Research

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Promoter elements of rice susceptibility genes are bound and activated by specific TAL effectors from the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae*

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Summary

- Plant pathogenic bacteria of the genus *Xanthomonas* inject transcription activator-like effector (TALe) proteins that bind to and activate host promoters, thereby promoting disease or inducing plant defense. TALes bind to corresponding *UPT* (up-regulated by TALe) promoter boxes via tandemly arranged 34/35-amino acid repeats. Recent studies uncovered the TALe code in which two amino acid residues of each repeat define specific pairing to *UPT* boxes.
- Here we employed the TALe code to predict potential *UPT* boxes in TALe-induced host promoters and analyzed these via β-glucuronidase (GUS) reporter and electrophoretic mobility shift assays (EMSA).
- We demonstrate that the *Xa13*, *OsTFX1* and *Os11N3* promoters from rice are induced directly by the *Xanthomonas oryzae* pv. *oryzae* TALes PthXo1, PthXo6 and AvrXa7, respectively. We identified and functionally validated a *UPT* box in the corresponding rice target promoter for each TALe and show that box mutations suppress TALe-mediated promoter activation. Finally, EMSA demonstrate that code-predicted *UPT* boxes interact specifically with corresponding TALes.
- Our findings show that variations in the *UPT* boxes of different rice accessions correlate with susceptibility or resistance of these accessions to the bacterial blight pathogen.

Introduction

Microbial plant pathogens deliver effector proteins into the host's cytoplasm to promote their virulence or to suppress plant innate immunity (Göhre & Robatzek, 2008; Boller & He, 2009; Hogenhout *et al.*, 2009). After delivery, microbial effectors are targeted to different subcellular compartments of the host cell. Recently it has become evident that the nucleus is targeted by effectors from various classes of plant microbial pathogens, including nematodes (Elling *et al.*, 2007), oomycetes (Kanneganti *et al.*, 2007), fungi (Kemen *et al.*, 2005) and bacteria (Deslandes *et al.*, 2003; Nissan *et al.*, 2006; Bai *et al.*, 2009). Transcription activator-like effectors (TALes) from the plant pathogenic bacterial genus *Xanthomonas* are among the most intensively studied class of nuclear-targeted microbial effectors (Kay & Bonas, 2009; White *et al.*, 2009). The most characteristic structural feature of TALes is the central repeat domain that is composed of a variable number of tandemly arranged, imperfect copies of a 34/35-amino acid motif (Schornack *et al.*, 2006). Differences between individual repeat units are found primarily at positions 12 and 13, the so-called repeat-variable diresidues (RVDs) (Moscou & Bogdanove, 2009). The repeat domain of the prototype TALe, AvrBs3, from *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), has been shown to interact with a corresponding promoter element,

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termed an *UPA* (up-regulated by AvrBs3) box, that is present in the promoter of the pepper transcription factor *UPA20*, a host susceptibility gene that appears to support bacterial spread (Kay *et al.*, 2007). The presence of a *UPA* box in a promoter results in AvrBs3-mediated expression of the given host gene (Kay *et al.*, 2009; Römer *et al.*, 2009b). The promoter of the pepper resistance (*R*) gene *Bs3* also contains a *UPA* box and thus is transcriptionally activated by AvrBs3 (Römer *et al.*, 2007, 2009b). Expression of *Bs3* triggers a cell death reaction, referred to as the hypersensitive response (HR), and results in resistance against *Xcv*. Thus, the *R* gene *Bs3* represents a 'promoter trap' that coopts AvrBs3's function in promoting virulence. Similarly, transcription of the rice *R* gene *Xa27* is specifically induced by AvrXa27, a TALe from the bacterial blight pathogen, *X. oryzae* pv. *oryzae* (*Xoo*) (Gu *et al.*, 2005). Recent studies uncovered that the *Xoo* TALe AvrXa27 binds to a matching promoter motif in the rice *Xa27* promoter (Römer *et al.*, 2009a). Thus the *R* genes *Bs3* and *Xa27* use identical mechanisms to detect their matching TALes. Promoter motifs that mediate TALe transcriptional activation have been collectively defined as *UPT* (up-regulated by TALes) boxes, with a subscript designation to define the specific TALe that targets the given *UPT* box (Römer *et al.*, 2009a).

