# *Xanthomonas* AvrBs3 Family-Type III Effectors: Discovery and Function

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## Key Words

TAL, T3S, plant pathogen, DNA-binding motif, transcription factor, biotechnology

## Abstract

Xanthomonads are bacterial plant pathogens that cause diseases on many plant species, including important crops. Key to pathogenicity of most *Xanthomonas* pathovars is a Hrp-type III secretion (T3S) system that translocates effector proteins into plant cells. Within the eukaryotic cell, the effectors are thought to perform a variety of tasks to support bacterial virulence, proliferation, and dissemination. We are only beginning to understand the host targets of different effectors. The largest effector family found in *Xanthomonas* spp. is the AvrBs3/PthA or TAL (transcription activator-like) family. TAL effectors act as transcriptional activators in the plant cell nucleus. Specificity of TAL effectors is determined by a novel modular DNA-binding domain. Here, we describe the discovery of TAL effectors and their structure, activity, and host targets.

## INTRODUCTION

**Hrp:** hypersensitive response and pathogenicity

**T3S:** type III secretion

*Xanthomonas* includes a diverse group of Gram-negative bacterial plant pathogens that together infect more than 200 different plant families. According to the relatively narrow host range, individual *Xanthomonas* strains are grouped into different pathovars (pv.). Typically, the bacteria infect plants through natural openings (e.g., stomata, hydathodes) and wounds. Some *Xanthomonas* pathovars [e.g., *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), and *Xanthomonas axonopodis* pv. *citri*] cause localized leaf spot or leaf streak; they invade and multiply extracellularly within the leaf mesophyll (apoplast; **Figure 1*a–c***). In contrast, other pathovars [e.g., *Xanthomonas campestris* pv. *campestris* (*Xcc*), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)] gain access to the plant vascular system (xylem), spread systemically throughout the plant, and cause black rot or leaf blight disease (1). Infection sites typically exhibit water-soaked lesions and/or chlorosis, and often become necrotic. Infected vascular systems can be plugged by the extrapolysaccharide xanthan produced by *Xanthomonas*, which leads to rapid wilting. Multiplying bacterial invaders feed from living plant cells, whereas rupture of necrotic areas or canker symptoms in later stages of the disease allow escape of the bacteria to the plant surface for dissemination (1, 21, 94).

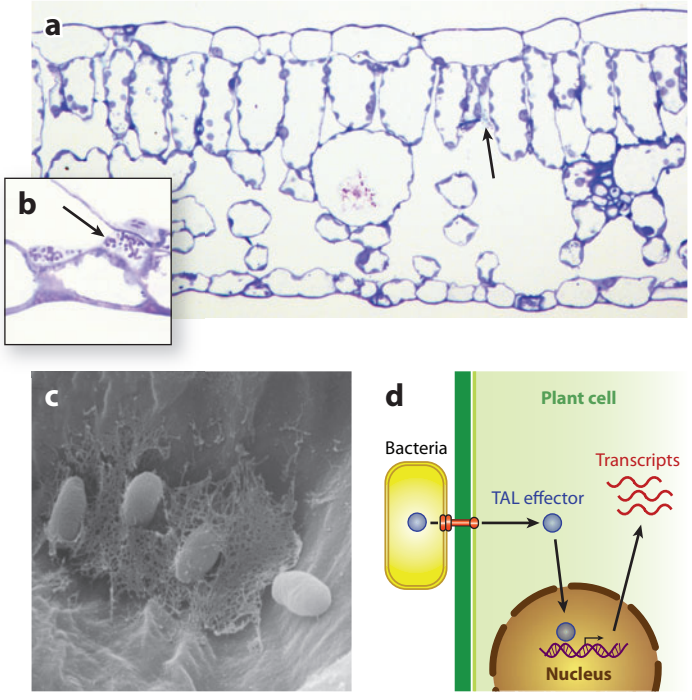



**Figure 1**

*Xanthomonas* pathovars inhabit the plant apoplast or xylem vessels and inject effector proteins into plant cells. (*a*) A microscopic image of a cross section through a pepper leaf infected by *X. campestris* pv. *vesicatoria* (*Xcv*). Note the large intercellular spaces and epidermal stomata. (*b*) Enlarged cross section of lower epidermis. (*a*, *b*) The arrows indicate bacterial microcolonies in the apoplast. (*c*) *Xcv* cells on pepper leaf cell surface inside the apoplast. The mesh corresponds to the exopolysaccharide xanthan. (*d*) *Xanthomonas* translocates a cocktail of effector proteins via a type III secretion system (*red*) into plant cells. Transcription activator-like (TAL) effectors localize to the plant cell nucleus, where they induce expression of specific target genes.

Comparative analyses of genome sequences revealed that *Xanthomonas* harbors all known types of protein secretion systems (type I to type VI) (11). The well-studied Hrp-type III secretion (T3S) system is essential for pathogenicity of most xanthomonads. So far, only the xylem-restricted *Xanthomonas albilineans* (sugarcane pathogen) lacks a Hrp-T3S system and instead harbors the distantly related SPI1-T3S. This T3S system is most similar to the SPI1-T3S system from *Salmonella*, and its role in *X. albilineans* virulence has not yet been clearly established (60). The genes encoding structural components of the *Xanthomonas* T3S system have first been cloned from *Xcv* (alternative names *X. euvesicatoria*, *X. axonopodis* pv. *vesicatoria*) (7) and are encoded in a 25-kb *hrp* gene cluster located on the chromosome (7, 9). Expression of *hrp* genes is controlled by two key regulatory genes, *hrpG* and *hrpX* (84, 85), and is modified by several regulatory networks (11). The T3S system spans both bacterial membranes and is extended by a hollow conduit, the Hrp pilus, that probably traverses the plant cell wall (83). Bacterial effector proteins are secreted through the T3S system and translocated into the plant cell cytoplasm via a bacterial translocon complex that inserts into the plant plasma membrane (10; **Figure 1*d***). The secretion and translocation signals are typically localized in the N-terminus

