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Plant Pathogen Recognition Mediated by Promoter Activation of the Pepper Bs3 Resistance Gene

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stages of flower development *L02* is expressed. The results indicate that *L02* RNA is present throughout style development (fig. S5A). Moreover, while *L02* is mainly expressed in developing stylar tissue, some expression can also be observed around the ovules (fig. S5, B to E). Further, the long-style NIL differs from the short-style NIL in that the former accumulates greater quantities of the *L02* transcript throughout style development (fig. S5A), a finding consistent with the prediction that the *Style 2.1* QTL is attributable to mutations in the 5′ regulatory portion of the *L02* gene rather than to changes in the amino acid sequence of the encoded protein.

In an effort to shed light on the mechanism by which *L02* modulates style elongation, we recorded cell number and size along the entire length of mature styles from both short-style and long-style genotypes (Fig. 3, A to C). Although the two genotypes differed significantly in style length, they did not differ with regard to the total number of cells in the long axis of each style, which suggests that increased cell elongation is responsible for the longer style length of the long-style genotypes (Fig. 3B). To investigate this hypothesis, each cell file along the longitude of individual styles was divided into consecutive sectors of 10 cells and measured (Fig. 3C). The average cell length of the two genotypes did not differ, except for the sector encompassing the 91st to 130th cells. In this distal region, cells of the long-style genotypes were significantly longer ($P < 0.01$) than their counterparts in the short-style genotypes (Fig. 3C). Thus, allelic variation at the *L02* gene modulates style length, and hence stigma exsertion, through localized, differential cell elongation in developing styles. These results also suggest that *L02* is a positive regulator of cell elongation, because greater accumulation of the *L02* transcript (as seen in the long-style genotype) is associated with greater cell elongation and hence with exserted styles.

The nucleotide sequence of the 5′ promoter region of *L02* (contained within the crossover interval that delimits the *Style2.1* QTL) (Fig. 2D and fig. S6) was compared between the long-style allele and the short-style allele. The results revealed a number of sequence differences, including 450-bp (base pair) and 750-bp deletions that were 4 kb and 8 kb upstream from the *L02* start codon, respectively (fig. S7). Sequence analysis from a broader cross section of tomato species revealed that only the 450-bp deletion is specific to the short-style allele found in the cultivated tomato and hence is a candidate for the cause of the down-regulation of *L02* associated with short styles (table S2). However, we cannot rule out the possibility that other, more subtle, sequence changes in the 5′ region of the *L02* promoter may be causal to the down-regulation of *L02* expression associated with the transition from long to short styles.

The evolution from allogamy to autogamy in plants is often associated with both a loss of self-incompatibility (mutation of the *S* locus) and a loss or reduction in stigma exsertion. Which occurs first is a matter of conjecture, but one would predict that self-incompatibility would be lost first (rendering the plants capable of self-pollination), followed by loss or reduction of stigma exsertion (making it more likely that the plants would automatically self-pollinate). If the loss of stigma exsertion occurred before the loss of self-incompatibility, the plants would be unable to either cross-pollinate or self-pollinate—a selective disadvantage. Examination of the phylogenetic tree of tomato and its wild relatives, with regard to mutations in the self-incompatibility (*S*) locus and short-style allele of *L02*, supports the previous order of events. Self-incompatibility was lost in the branch leading to the clade of five self-compatible species (fig. S8). However, the short-style allele of *L02* apparently occurred later in the branch of the phylogenetic tree leading to the cultivated tomato (fig. S8 and table S2). However, it must be recognized that this conjecture is based on genetic studies and sequencing on a relatively small subset of tomato species accessions. A fuller understanding of the evolution of the *Style 2.1* gene throughout the clade of tomato species must await further studies.

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Plant Pathogen Recognition Mediated by Promoter Activation of the Pepper *Bs3* Resistance Gene

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Plant disease resistance (R) proteins recognize matching pathogen avirulence proteins. Alleles of the pepper *R* gene *Bs3* mediate recognition of the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) type III effector protein AvrBs3 and its deletion derivative AvrBs3Δrep16. Pepper *Bs3* and its allelic variant *Bs3-E* encode flavin monooxygenases with a previously unknown structure and are transcriptionally activated by the *Xcv* effector proteins AvrBs3 and AvrBs3Δrep16, respectively. We found that recognition specificity resides in the *Bs3* and *Bs3-E* promoters and is determined by binding of AvrBs3 or AvrBs3Δrep16 to a defined promoter region. Our data suggest a recognition mechanism in which the Avr protein binds and activates the promoter of the cognate *R* gene.