Although it was long known that TALe target specificity is defined by the number and order of repeat units that together form the repeat domain (Herbers *et al.*, 1992), it was not clear how the repeat domain conferred target specificity at the molecular level. Recent studies demonstrated that RVDs specify the nucleotide target site of a given TALe with one RVD pairing to one specific *UPT* box nucleotide (Boch *et al.*, 2009; Moscou & Bogdanove, 2009). This pairing code defined the interaction of TALes to colinear binding sites and was used to deduce functional *UPT* boxes for such TALes for which a corresponding host target promoter was not available (Boch *et al.*, 2009).

Recently, a number of genes have been identified in rice that are targeted and transcriptionally activated by specific *Xoo* TALes to promote virulence of *Xoo* (Chu *et al.*, 2006; Yang *et al.*, 2006; Sugio *et al.*, 2007; Antony *et al.*, 2009; Yuan *et al.*, 2009). The *UPT* boxes in the promoters of these rice genes have been predicted (Boch *et al.*, 2009; Moscou & Bogdanove, 2009) by the use of the TAL code but not functionally validated. In the present study we analyzed if code predicted *UPT* boxes in the TALe-induced rice promoters of *Xa13* (also known as *Os8N3*), *OsTFX1* and *Os11N3* are crucial to transcriptional activation by matching TALes. Furthermore we tested via electrophoretic mobility shift assay (EMSA) if TALes physically interact with the corresponding *UPT* boxes and how box mutations affect the TALe–DNA interaction. Our results show that resistance and susceptibility to *Xoo* in rice are influenced by *UPT* box sequences.

Materials and Methods

Generation of the promoter *uidA* fusion constructs

Promoter regions of *OsTFX1*, *Os11N3*, and *Xa13* were PCR-amplified from genomic rice (*Oryza sativa*) DNA of cv IR24. The *xa13* promoter region was amplified from genomic rice DNA of cv IRBB13. Amplification was carried out with Phusion high-fidelity DNA polymerase (New England Biolabs, Frankfurt, Germany) and primers provided in Supporting Information (Fig. S1). The PCR fragments were cloned into pENTR-D (Invitrogen GmbH, Karlsruhe, Germany), sequenced and transferred into the T-DNA vector pGWB3 (Nakagawa *et al.*, 2007) by LR recombination (Invitrogen). pGWB3 derivatives were transformed into *Agrobacterium tumefaciens* GV3101 (Koncz & Schell, 1986) for *in planta* analysis.

Generation of the TALe constructs

For the generation of T-DNA vectors that contain *avrXa7*, *pthXo1* or *pthXo6*, we used the vector pENTR-D-*Bam*HI-*avrXa27* (Römer *et al.*, 2009a). The *Bam*HI fragments of *avrXa7*, *pthXo1* and *pthXo6* were transferred into pENTR-D-*Bam*HI-*avrXa27*, resulting in the pENTR-D-*avrXa7*, pENTR-D-*pthXo1* and pENTR-D-*pthXo6*, respectively. The TALe genes were transferred via LR recombination in the binary vectors pGWB2 or pGWB5 (Nakagawa *et al.*, 2007). pGWB2 and pGWB5 derivatives were transformed into *A. tumefaciens* GV3101 for *in planta* analysis. For EMSA we transferred *pthXo1* and *pthXo6* from pENTR-D-*pthXo1* and pENTR-D-*pthXo6* via LR recombination into pDEST17 (Invitrogen).

Insertion of *UPT* boxes in the *Bs3* promoter

For the insertion of the predicted *UPT* boxes in the *Bs3* promoter 5′ upstream of the UPT_{AvrBs3} box we used primers Xa13in30R-fwd-01-PR GATATNCATCTCCCCCTACTGTACACCACCAACTGGTTAAACAATGAACACGTTTGC, Xa13in30R-fwd-02-T-PR GATAGCATCTCCCCCTACTGTACACCACCAACTGGTTAAACAATGAACACGTTTGC, OsTFX1in30R-fwd-01-PR ACCCTATAAAAGGCCCTCACCAACCCATCGCCTGGTTAAACAATGAACACGTTTGC, OsTFX1in30R-fwd-02-T-PR ACCCATAAAAGGCCCTCACCAACCCATCGCCTGGTTAAACAATGAACACGTTTGC, Os11N3in30R-fwd-03-PR GCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTCCTGGTTAAACAATGAACACG, Os11N3in30R-fwd-04-T-PR GCACATATAAACCCCCTCCAACCAGGTGCTAAGCTCCTGGTTAAACAATGAACACG in combination with the primer 4in30R-rev-02-PR GGTGTGCAAATTGTGGTTTAACCC. All primers used are phosphorylated at their 5′ termini. Insertion was

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done using the Phusion site directed mutagenesis kit (New England Biolabs). As a template, we used pENTR-D containing 343 bp 5′ of the ATG start codon of the *Bs3* gene. The promoter was amplified from genomic DNA of ECW-30R pepper plants using the Phusion high-fidelity DNA polymerase. After sequencing, the promoter constructs were transferred by LR recombination in the binary vector pGWB3 (Nakagawa *et al.*, 2007). pGWB3 derivatives were transformed into *A. tumefaciens* GV3101 (Koncz & Schell, 1986) for *in planta* analysis.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were carried out as described earlier (Römer *et al.*, 2009a).