**Table 1** *Xanthomonas*-related webpages

| Description | Link |
|---|---|
| Effectors, genomes, meetings, Web tools, papers | **http://www.xanthomonas.org** |
| *Xoo* genome database (Japan) | **http://microbe.dna.affrc.go.jp/*Xanthomonas*/** |
| *Xanthomonas axonopodis* pv. *phaseoli* project | **http://www.cns.fr/spip/*Xanthomonas*-axonopodis-pathovar.html** |
| *Xanthomonas albilineans* project | **http://www.cns.fr/spip/*Xanthomonas*-albilineans-threatens.html** |
| French *Xanthomonas* network | **http://www.reseau-xantho.org** |
| *Xanthomonas* genome browser | **http://xgb.fli-leibniz.de/cgi/index.pl** |



of *Xanthomonas* effector proteins, of which several require the general *Xcv* T3S chaperone HpaB for secretion (12). Bacterial effectors target different plant pathways, leading to suppression of plant defense and modulation of the host transcriptome to support bacterial virulence (for excellent recent reviews we refer the reader to 4, 11, 33, 43, 86, 87). *Xanthomonas* strains express a cocktail of typically 20–40 type III effectors, which can be grouped into different families based on sequence similarity and biochemical activity. The rapidly increasing *Xanthomonas* effector repertoire is fueled by bioinformatic analyses of, so far, nine published *Xanthomonas* genome sequences (11, 60, 86) and five complete or draft genome sequences in public databases. More than 20 additional *Xanthomonas* genomes are not yet publically available or currently being sequenced. These will significantly increase the available effector sequences. Further information on *Xanthomonas* genomes and effectors can be found online (**Table 1**).

Transcription activator-like (TAL) effectors represent the largest effector family and function as transcriptional activators of plant genes (43, 68, 86–88) (**Figure 1*d***, **Figure 2*a***). Their peculiar structure encompassing a novel DNA-binding domain has given them special attention. TAL effectors have predominantly been found in *Xanthomonas*, but less related homologs exist also in *Ralstonia solanacearum* (19, 31). Here, we describe the discovery of this unique effector family, the identification of plant targets, and details on the DNA-binding specificity of *Xanthomonas* TAL effectors.

## DISCOVERY OF TAL EFFECTORS

### Isolation of *avrBs3*, the Type Member of a Large Effector Family: Not a Hot Start but a Fortune Cookie

The *avrBs3* gene was the first isolated member of what turned out to be a large family of type III effector proteins in *Xanthomonas* spp. *avrBs3* was identified genetically at the University of Florida, Gainesville by Robert Stall and coworkers, who inoculated different *Xcv* strains on susceptible (Early Cal Wonder; ECW) and resistant pepper (*Capsicum annuum*) cultivars. The resistant cultivars ECW-10R and ECW-30R carried the dominant resistance (*R*) genes *Bs1* and *Bs3*, respectively, which were introduced into the ECW background to generate near-isogenic lines (53). *Xcv* strains 71–21 and 82–8 specifically induced a hypersensitive reaction (HR), a rapid, local plant cell death that halts bacterial ingress, in ECW-30R. This suggested the presence of a corresponding avirulence (*avr*) gene, termed *avrBs3*, in the two strains. Pathogen *avr* genes render the interaction with a plant carrying a matching *R*-gene incompatible, whereas the strain is still virulent on susceptible plants, i.e., in compatible interactions (23, 33, 41). In most cases, specific recognition of the pathogen induces the HR.

The *avrBs3* gene is localized on pXV11, a self-transmissable plasmid, and was isolated from *Xcv* strain 71–21 in the laboratory of Brian Staskawicz at the University of California, Berkeley (8). The *avrBs3* gene was much larger than expected for a typical bacterial gene: a 5-kb fragment carrying *avrBs3* was identified

**TAL:** transcription activator-like

***avr*:** avirulence

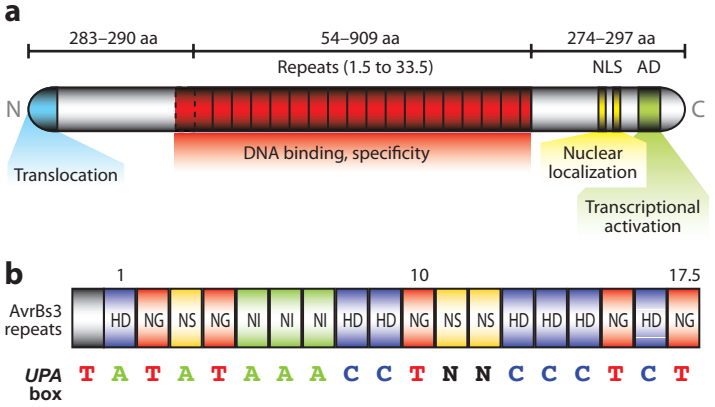
**HR:** hypersensitive reaction

***R* gene:** resistance gene

aa: amino acid

by complementation of *Xcv* strain 85–10, which is virulent on pepper ECW-30R. As previously shown for other *avr* genes (70), *avrBs3* was dominant over the virulence functions in *Xcv* strain 85–10, resulting in the HR induction in leaves of pepper ECW-30R plants (8).

DNA sequence analysis of *avrBs3* was difficult not least because of its high G+C content (64%). First, staggered deletion clones using DNaseI were generated and sequenced by the Sanger and Maxam-Gilbert methods. Surprisingly, the sequences of different deletion clones appeared identical. Indeed, the central part of the *avrBs3* gene was repetitive, consisting of nearly identical 102 bp-direct repeats, present 17.5 times (**Figure 2*a***). Each repeat encoded 34 amino acids (aa) (8). Expression analyses using *avrBs3* promoter fusions and Western blot analyses demonstrated that the gene was constitutively expressed, resulting in a 122-kDa protein (1164 aa), which was detectable using a specific polyclonal antibody (47).