Resistance (R) proteins, a class of plant immune receptors that mediate recognition of pathogen-derived avirulence (Avr) proteins, are a well-studied facet of the plant defense system (*1*). The bacterial plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) uses a type III secretion (T3S) system to inject an arsenal of about 20 effector proteins into the host cytoplasm that collectively promote virulence (*2*). R protein–mediated defense in response to *Xcv* effector proteins is typically accompanied by a programmed cell death response referred to as the hypersensitive response (HR).

One Avr protein that R proteins recognize is AvrBs3, a member of a *Xanthomonas* family of highly conserved proteins (*3*). The central region of AvrBs3 consists of 17.5 tandem near-perfect

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34–amino acid repeat units that determine avirulence specificity (*4*). AvrBs3 also contains nuclear localization signals (NLSs) and an acidic transcriptional activation domain (AD) (*5*, *6*), similar to eukaryotic transcription factors, and induces host gene transcription (*7*). Mutations in the NLS or AD of AvrBs3 abolish pathogen recognition by the matching pepper *R* gene *Bs3* (*5*, *8*), which suggests that recognition involves the transcriptional activation of host genes.

Previously we identified bacterial artificial chromosome (BAC) clones derived from the pepper (*Capsicum annuum*) cultivar Early California Wonder 30R (ECW-30R) that cover the *Bs3* gene (*9*). For complementation-based identification, fragments of a *Bs3*-containing BAC (*9*) were cloned into a plant transformation vector and were delivered into *Nicotiana benthamiana* leaves via *Agrobacterium tumefaciens*–mediated transient transformation. Two nonidentical clones carrying the same coding sequence triggered an HR in *N. benthamiana* when cotransformed with *avrBs3*. A genomic DNA fragment containing only the predicted coding sequence and ~1 kb of sequence upstream of the ATG mediated AvrBs3 recognition, confirming that this gene is *Bs3* (Fig. 1A).

AvrBs3 mutants lacking the AD (AvrBs3ΔAD) or repeat units 11 to 14 (AvrBs3Δrep16) did not trigger HR in pepper *Bs3* plants (*4*, *5*) and also failed to trigger HR in *N. benthamiana* when coexpressed with the cloned *Bs3* gene (Fig. 1A). AvrBs4, which is 97% identical to AvrBs3 but is not recognized by pepper *Bs3* genotypes (*10*), also did not trigger HR in *N. benthamiana* when coexpressed with *Bs3* (Fig. 1A). Therefore, *Bs3* mediates specific recognition of wild-type AvrBs3 in both pepper and *N. benthamiana*, but not when AvrBs3 lacks the AD or repeat units 11 to 14; nor does *Bs3* mediate recognition of the AvrBs3-like AvrBs4 protein (Fig. 1C).

The *Bs3* gene has three exons and two introns (Fig. 1D), is 342 amino acids long (fig. S1), and is homologous to flavin-dependent mono-