β-Glucuronidase (GUS) measurements

Leaves of three *Nicotiana benthamiana* plants were inoculated with a mixture of *Agrobacterium* delivering constructs for expression of TALes and the promoter-GUS reporter. Twenty-seven or 48 h postinoculation, two leaf discs (1 cm diameter) from separate infiltration spots of the same constructs on one plant were combined, ground in liquid nitrogen, and GUS assays were done as described previously (Kay *et al.*, 2007). Samples were measured in a plate reader at 360 nm (excitation) and 465 nm (emission) with 4-methyl-umbelliferon (MU) (Carl Roth, Karlsruhe, Germany) dilutions as standard. Proteins were quantified using Bradford assays (Bio-Rad). Triplicate samples from three different plants were combined into one data point. In parallel, leaf discs from inoculated areas were sampled and incubated overnight in X-Gluc staining solution (Schornack *et al.*, 2005). Leaf discs were cleared in 100% ethanol and dried using cellulose foil. Experiments were performed at least twice with similar results.

Results

The promoters of the rice genes *Xa13*, *OsTFX1* and *Os11N3* are direct targets of the *Xoo* TALes PthXo1, PthXo6 and AvrXa7, respectively

Recent studies uncovered that the *Xoo* TALes PthXo1, PthXo6 and AvrXa7 transcriptionally activate the rice *Xa13* (synonym: *Os8N3*), *OsTFX1* and *Os11N3* genes, respectively (Chu *et al.*, 2006; Yang *et al.*, 2006; Sugio *et al.*, 2007; Antony *et al.*, 2009; Yuan *et al.*, 2009). To test if the rice *OsTFX1*, *Os11N3* and *Xa13* promoters are direct TALe targets, we amplified the corresponding promoter fragments from rice genomic DNA and cloned these in a T-DNA vector in front of an *uidA* reporter gene (Figs 1a, S1). The promoter::*uidA* fusion constructs were delivered into *N. benthamiana* leaves via transient *A. tumefaciens*-mediated