## Isolation of *avrBs3* Homologs

Shortly after the first *avrBs3* paper, three homologs (*avrXa5*, *avrXa7*, and *avrXa10*) were

**Figure 2**

Functional domains of *Xanthomonas* TAL effectors. (*a*) The N-terminal region contains the type III translocation signal. The number of repeats in TAL effectors varies between 1.5 and 33.5. The predicted repeat zero has a different amino acid (aa) sequence, but contributes to DNA binding and is indicated with dashed lines. NLS, nuclear localization signal; AD, activation domain. (*b*) The 17.5 repeats of AvrBs3 are classified into repeat types according to amino acids 12 and 13 per repeat and displayed with repeat-specific colors. The consensus *UPA* box matches to the repeat types of AvrBs3. (*c*) Repeats of different length and the frequencies of repeats with this length in 113 known TAL effectors (see **Supplemental Table 1**, follow the **Supplemental Material link** on the Annual Reviews home page at **http://www.annualreviews.org**) are shown. The general repeat length is 34 aa. The 19-aa and 20-aa repeats correspond to the last half repeat in TAL effectors. The 30-aa repeat is found in XOCORF_4248 (*Xoc* BLS256). The 40-aa repeat is present in XOO2866 (*Xoo* MAFF 311018), AvrXa7-3M (*Xoo* PXO356) and XOO3014 (*Xoo* KACC10331). The 42-aa repeat is present in XOO1136 (*Xoo* MAFF 311018) and TAL-C9b (*Xoo* PXO99A). The hypervariable aa at positions 12 and 13 that confer DNA target specificity are shaded in gray. Repeat zero (Rep. 0) is aligned, but the aa that contribute to DNA binding are not known. (*d*) An aa consensus sequence logo and the aa polymorphisms (in decreasing frequency from top to bottom) of 2023 TAL repeats from 113 TAL effectors are projected onto a typical 34-aa repeat. Not all combinations are found in nature.

cloned from the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) PXO99$^{A}$ using *avrBs3* as a probe (35). The predicted proteins were highly conserved to AvrBs3 and contained similar tandem repeats; however, number, order, and length of repeats varied (**Figure 2**). Comparison of the AvrBs3 and AvrXa10 repeats revealed for the first time that variation occurs predominantly at positions 12 and 13 (termed hypervariable) (35). Surprisingly, *X. campestris* pv. *malvacearum* and Asian *Xoo* strains contain multiple *avrBs3* homologs (35, 96). This was supported by recent genome sequence analyses (50, 59).

At the time, *avrBs3* from *Xcv* and homologs from *Xoo* shared homology to a partial sequence of *pthA* from *X. citri* (73). The *pthA* gene was isolated as a virulence determinant and causes canker-like symptoms in citrus (21, 72). The canker is due to hyperplasia (cell multiplication) and hypertrophy (cell enlargement), which lead to epidermal rupture and bacterial release. In addition, multiple *avrBs3*-family members present in the cotton pathogen *Xanthomonas campestris* pv. *malvacearum* contribute to increased release of the pathogen to cotton leaf surfaces (94, 96).

## Truncated AvrBs4 from *Xcv* Induces the HR in Tomato

In contrast to Asian rice pathogenic *Xoo* strains (35), the presence of *avrBs3* homologs in *Xcv* strains is rare (1–2 copies). *Xcv* strain 82–8 carries an *avrBs3* homolog on its second plasmid pXV12. From genetic studies, it was clear that this homolog had no AvrBs3 activity (8). In the laboratory of R. Stall, an incomplete *avrBs3*-homolog (termed *avrBsP*) (13) from a different *Xcv* strain (strain 87–7) was isolated that had avirulence activity on tomato (*Solanum lycopersicum*) cultivar Bonny Best. Similarly, when the *avrBs3* homolog from *Xcv* 82–8 was cloned on a 15-kb fragment and tested in a tomato pathogenic *Xanthomonas* strain, it induced the HR in Bonny Best and Money Maker; i.e., the clone had avirulence activity on tomato. As a result of its presumed allelism to *avrBs3*, it was first termed *avrBs3–2* and later renamed *avrBs4* because it mediates recognition by the *Bs4* resistance gene in tomato (2, 6, 67).

DNA-sequence analysis of *avrBs4* revealed a strikingly similar sequence compared to *avrBs3* and identity to the truncated *avrBsP* version (13): the promoter sequence and the N-terminal amino acids are identical, and the gene contains 17.5 repeats but in a different order than *avrBs3*. The comparison of AvrBs3 and AvrBs4 demonstrated again that the repeats are highly conserved, and that positions 12 and 13 in each repeat are hypervariable. The C-terminal region of *avrBs4* had suffered a 12-bp deletion, including a highly conserved *Bam*HI site (6).

Intriguingly, and in contrast to all other AvrBs3 homologs analyzed to date, the avirulence activity of AvrBs4 was maintained in a series of deletion constructs lacking C-terminal sequences. The minimal construct sufficient to induce the HR consists of the N-terminal part plus 3.5 repeats; i.e., the C-terminal region of AvrBs4 is not required for the Bs4-mediated HR in tomato (6, 67). Bs4 is a TIR-NB-LRR protein that localizes to the plant cell cytoplasm, where it directs recognition of AvrBs4 (67). To date, Bs4 is the only characterized example of this type of resistance protein for TAL effectors (see below).

## The Repeats Determine the Specificity of Action

As the repeats in *avrBs3* were nearly identical, it was unclear if they were all needed for the HR induction. Deletion derivatives in *avrBs3* lacking various repeats revealed that most derivatives lost activity. They no longer induced the HR in resistant plants, but some *avrBs3* derivatives had gained a new specificity (30). The most interesting example was *avrBs3Δrep16* (lacks 4 repeats, 11–14), which lost the avirulence activity in pepper ECW-30R (no recognition by *Bs3*), but gained recognition in pepper ECW. Genetic studies demonstrated that recognition was due to an allele at the pepper *Bs3* locus [*bs3* (30), now termed *Bs3-E* (62)]. The fact that

NLS: nuclear localization sequence

AD: activation domain

*avrBs3Δrep109* (lacks repeats 13–15) retained the *avrBs3*-mediated HR in ECW-30R, but *avrBs3Δrep9*, a deletion derivative also lacking three repeats (5–7), was inactive demonstrated that it is the order and thus specific sequence of the repeats that determines activity. Subsequently, this notion was confirmed by repeat domain swaps (80, 93–95).