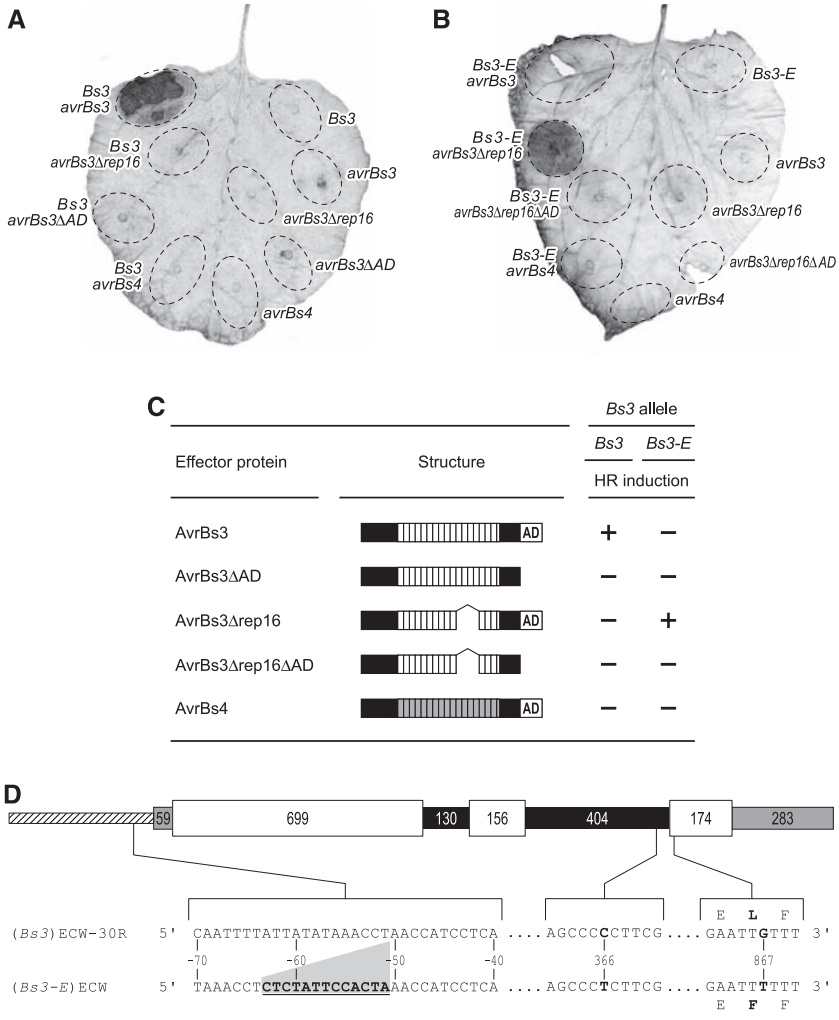

Fig. 1. (**A**) Recognition specificity of the *Bs3* allele from ECW-30R. The *Bs3* gene and/or *avr* genes were expressed transiently in *N. benthamiana* leaves via *A. tumefaciens* (OD_{600} = 0.8). Dashed lines mark the inoculated areas. Four days after infiltration, the leaves were cleared to visualize the HR (dark areas). (**B**) *Bs3-E* and/or *avr* genes were transiently expressed in *N. benthamiana* leaves. (**C**) The relationship between domain structure and activity of AvrBs3, AvrBs3 derivatives, and AvrBs4. Plus and minus signs indicate presence or absence of the HR in *N. benthamiana* upon coexpression of the pepper *Bs3* or *Bs3-E* allele, respectively. For details, see Fig. 1A. White- and gray-boxed areas in the central part of the protein represent the repeat region of AvrBs3 and AvrBs4, respectively. AD refers to the C-terminal acidic transcriptional activation domain. (**D**) Gene structure of the ECW-30R *Bs3* and the ECW *Bs3-E* alleles. Exons, introns, untranslated regions, and promoter regions are displayed to scale as white, black, gray, and hatched boxes, respectively. The length of these elements (in base pairs) is indicated within the boxes. Differences between the *Bs3* alleles are marked in boldface. A 13-bp insertion in the *Bs3-E* promoter relative to the *Bs3* promoter is underlined. Nucleotide positions of the promoter and exon 3 polymorphisms are relative to the transcriptional and translational start sites, respectively. Amino acids encoded by the polymorphic region in exon 3 (E, Glu; L, Leu; F, Phe) are depicted above and below the nucleotide sequences.

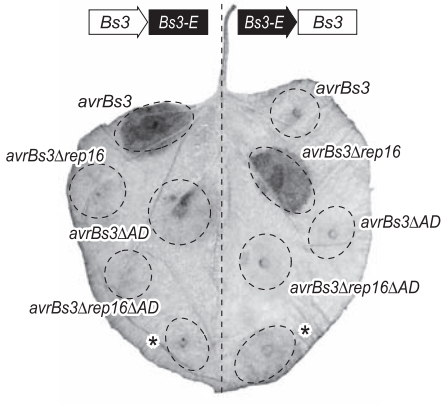

Fig. 2. Chimeras containing the promoter (arrow) of the *Bs3* allele (white) and the coding region (box) of the *Bs3-E* allele (black) or the reciprocal combination (right side of the leaf) were expressed together with *avrBs3*, *avrBs3Δrep16*, and derivatives as indicated. Asterisks mark areas in which only *A. tumefaciens* delivering the chimeric constructs was infiltrated. Dashed lines mark the inoculated areas. Four days after inoculation, leaves were cleared to visualize the HR (dark areas).

