposed way of action of XopD, a member of the C48 family of cysteine proteases. After secretion and translocation of XopD into the plant cell (2), it probably binds to importin $\alpha$ via its nuclear localization signal (NLS, 3). Importin $\beta$ binds to the complex (4) and mediates the nuclear import of XopD (5). Inside the nucleus, XopD localizes to nuclear foci, where it deSUMOylates yet unidentified plant proteins (6; Hotson et al., 2003). (B) Proposed way of action of AvrBs3. AvrBs3 is constitutively expressed in *Xcv* (Bonas et al., 1989). The TTS chaperone HpaB physically interacts with the N-terminal 1–50 amino acids of AvrBs3 and promotes its secretion through the TTS system (Büttner et al., 2004). AvrBs3 is translocated into the plant cell (Szurek et al., 2002), where it dimerizes (Gürlebeck et al., 2005). Importin $\alpha$ specifically interacts with the NLSs of AvrBs3 (Szurek et al., 2001), importin $\beta$ probably binds to this complex and mediates transport into the nucleus (6). Here, AvrBs3 either binds directly to DNA (7a) or in a complex with other plant proteins (7b) and modifies the host cell transcriptome resulting in hypertrophy symptoms (Marois et al., 2002) or the HR.

a role in modification of the plant cell wall architecture which is in accordance with the hypertrophy triggered by AvrBs3. Of special interest are *upa* genes that are induced independently of de novo protein synthesis and thus could be direct targets of AvrBs3 (Marois, Kay and Bonas, unpublished). Several *upa* genes encode proteins with homology to enzymes and to transcriptional regulators. The latter are of special interest because they could be primary targets of AvrBs3 at the top of the cascade leading to disease. Detailed analysis of the *upa* genes is in progress and will give important insights into the mechanism by which AvrBs3-like proteins trigger host gene expression. Interestingly, for AvrXa7 from *Xoo* binding to AT-rich DNA was shown (Yang et al., 2000). However, no specific DNA sequence was defined. Although a known type of DNA-binding motif is missing in AvrBs3-like proteins, we cannot exclude that AvrBs3 and its homologs bind directly to promoter elements. Alternatively, AvrBs3 might interact with a transcription factor that itself can bind to DNA and whose activity is

modulated by AvrBs3 via its AAD (Fig. 5). Strikingly, in recent yeast two-hybrid screens, cDNAs were isolated from tomato, which indeed encode putative transcription factors (Gürlebeck, Raschke and Bonas, unpublished).

## Future prospects

Due to the growing amount of bacterial genome data and the possibility of in silico prediction and high-throughput analyses the number of effector proteins from phytopathogens increases constantly. The major challenge for the future is to assign molecular functions to individual effector proteins. The contribution of putative virulence factors to the host–pathogen interaction will be assayed by genetic and phenotypic analyses. The generation of multiple knockout mutants might be required to eliminate functional redundancy and to reveal the contribution of type III effectors to disease development. Identification of plant virulence targets by protein interactor screens using yeast two-hybrid studies and biochemical approaches might help to get a deeper insight into the molecular function of a given effector protein. The comparison of identified virulence targets, especially for large effector families, could reveal common strategies of phytopathogens to conquer their hosts. And, finally, the identification and analysis of corresponding R proteins will illustrate which diverse or common mechanisms plants use to defend themselves.

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