## The AvrBs3 Family Contains Eukaryotic Motifs: NLSs and AD

Functional monopartite nuclear localization sequences (NLSs) in the C-terminal region of the AvrBs3 family (**Figure 2*a***) and their importance for activity were shown by nuclear localization of fusion proteins in onion bombardment experiments and genetic studies (80, 95). Moreover, the NLS AvrBs3-family effectors contain an acidic activation domain (AD) (**Figure 2*a***) at their C-terminal end. This AD was first functionally described for AvrXa10 from *Xoo* (99, 100) and subsequently also for AvrBs3 (74).

The presence of NLSs and AD in AvrBs3 was intriguing because both features are typical eukaryotic motifs, which suggested an activity of AvrBs3 in plant cells. Sequence analysis of *avrBs3* homologs from other xanthomonads showed that these motifs are highly conserved.

Twenty years ago, it was unknown that effector proteins like AvrBs3 act inside plant cells. To test this, AvrBs3 was expressed via *Agrobacterium*-mediated gene transfer into leaf cells of susceptible and resistant pepper lines. Only in resistant ECW-30R was the HR induced, and this was dependent on at least one functional NLS. The entire NLS region could be functionally replaced by the heterologous NLS from the SV40 large T-antigen, indicating that its sole role was nuclear targeting of the protein (80). That AvrBs3 indeed localizes to the plant cell nucleus was shown by *Xcv* T3S system-dependent delivery in pepper leaf tissue using immunocytochemistry (75). In addition, protein-protein-interaction studies showed that AvrBs3 dimerizes in the plant cytoplasm via its repeat region before it is imported into the nucleus (29). As AvrBs3 interacts with pepper importin α, nuclear import is most likely mediated by the importin α/β machinery in the plant cell (74). The importance of NLSs was confirmed for AvrBs3 homologs, e.g., for AvrXa7 from *Xoo* (93).

The facts that mutations and deletions in the AD rendered the proteins inactive and that the AD could, at least partially, functionally be replaced by the heterologous AD of *Herpes simplex* virus VP16 demonstrated the functional importance of this domain (74, 100). Together with the fact that nuclear targeting is essential for function and that AvrXa7 binds to DNA (93), it has been suggested that AvrBs3 family members are TAL proteins.

## *avrBs3* and *avrBs4* Are Flanked by Conserved Inverted Repeats

An additional interesting feature also in the light of the evolution of the *avrBs3* family is the fact that both *avrBs3* and *avrBs4* are flanked by nearly perfect inverted repeats (IR) of 62 bp that with the exception of one nucleotide in the left IR are identical in both alleles. Homologous sequences were identified by hybridization in other *Xcv* strains lacking *avrBs3* homologs, e.g., *Xcv* 85–10 (6), and in the flanking regions of type III effector genes in this strain (58, 77). This suggests that TAL effector genes evolved by horizontal gene transfer of mobile cassettes.

# PLANT TARGET GENES

## TAL Virulence Targets

**Targets of AvrBs3.** Early on, it was shown that some TAL effectors are important virulence factors. The first examples were PthA from *X. citri* and Avrb6 from *X. campestris* pv. *malvacearum*, which cause citrus canker (72) and enhance water-soaking (94, 96), respectively. AvrBs3 contributes to bacterial fitness in the field (89), but bacterial counts are not affected. In contrast, several TAL effectors from *Xoo* are

**Table 2 Examples of TAL effector targets**

| TAL effector[a] | Plant target | Description | Reference |
|---|---|---|---|
| **TAL effectors target same gene at same (overlapping) DNA target boxes** | | | |
| *Xcv* AvrBs3 | *Bs3* (pepper) | Resistance gene | (62) |
| *Xg* AvrHah1 | *Bs3* (pepper) | Resistance gene | (69) |
| **TAL effectors target same gene via different DNA target boxes** | | | |
| *Xoc* Tal-C1c | *OsHEN1* (rice) | sRNA biogenesis | (54) |
| *Xoo* Tal9a | *OsHEN1* (rice) | sRNA biogenesis | (54) |
| **TAL effector target different members of same gene family** | | | |
| *Xcv* AvrBs3 | *UPA16* (pepper) | nodulin MtN3 family | (45) |
| *Xoo* PthXo1 | *Os8N3* (rice) | nodulin MtN3 family | (91) |
| *Xoo* AvrXa7 | *Os11N3* (rice) | nodulin MtN3 family | (64) |
| **TAL effectors manipulate transcription machinery** | | | |
| *Xoo* PthXo7 | *OsTFIIAγ1* (rice) | small subunit of TFIIA; compensates rice *xa5* mutation in *OsTFIIAγ5* | (71) |

[a]*Xcv*, *Xanthomonas campestris* pv. *vesicatoria*; *Xg*, *Xanthomonas gardneri*; *Xoc*, *Xanthomonas oryzae* pv. *oryzicola*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.



essential virulence factors for infection of rice (82, 86).

Surprisingly little, however, is known about the corresponding plant virulence targets of TAL effectors and their role for the pathogen (**Table 2**). The first TAL targets in plants were identified for AvrBs3 in pepper (52) and termed *UPA* (upregulated by AvrBs3). AvrBs3 causes hypertrophy, which is due to an enlargement of the mesophyll cells in infected tissue and might help the bacteria to escape from infection sites to facilitate bacterial spreading (44, 52; S. Hahn & U. Bonas, unpublished data). Not surprisingly, auxin-induced genes and α-expansins are among the *UPA* genes (52). Only four *UPA* genes were putative direct AvrBs3 targets because their induction required no de novo protein synthesis (52). *UPA20*, the key regulator of the plant cell hypertrophy phenotype, was identified in a second screen as a direct target of AvrBs3 (44). *UPA20* encodes a basic helix-loop-helix (bHLH) transcription factor that controls cell enlargement, and expression of at least one of the later induced *UPA* genes, *UPA7* (encodes α-expansin) (44). A conserved promoter element (*UPA* box) was found in several directly induced *UPA* genes, and, remarkably, AvrBs3 directly binds to the *UPA* box (44, 45). AvrBs3 specifically binds the target sequence both in vitro and in vivo. Even more intriguing is the fact that the repeats alone bind to the DNA, thus representing a novel DNA-binding motif (44).