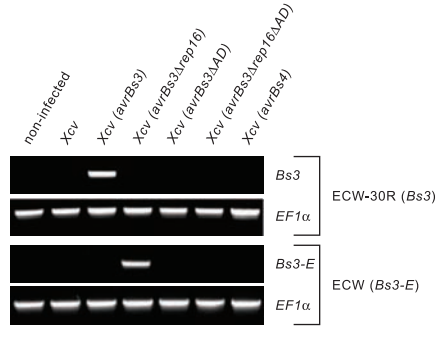

Fig. 3. Semiquantitative RT-PCR on cDNA of noninfected and *Xcv*-infected pepper ECW-30R (*Bs3*) and ECW (*Bs3-E*) leaves 24 hours after infection. The *avrBs3*-like genes that are expressed in the given *Xcv* strains are indicated in parentheses. Elongation factor 1α (EF1α) was amplified as a control.

oxygenases (FMOs) (fig. S2) (*11*). Bs3 is most closely related to FMOs of the *Arabidopsis* YUCCA family (fig. S3) but lacks a stretch of ~70 amino acids present in all related FMOs (fig. S4).

The AvrBs3 derivative AvrBs3Δrep16, which lacks repeat units 11 to 14, triggers HR in the pepper cultivar ECW but not in the near-isogenic *Bs3*-resistant cultivar ECW-30R (*4*). We transformed *N. benthamiana* with the ECW *Bs3* allele (termed *Bs3-E*) including ~1 kb of the promoter and showed that it mediated recognition of AvrBs3Δrep16 but not AvrBs3 (Fig. 1B). Furthermore, AvrBs3Δrep16 lacking the C-terminal AD did not trigger HR when coexpressed with *Bs3-E* (Fig. 1B), and *Bs3-E* did not mediate recognition of AvrBs4. Thus, *Bs3* and *Bs3-E* represent functional alleles with distinct recognition specificities (Fig. 1C). The coding sequences of the two *Bs3* alleles differ by a single nucleotide conferring a nonsynonymous change in exon 3, resulting in a leucine-phenylalanine difference (Fig. 1D and fig. S1). The promoter regions also differed by a 13–base pair (bp) insertion in *Bs3-E* compared to *Bs3*, at position –50 relative to the transcription start site.

We fused the *Bs3* promoter to the *Bs3-E* coding sequence and vice versa, then cotransformed *N. benthamiana* with these chimeras in combination with *avrBs3*, *avrBs3Δrep16*, or the corresponding AD mutant derivatives. The *Bs3* promoter fused to the *Bs3-E* coding sequence mediated exclusively AvrBs3 recognition, whereas the reciprocal chimera (*Bs3-E* promoter fused to the *Bs3* coding sequence) mediated exclusively recognition of AvrBs3Δrep16 (Fig. 2). Thus, the promoter and not the coding region determines recognition specificity of the pepper *Bs3* alleles.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) revealed strongly increased *Bs3* transcript levels in pepper ECW-30R *Bs3* plants upon infection with *avrBs3*-expressing, but not *avrBs3Δrep16*- or *avrBs4*-expressing, *Xcv* strains (Fig. 3). Likewise, *Bs3-E* levels in ECW *Bs3-E* plants increased upon infection with *avrBs3Δrep16*-expressing *Xcv* strains, but not when infected with *avrBs3*- or *avrBs4*-expressing *Xcv* strains. AD-mutant derivatives of *avrBs3* and *avrBs3Δrep16* did not induce accumulation of *Bs3* or *Bs3-E* mRNA. Expression patterns were unaltered in the presence of the translation inhibitor cycloheximide (fig. S5), which indicates that accumulation of the *Bs3* and *Bs3-E* transcripts was independent of de novo protein synthesis. *Agrobacterium*-mediated transient coexpression of *avrBs3* and a *Bs3-GFP* (green fluorescent protein) fusion construct under the control of the *Bs3* promoter caused GFP emission, whereas delivery of *Bs3-GFP* on its own did not result in GFP emission (fig. S6). Together these data indicate that AvrBs3 and AvrBs3Δrep16 induce transcription of the respective *R* genes *Bs3* and *Bs3-E*, and that the subsequent accumulation of these R proteins triggers HR. In agreement with this result, constitutive expression of *Bs3* or *Bs3-E* under the cauliflower mosaic virus 35S promoter triggered an *avr*-independent HR (fig. S7). We identified *Bs3* mutants with single amino acid replacements that were not compromised in protein stability but no longer triggered HR when expressed in *N. benthamiana* (fig. S8), indicating that the enzymatic activity of Bs3 is crucial to its function as a cell death inducer.

Electrophoretic mobility shift assays (EMSAs) with GST-AvrBs3 fusion protein and biotin-labeled *Bs3* and *Bs3-E* promoter fragments (Fig. 4A) showed that AvrBs3 bound to both *Bs3*- and *Bs3-E*–derived promoter fragments containing the polymorphism, although affinity appeared higher for the *Bs3*-derived fragment (Fig. 4B). Competition assays with labeled *Bs3*-derived promoter fragments and nonlabeled *Bs3*- and *Bs3-E*–derived promoter fragments, and vice versa, confirmed that AvrBs3 binds with high affinity to the *Bs3* promoter fragment and with low affinity to the *Bs3-E* promoter fragment (Fig. 4C). In contrast, AvrBs3 did not bind to a DNA fragment from a nonpolymorphic region of the *Bs3* promoter (Fig. 4B). Furthermore, EMSA studies showed that both AvrBs3 and AvrBs3Δrep16 have a higher affinity for the *Bs3* promoter than for the *Bs3-E* promoter (Fig. 4 and fig. S9). Therefore, promoter binding per se of AvrBs3 or AvrBs3Δrep16 is not the basis for promoter activation specificity.

We performed chromatin immunoprecipitation assays by infiltrating pepper ECW-30R (*Bs3*) and ECW (*Bs3-E*) leaves either with *avrBs3*-expressing *Xcv* wild-type strains or with an iso-