**Targets of other TAL effectors.** The TAL effectors AvrXa7, PthXo1, PthXo2, PthXo3 from *Xoo*, and PthA and PthB from *X. citri* pv. *citri* are major virulence determinants (22, 72, 87). It may well be that other TAL effectors contribute in a more subtle way to bacterial virulence and that a corresponding phenotype has not been obvious so far, e.g., because of redundancies in effector functions.

*Os8N3/Xa13* is a rice target gene induced by PthXo1 (16, 17, 91) (**Table 2**). The recessive *xa13* allele acts as an *R* gene against *Xanthomonas* infections (16, 17). Resistance is based on lack of PthXo1-mediated *Os8N3* expression in *xa13* homozygous plants (98), which implies that *Os8N3* is a genuine susceptibility gene (virulence target) of PthXo1. Lack of PthXo1 inducibility is proposed to be based on mutations in the *xa13* promoter that prevent binding of PthXo1 (5, 17, 54, 91; see below). Interestingly, expression of *Os8N3* is not essential for plant susceptibility to *Xoo* if the bacteria deliver AvrXa7, PthXo2, or PthXo3 into the plant (91). As AvrXa7, PthXo2, and PthXo3

fail to induce *Os8N3* expression, they likely enhance virulence by targeting alternative plant susceptibility genes (87).

How does expression of *Os8N3* trigger susceptibility of rice towards *Xanthomonas* infections? *Os8N3* is involved in pollen development (16, 17) and encodes a protein with similarity to *MtN3*, a nodulin of *Medicago truncatula* (24). *MtN3* and *Os8N3* belong to a family of 17–20 members in rice with homologs in mammals, insects, nematodes, and filamentous fungi (17, 28, 91). *MtN3*, the type member of this family, is induced in early stages of symbiosis (24), suggesting that common plant genes are required for symbiosis and susceptibility. Two other members of the plant nodulin family are induced by additional TAL effectors (AvrXa7, AvrBs3) (**Table 2**). This suggests an important role of the nodulin 3(N3) family in plant susceptibility to *Xanthomonas* infections and demonstrates that evolutionary TAL effector specificity selection can converge on similar host targets.

*OsHen1* seems to be another important virulence target because the gene is induced by two different TAL effectors, Tal1c from *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) BLS256 and Tal9a from *Xoo* PXO99$^{A}$ (54) (**Table 2**). The two effectors have different repeat domains and bind to different target sites in the *OsHen1* promoter, suggesting independent evolutionary selection histories for both effectors. *OsHen1* encodes a protein with a predicted methyltransferase domain and similarity to HUA ENHANCER 1 (Hen1) and homologs from different eukaryotes (15, 79). Hen1 is involved in microRNA (miRNA) maturation by transferring a methyl group to the 3′ terminal nucleotide of miRNA duplexes (36, 97). Mature miRNAs are assembled into RISC complexes and guide cleavage and translational repression of target mRNAs (14). miRNAs are also targets of other T3S effectors (55), but so far, the role of *OsHen1* for bacterial pathogenesis is unclear.

## TAL Resistance Mousetraps

In the coevolution with bacterial pathogens, plants have evolved *R* genes to detect the activity of type III effectors and mount a defense reaction. Some effector proteins are recognized by protein components of the plant surveillance system, i.e., by direct binding to the R protein or indirectly and, more often observed, through their activity within the plant cell (33, 41, 76, 81). With the sole exception of *Bs4* (67), *R* genes that detect known TAL effectors require an NLS and the AD in the corresponding effector, indicating the need for nuclear localization and transcriptional activation of plant genes (27, 74, 80, 93, 99, 100). For this mode of recognition, resistant plants have established molecular traps that have been termed decoys (81) or mousetraps (4) to detect effector activity. Accordingly, TAL effectors are lured into expressing genes that induce plant defences.

The first example of such a resistance gene recognizing a TAL effector was *Xa27* (26, 27). Identical *Xa27* open reading frames are present in resistant and susceptible rice cultivars, but *Xa27* is only expressed in resistant lines following infection with *Xanthomonas* strains delivering AvrXa27 (27). Xa27 is a protein of unknown function that is secreted into the leaf apoplast and induces plant defense reactions but not cell death (78, 90).

In contrast, pepper *Bs3*-mediated resistance against *Xcv* expressing *avrBs3* leads to the HR (8, 53). Cloning of the *Bs3* gene from pepper ECW-30R revealed that *Bs3* expression and the HR depend on binding of AvrBs3 to a specific DNA element (*UPA* box) in the *Bs3* promoter (62, 65) (**Table 2**). Importantly, the *Bs3-E* promoter from pepper ECW contains a 13-bp deletion in the *UPA* box of the *Bs3* promoter, emphasizing the importance of this element. In the absence of AvrBs3, no *Bs3* expression was detected in different plant tissues (62, 65), suggesting that the *Bs3* gene product is not required for normal cellular activities and functions as a mousetrap for AvrBs3. *Bs3* encodes a unique flavin monooxygenase (FMO) with homology to *Arabidopsis* YUCCA proteins (62). Whether Bs3 triggers cell death because of toxin production or via induction of defense signaling is not clear. At least two additional *R* genes from rice (*Xa7*, *Xa10*) that yet await isolation also require

NLSs and functional ADs in the corresponding TAL effectors AvrXa7 and AvrXa10, respectively, for resistance (93, 99). This suggests that they, too, function as mousetraps. The rice allele *xa5* has been classified as a recessive resistance gene (25, 37, 40) and is discussed below.

## THE DNA-BINDING ACTIVITY OF TAL EFFECTORS

Since discovery of AvrBs3, the highly repetitive central domain of this protein family has puzzled scientists (8, 35). Early data have already indicated that the repeat domain controls the specificity of TAL effectors (30, 92, 93) and that TAL effectors have an affinity for DNA (93). Finally, the first identification of a target DNA sequence that is directly bound by the TAL effector AvrBs3 was a breakthrough in its functional analysis (44, 62). The in vitro DNA-binding capacity of AvrBs3 demonstrated that no host factors are required for its interaction with DNA. DNA footprint analyses have shown that AvrBs3 and derivatives protect the *UPA*-box plus flanking DNA regions of 5–10 bp each (5, 45), indicating that no additional DNA sequences beyond the target box are bound by TAL effectors.

### A Novel Type of DNA-Binding Domain

The TAL effector repeat domain constitutes a novel DNA-binding domain (44) that shows no similarity to known DNA-binding elements. Therefore, it was difficult to identify amino acids involved in base pair recognition. The breakthrough in cracking the code was shown in two independent studies (5, 54). Key was the observation that the length of the *UPA* box roughly equals the number of AvrBs3 repeats (**Figure 2*b***). This prompted establishment of a model that one repeat determines recognition of one DNA base pair (5, 54). Classification of AvrBs3 repeats into repeat types according to the hypervariable residues revealed a correlation of certain repeat types with certain base pairs in the *UPA* box (5, 54) (**Figure 2*b***). The N-terminally localized repeats correspond to the 5′ end, whereas the C-terminal repeats correspond to the 3′ end of the DNA box. According to the model, the array of repeats in the repeat domain of a TAL effector corresponds to a consecutive DNA sequence. The specificity of each repeat is determined by a di-amino acid motif; e.g., NI repeats correspond to adenine (in the upper, coding strand), NG repeats to T, and HD repeats to C (5, 54) (**Figures 2*b***, **4**), etc. The allocation of repeat-type specificities enabled the correct prediction of target boxes for TAL effectors with hitherto unknown specificity in promoters of induced genes. It also provided a molecular explanation as to why the *Bs3-E*, but not the *Bs3* promoter, is induced by AvrBs3Δrep16 (5, 54). Using the code, the target DNA specificities of four TAL effectors [Hax2, Hax3, Hax4 (42), AvrXa10 (35)] were predicted. The TAL effectors specifically induced a reporter gene that was under the control of the corresponding target DNA sequences (5). In a parallel study, bioinformatic analyses provided evidence that the DNA base-pair specificities of TAL effector repeats are not affected by repeat neighbors (54). The specificities of several repeat types were experimentally verified, demonstrating that certain repeats (NG, NI, HD repeat) have a strong base-pair preference. In contrast, other repeats recognize two different base pairs (NN repeat: G, A) or are apparently nonselective (NS repeat: A, C, G, T) (see **Figure 4**) (5). Repeats contain amino acid substitutions at other positions than positions 12 and 13 (**Figure 2*d***). However, they appear to not influence the specificity of DNA recognition. Interestingly, the target box of TAL effectors is 1 bp longer in the 5′ end than the region specified by the repeats (5, 54, 65). This specificity is probably encoded in a repeat zero adjacent to repeat 1 (**Figure 2*a*,*b***) that has only low amino acid conservation (**Figure 2*c***) and has not been considered to be part of the repeat region, previously (5). An important consequence of the TAL effector code is that artificial effectors with novel repeat orders, and thus novel DNA target preferences, can be constructed (5).

Taken together, experimental evidence and bioinformatics solved the DNA-target specificity of TAL effectors. This highly modular method of encoding a DNA-binding specificity is unparalleled in its simplicity.

Supplemental Material

The DNA-recognition code of TAL effectors now presents an explanation as to how a specific set of genes can be induced by different TAL family members. The DNA recognition is therefore mediated by the repeat region, which varies between TAL family members, and other TAL protein domains probably facilitate the initiation of transcription. The set of induced genes then determines the virulence function of the corresponding effector.

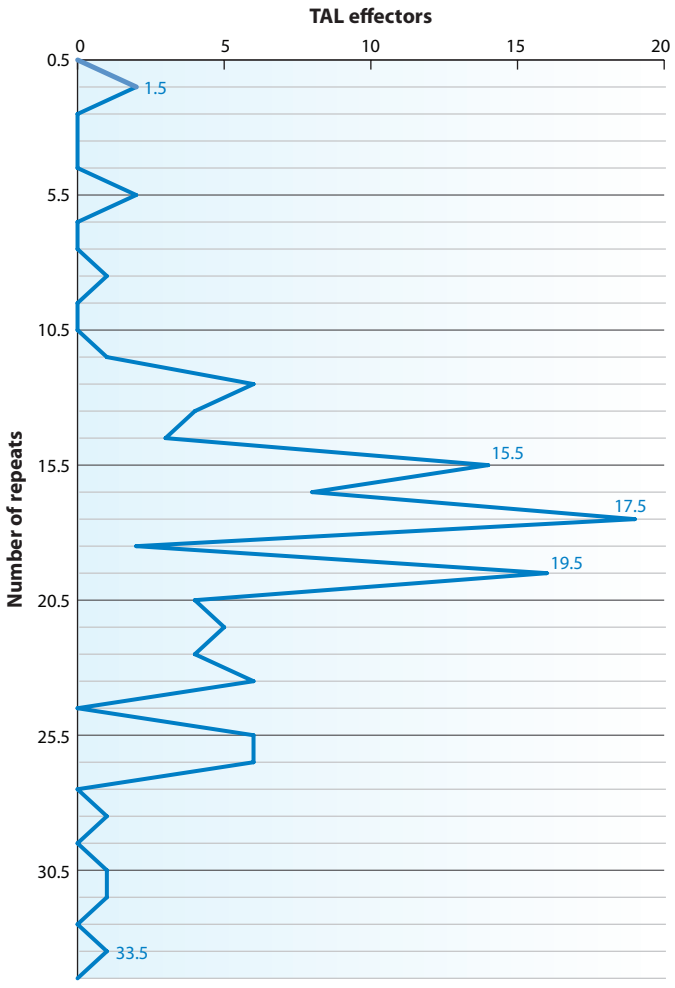



**Figure 3**

Number of repeats in *Xanthomonas* TAL effectors. Repeat domains from 113 TAL effectors (see **Supplemental Table 1**) were analyzed. TAL effectors with 17.5 repeats are most frequent.

## TAL Repeat Structure and the Protein-DNA Interface

The collection of 113 (nonredundant, no pseudogenes) TAL effector sequences currently present in databases (see **Supplemental Table 1**) reveals that TAL effectors contain repeat domains of different lengths. The number of repeats varies between 1.5 and 33.5 (**Figure 3**), with the short genes probably being nonfunctional, because a minimum number of 6.5 repeats is necessary to induce target gene expression (5).