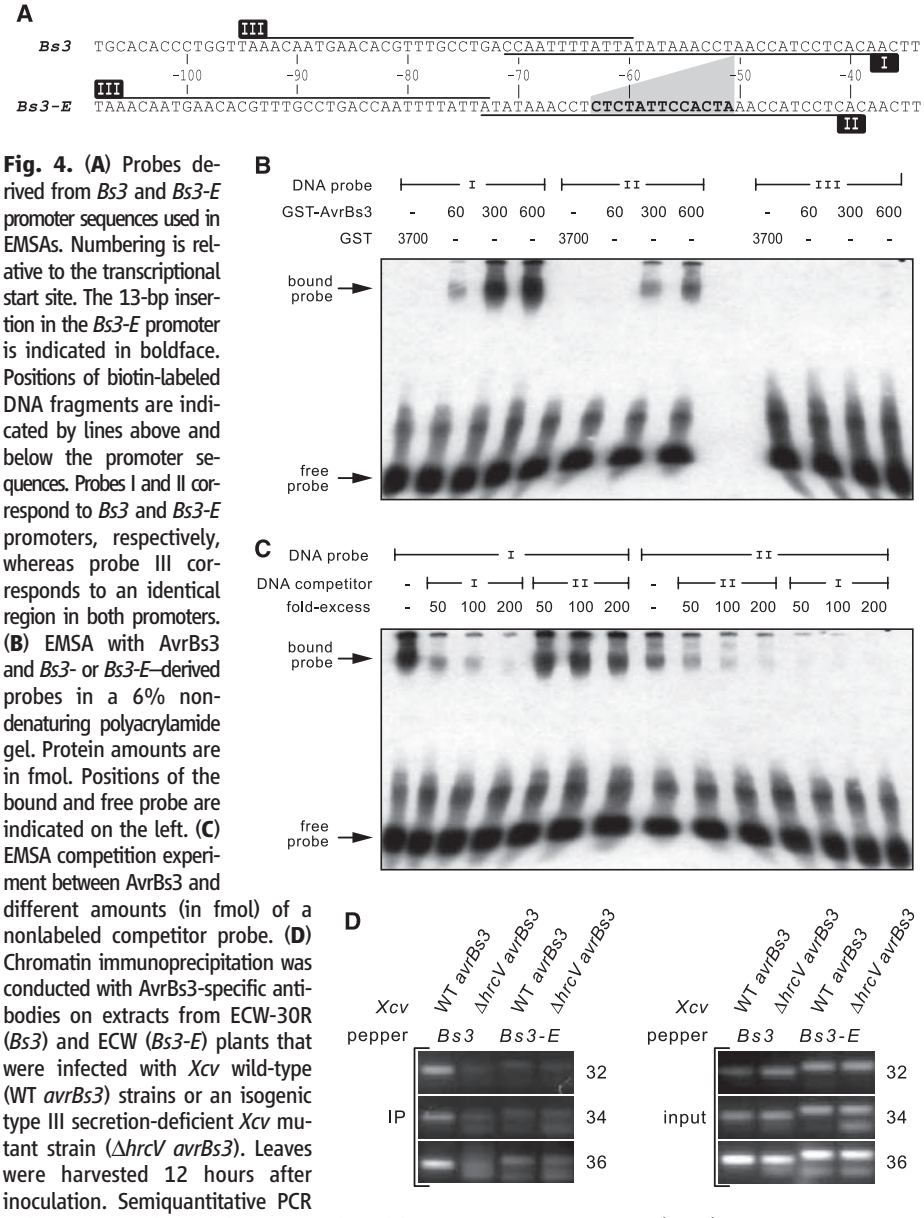

Fig. 4. (**A**) Probes derived from *Bs3* and *Bs3-E* promoter sequences used in EMSAs. Numbering is relative to the transcriptional start site. The 13-bp insertion in the *Bs3-E* promoter is indicated in boldface. Positions of biotin-labeled DNA fragments are indicated by lines above and below the promoter sequences. Probes I and II correspond to *Bs3* and *Bs3-E* promoters, respectively, whereas probe III corresponds to an identical region in both promoters. (**B**) EMSA with AvrBs3 and *Bs3*- or *Bs3-E*–derived probes in a 6% nondenaturing polyacrylamide gel. Protein amounts are in fmol. Positions of the bound and free probe are indicated on the left. (**C**) EMSA competition experiment between AvrBs3 and different amounts (in fmol) of a nonlabeled competitor probe. (**D**) Chromatin immunoprecipitation was conducted with AvrBs3-specific antibodies on extracts from ECW-30R (*Bs3*) and ECW (*Bs3-E*) plants that were infected with *Xcv* wild-type (WT *avrBs3*) strains or an isogenic type III secretion-deficient *Xcv* mutant strain (Δ*hrcV avrBs3*). Leaves were harvested 12 hours after inoculation. Semiquantitative PCR with 32, 34, and 36 cycles was conducted before immunoprecipitation (input) or on immunoprecipitated material (IP). ECW-30R (*Bs3*) and ECW (*Bs3-E*) derived PCR products differ in size because of a 13-bp insertion in the *Bs3-E* promoter.

genic *hrcV* mutant strain. HrcV is a conserved protein of the core T3S system with mutants incapable of delivering T3S effector proteins (*12*). After immunoprecipitation with an antibody to AvrBs3 (*13*), enrichment of the *Bs3* but not the *Bs3-E* promoter region was detected by semi-quantitative PCR (Fig. 4D). This demonstrates that *Xcv*-delivered AvrBs3 binds to the *Bs3* promoter in vivo with higher affinity than to the *Bs3-E* promoter. Given that *Bs3* promoter enrichment was detected in leaf material inoculated with the wild type but not with the *hrcV* mutant strain, we conclude that the *Bs3* promoter is bound before cell lysis.

We also infected the pepper cultivar ECW-123R containing the *R* genes *Bs1*, *Bs2*, and *Bs3* with xanthomonads delivering either the structurally unrelated AvrBs1, AvrBs2, or AvrBs3 protein or none of these Avr proteins. RT-PCR showed that the *Bs3*-derived transcripts were detectable only upon infection with *avrBs3*-expressing *Xcv* strains (fig. S10). Therefore, *Bs3* is not transcriptionally activated in the course of the *Bs1*- or *Bs2*-mediated HR.

Isolation of the pepper *Bs3* gene uncovered a mechanistically novel type of recognition mechanism and a structurally novel type of R protein that shares homology to FMOs. Recently, FMO1, an *Arabidopsis* protein that is sequence-related to Bs3 (fig. S2), was shown to be involved in pathogen defense (*14*–*16*). Thus, FMO1 and Bs3 may have similar functions. However, *FMO1* is transcriptionally induced by a variety of stimuli including virulent and avirulent microbial pathogens (*14*, *16*, *17*). In contrast, *Bs3* was not induced by virulent *Xcv* strains (Fig. 3), nor by resistance reactions mediated by the pepper *R* genes *Bs1* and *Bs2* (fig. S10). Moreover, 35S-driven *Bs3* alleles triggered an HR reaction (fig. S7), whereas a 35S-driven *FMO1* gene mediates broad-spectrum resistance but not HR (*14*, *15*). Thus, *Arabidopsis FMO1* and pepper *Bs3* differ with respect to their transcriptional regulation and function.

Our results show that the bacterial effector protein AvrBs3 binds to and activates the promoter of the matching pepper *R* gene *Bs3*. Analysis of host genes that are up-regulated by AvrBs3 ("*upa*" genes) in a compatible *Xcv*-pepper interaction (*7*, *18*) led to the identification of the *upa*-box (TATATAAACCN$_{2\text{-}3}$CC), a conserved DNA element that was shown to be bound by AvrBs3 and that is also present in the *Bs3* promoter (Fig. 1D) (*18*). This suggests that binding of AvrBs3 to the *upa*-box is crucial for activation of corresponding promoters. However, binding of an AvrBs3-like protein does not necessarily result in promoter activation, because AvrBs3Δrep16 bound with higher affinity to the *Bs3* than to the *Bs3-E* promoter (fig. S9) but only activated the *Bs3-E* and not the *Bs3* promoter (Fig. 3). Because AvrBs3Δrep16 and AvrBs3 differ in their structure, we postulate that upon DNA binding, their functional domains (e.g., AD) are exposed at different promoter locations, which may define whether AvrBs3Δrep16 and AvrBs3 are able to activate a given promoter. Additionally, given that the *Bs3* promoter determines recognition specificity, the *Bs3* promoter might be coevolving to maintain compatibility with rapidly changing AvrBs3-like proteins, similar to that seen in the NB-LRR proteins (*19*, *20*).

We consider it likely that not only AvrBs3 but also other AvrBs3 homologs bind to and activate promoters of matching *R* genes. The recently isolated rice *R* gene *Xa27*, which mediates recognition of the AvrBs3-like AvrXa27 protein from *Xanthomonas oryzae* pv. *oryzae* (*21*), is transcriptionally induced by AvrXa27, and thus it is tempting to speculate that the *Xa27* promoter is a direct target of AvrXa27. However, whether AvrXa27 acts directly at the *Xa27* promoter remains to be clarified.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S10
References

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A Bacterial Effector Acts as a Plant Transcription Factor and Induces a Cell Size Regulator

Sabine Kay,[1] Simone Hahn,[1] Eric Marois,[1]* Gerd Hause,[2] Ulla Bonas[1]†

Pathogenicity of many Gram-negative bacteria relies on the injection of effector proteins by type III secretion into eukaryotic cells, where they modulate host signaling pathways to the pathogen's benefit. One such effector protein injected by *Xanthomonas* into plants is AvrBs3, which localizes to the plant cell nucleus and causes hypertrophy of plant mesophyll cells. We show that AvrBs3 induces the expression of a master regulator of cell size, *upa20*, which encodes a transcription factor containing a basic helix-loop-helix domain. AvrBs3 binds to a conserved element in the *upa20* promoter via its central repeat region and induces gene expression through its activation domain. Thus, AvrBs3 and likely other members of this family provoke developmental reprogramming of host cells by mimicking eukaryotic transcription factors.

Gram-negative phytopathogenic bacteria of the genus *Xanthomonas* cause a broad variety of diseases in crop plants (*1*). Pathogenicity depends on the translocation of effector proteins directly into the plant cell cytosol by a type III secretion (T3S) system (*2*). The AvrBs3 family is a prominent effector class in *Xanthomonas* spp. (*3*), comprising major virulence determinants (*4*–*6*). These effectors are characterized by a central repeat region, nuclear localization signals (NLSs), and an acidic transcriptional activation domain (AD) (*3*). AvrBs3 was isolated from *X. campestris* pv. *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease on pepper and tomato (*7*). In susceptible host plants and other solanaceous species, AvrBs3 elicits hypertrophy (i.e., enlargement) of mesophyll cells (*8*) and also contributes to the dispersal of *Xcv* between pepper plants under field conditions (*9*). Cell enlargement is also found in other complex disease phenotypes [e.g., citrus canker elicited by the AvrBs3-like effector PthA from *X. axonopodis* pv. *citri* (*10*) and *Pantoea agglomerans*–induced gall formation (*11*)].