Overall, the individual repeat units are highly conserved apart from position 4, positions 12 and 13 (hypervariable residues), and position 32 (**Figure 2*d***). Other amino acid substitutions throughout the repeat can be found, but these are rare (**Figure 2*d***). The predominant length of TAL repeats is 34 aa, but also 30-aa to 42-aa repeats exist (**Figure 2*c***). It is unclear, so far, whether all repeat types are functional.

The DNA-binding specificity of TAL effector repeats is apparently solely based on the nature of a di-amino acid motif at the hypervariable positions 12 and 13 in a typical 34-aa repeat (**Figure 2**) (5, 54). This suggests that the amino acids at position 12 and 13 probably interact directly with DNA bases. Different combinations of hypervariable amino acids can be found in TAL effectors from *Xanthomonas*, but only a few of them are predominantly used (**Figure 4**). It is still unknown whether the rare repeats are functional or contribute to the same extent to DNA-binding. Thirty five–aa repeats (e.g., in Hax2, AvrHah1) have the same DNA-target preference as 34-aa repeats (5), indicating that the additional proline at amino acid position 33 in a 35-aa repeat (42, 69) does not influence repeat specificity. In contrast, 33-aa

repeats present in some TAL effectors contain a deletion of the amino acid at position 13 in comparison to a 34-aa repeat (**Figure 2*c***). Amino acid position 13 corresponds to the second hypervariable amino acid involved in DNA-target specificity. These types of repeats are often referred to as N* or H* (the asterisk represents the missing amino acid). As the amino acid following the deletion is a glycine (G), one might assume that an N* repeat (33 aa) equals an NG repeat (34 aa), but this does not seem to be the case. A statistical analysis of 20 putative DNA-target sites of different TAL effectors revealed the presence of C or T bases with similar frequencies at positions corresponding to N* repeats (54). In contrast, the predicted (5, 54) and experimentally verified NG repeat preference is exclusively for T (5). Thus, the deletion of amino acid 13 in a 33-aa repeat does not simply shift the following amino acid to substitute for the missing specificity-determining amino acid. Therefore, we postulate that other amino acids (e.g., in predicted α-helices) are involved in positioning the specificity-determining amino acids in respect to the DNA bases.

Secondary structure predictions revealed that each AvrBs3 repeat contains two conserved α-helices, flanking a loop-domain that includes the hypervariable residues (68). This predicted structure of the repeat region shows similarity to the superfamily of solenoid proteins, which contain repeat structural units that fold into a superhelical structure (48). This superfamily of proteins include tetratricopeptide repeat (TPR) proteins (20), of which several protein structures have been solved, and pentatricopeptide repeat (PPR) proteins (66). TPR and PPR proteins contain degenerated tandem 34-aa and 35-aa repeats, respectively (20, 66). In contrast to TAL effectors, TPR proteins interact with other proteins via their repeat domain (20), whereas PPR proteins interact with single-stranded RNA (66).

In the predicted helical superstructure of the AvrBs3 repeat region, the hypervariable amino acids are positioned like a string at the inner side of the helix facing its axis (68). Therefore, we predict that a right-handed helically arranged TAL repeat domain is wrapped around the DNA double helix with the hypervariable amino acids facing towards the DNA bases.

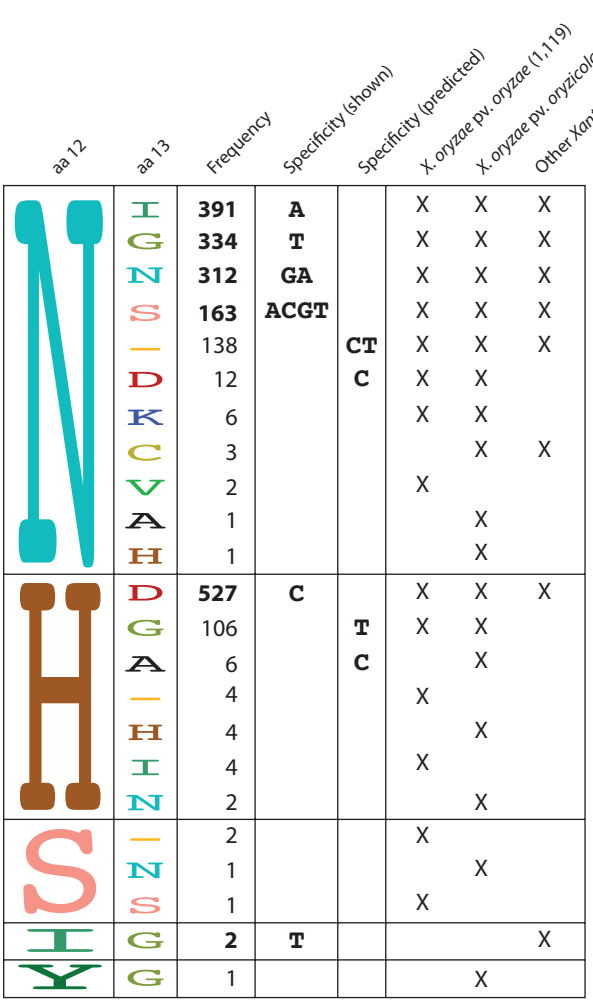



**Figure 4**

Repeat types in *Xanthomonas* TAL effectors based on the hypervariable amino acids 12 and 13 per repeat. Di-amino acid motifs at positions 12 and 13 and their frequencies from 113 TAL effectors (see **Supplemental Table 1**) are shown. A dash (-) refers to a deletion of amino acid 13. Specificities verified experimentally (*bold*) (5), predicted specificities (54), and the occurrence (X) of repeat types in TAL effectors from different xanthomonads (total number of repeats) are indicated. Note the high relative frequencies of a few major repeat types.

## The Stoichiometry of TAL-DNA Complexes

AvrBs3 and derivatives form dimers in the cytosol of plant cells, which are transported into the cell nucleus (29). Because of transcomplementation of two nonfunctional constructs, it has been speculated that AvrBs3 dimers associate with target DNA (29). Often transcription factors bind DNA as a dimer (49). In contrast, the TATA-box binding protein (TBP) forms inactive dimers and monomerizes to bind to the promoter (46, 56, 57). The predicted superhelical structure of TAL effector repeats (68) does not support an intuitive model for the way a TAL dimer could interact with target DNA sequences, and possibly TAL proteins bind as monomers to DNA. 3D-structure analysis is needed to solve the structure of the TAL-DNA complex.

## Interaction with the Plant Transcription Machinery

Although TAL effectors induce and modulate the transcription of plant genes, it is enigmatic which plant factors are involved. The presence of the TAL effector directs the transcriptional start to a position 44–61 bp downstream of the effector DNA-binding sequence (27, 44, 45, 62, 63, 65). In case of *UPA* genes with basal expression, AvrBs3 activation changes the transcriptional start site (44, 45), possibly because AvrBs3 binding to the promoter blocks the natural TATA box, which is part of the *UPA* box. The TAL target box can be positioned at different sites in a promoter, which shifts the transcriptional start site accordingly (63). This suggests that the TAL protein directs assembly of the transcription initiation complex at a position close to it. In addition, this indicates that TAL function does not require a defined distance to other possible promoter elements.

How does a TAL effector interact with the basal plant transcription machinery? Yeast-two-hybrid studies have not revealed interacting components of the plant transcription machinery (D. Gürlebeck & U. Bonas, unpublished data). Although this is still an open question, there are a few clues.

The recessive *R* gene *xa5* from rice is an allele of a gene encoding the γ (small) subunit of transcription factor TFIIA (37, 40). Rice contains two genes encoding TFIIAγ (*TFIIAγ5* and *TFIIAγ1*), with TFIIAγ5 being the predominantly expressed gene (37, 40, 71). The *xa5* allele contains a missense mutation ($Val_{39}Glu$) that does not seem to compromise its function. In eukaryotic cells, TFIIA (α-, β-, γ-subunits) stabilizes binding of the TBP-TFIID complex to the TATA box (32). TFIIA is highly conserved among eukaryotes, but not generally required for transcription (34). The occurrence of the *xa5* resistance allele implies that TAL effector function in rice requires TFIIAγ and that the *xa5* mutation is an adaptation to evade TAL virulence functions and thereby *Xanthomonas* infections (37–39). Accordingly, *xa5* confers resistance to *Xoo*, but also attenuates *Xa27*-mediated resistance that requires induction by AvrXa27 (25). However, how TFIIAγ is involved in TAL-mediated gene expression and whether the *xa5* mutation interferes with TAL effector function in general is not yet clear. Interestingly, the TAL effector PthXo7 induces expression of *TFIIAγ1*, the lower expressed gene (71) (**Table 2**). PthXo7 might compensate the *xa5* mutation by increasing the abundance of a TAL-compatible TFIIAγ. Indeed, PthXo7 increases the virulence of *Xoo* strain PXO86, which otherwise is not virulent on *xa5* rice lines (71, 87). Future studies will clarify which parts of the plant basal transcription machinery are usurped by TAL effectors.

## BIOTECHNOLOGY PERSPECTIVES

Knowing the code *Xanthomonas* TAL effectors use to manipulate plant gene expression, one can now use this information against the pathogen. Several TAL target boxes can be combined into one promoter (63). Accordingly, transgenic plants can be generated by inserting

target boxes of different TAL effectors in front of a resistance gene (e.g., *Bs3*) (63). These plants will be resistant to infection by bacteria delivering matching TAL effectors.

In addition, the modular DNA-binding specificity of the TAL effector repeat domain is highly amenable for biotechnology. Individual repeat units can be assembled into any order to generate novel repeat domains and thereby novel DNA-binding specificities (5). Because the specificity of individual repeats does not seem to be influenced by neighboring repeats, specificities are clearly predictable, which is a unique feature for DNA-binding domains.

Tandemly arranged zinc-finger domains with different binding preferences have been used to construct proteins with custom-designed DNA-binding specificities (18). Nevertheless, target specificities of zinc-finger proteins is not completely predictable, and laborious screening efforts are needed to identify proteins with a given DNA-binding specificity. Custom-designed DNA-binding domains have a high potential for biotechnology; e.g., fusions of zinc-finger domains with nucleases have generated highly specific nucleases with designed specificity (51). DNA strand cleavage assists localized insertion of DNA fragments in cells of complex eukaryotes, including humans (3, 61). Highly localized nucleolytic cleavage can, therefore, be used to insert DNA fragments at specific chromosomal positions, e.g., in gene therapy. In addition, fusion of designed DNA-binding domains to functional protein domains that can regulate gene expression might allow targeted silencing of genes. Custom-designed TAL effectors are per se specific gene activators and might be modified to function not only in plants, but also in other organisms, including humans. The ingenious DNA-binding mechanism of *Xanthomonas* TAL effectors renders these proteins important for potentially many applications beyond the manipulation of plant host cells.

## SUMMARY POINTS LIST

1. *Xanthomonas* translocates members of a large family of TAL effectors that function as transcriptional activators in plant cells. They localize to the plant cell nucleus and modulate expression of plant genes.
2. In susceptible plants, TAL effectors activate the expression of susceptibility genes to support bacterial virulence. In resistant plants, TAL effectors are lured to induce expression of resistance genes, leading to the induction of specific defense reactions.
3. TAL effectors contain NLSs, that mediate import into the plant cell nucleus, a C-terminal AD needed to activate plant gene expression, and a central repeat domain that directly binds to DNA.
4. The specificity of TAL effectors is encoded in the central domain of consecutive repeats. Each repeat corresponds to one DNA base pair. Two variable amino acids at repeat positions 12 and 13 determine the DNA base-pair recognition specificity of each repeat. The consecutive array of repeats binds to a consecutive DNA sequence.
5. Rearranging repeat units generates novel custom-designed DNA-binding specificities with high potential for biotechnology.

## FUTURE ISSUES LIST

1. What are the virulence targets of TAL effectors, and what are their roles in the pathogenicity of *Xanthomonas*?

2. How do TAL effectors cooperate with the general transcription machinery of the plant cell to induce gene expression?
3. Do TAL effectors function in other kingdoms of life?
4. Can the TAL repeat domain be used to construct sequence-specific DNA-binding proteins for biotechnology?

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**Errata**

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