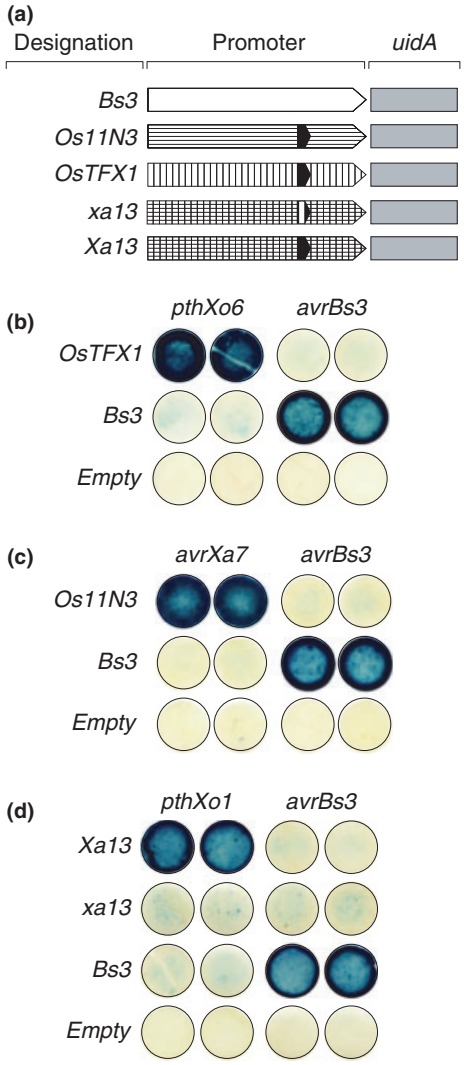

Fig. 1 Promoters of *Xoo* susceptibility genes in rice are transcriptionally activated by their matching transcription activator-like effector (TALe) proteins. (a) Graphical display of the studied promoter::*uidA* reporter constructs. Arrows represent the rice promoters *Os11N3*, *OsTFX1*, *Xa13*, *xa13* and the pepper *Bs3* gene. Nucleotide sequences of the rice promoters are provided in Supporting Information, Fig. S1. The corresponding UPT_{AvrXa7}, UPT_{PthXo6}, UPT_{PthXo1} and UPT_{AvrBs3} boxes are shown as black boxes. A black box with a white bar represents the nonfunctional UPT_{PthXo1} box of the *xa13* promoter from the rice cv IRBB13. A gray box represents the *uidA* reporter gene, encoding the β-glucuronidase (GUS) protein. (b–d) *In planta* functional analysis of rice promoters and their matching TALes. *uidA* T-DNA constructs under transcriptional control of the depicted plant promoters were delivered via *Agrobacterium tumefaciens* into *Nicotiana benthamiana* leaves in combination with either an empty T-DNA vector (empty) or the *35S*-promoter-driven TALe genes of *avrBs3*, *pthXo6*, *avrXa7* and *pthXo1*. Leaf discs were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc) to visualize activity of the GUS reporter. Samples were taken at 27 hpi (b, d) or 48 hpi (c).

T-DNA transformation (agroinfiltration) in combination with the cauliflower mosaic virus *35S* (*35S*) promoter-driven TALe genes *pthXo1*, *pthXo6*, *avrXa7* and *avrBs3*. GUS

assays showed that the rice *OsTFX1* and *Os11N3* promoters are activated specifically by the matching *Xoo* TALes PthXo6 and AvrXa7, respectively, but not by the related *Xcv* TALe AvrBs3 (Fig. 1b,c). Furthermore, the GUS assays showed that the *Xoo* TALe PthXo1 transcriptionally activates only the rice *Xa13* promoter from the rice cv IR24 but not the allelic *xa13* promoter from the *Xoo*-resistant rice cv IRBB13 (Fig. 1d). Our GUS assays are in agreement with previous studies showing that *Xoo* delivering PthXo1 activates only expression of *Xa13* but not *xa13* alleles (Chu *et al.*, 2006; Yuan *et al.*, 2009). In our GUS assays, the pepper *Bs3* promoter was not activated by any of the *Xoo* TALes but it was activated by the *Xcv* TALe AvrBs3 (Fig. 1b–d). These data demonstrate that the *OsTFX1*, *Os11N3* and *Xa13* promoters are direct targets of the *Xoo* TALes PthXo6, AvrXa7 and PthXo1, respectively.

TALes target the *in silico* predicted *UPT* boxes

We used the TALe code (Boch *et al.*, 2009; Moscou & Bogdanove, 2009) to predict the UPT_{PthXo6}, UPT_{AvrXa7} and UPT_{PthXo1} boxes of the rice *OsTFX1*, *Os11N3* and *Xa13* promoters, respectively (Figs S1, S2, Table S1). Regions potentially encompassing the distinct *UPT* boxes were introduced into the pepper *Bs3* promoter and cloned in front of an *uidA* reporter gene. The *Bs3* promoter-embedded *UPT* boxes were agroinfiltrated into *N. benthamiana* leaves in combination with the *35S* promoter-driven TALe genes *pthXo1*, *pthXo6*, *avrXa7* or *avrBs3*. GUS assays showed that a *Bs3* promoter derivative containing a given *UPT* box is transcriptionally activated only by the matching *Xoo* TALe (Figs 2–4). For example, insertion of the UPT_{PthXo6} box from the rice *OsTFX1* into the pepper *Bs3* promoter (*OsTFX1* in *Bs3*, Fig. 2b) made this promoter PthXo6- but not PthXo1-inducible. By contrast, the *Bs3* wild-type promoter (*Bs3*) that lacks the UPT_{PthXo6} box was only AvrBs3- but not PthXo6-inducible. Similarly, insertion of the UPT_{AvrXa7} and UPT_{PthXo1} boxes into the *Bs3* promoter resulted in promoter constructs that were AvrXa7- and PthXo1-inducible, respectively (Figs 3b, 4b). All *Bs3* promoter derivatives contain the UPT_{AvrBs3} box and thus were AvrBs3-inducible, irrespective of whether or not a *Xoo* TALe box was present (Figs 2b, 3b, 4b). In summary, the TALe code enabled the identification of *UPT* boxes from rice promoters that are transcriptionally up-regulated by corresponding *Xoo* TALes.

Mutation of the conserved 5′ terminal T nucleotide of *UPT* boxes results in reduced TALe-mediated inducibility

All *UPT* boxes that have been predicted with the TALe code are preceded by a 5′ terminal T nucleotide (Boch *et al.*, 2009; Moscou & Bogdanove, 2009). Mutations in

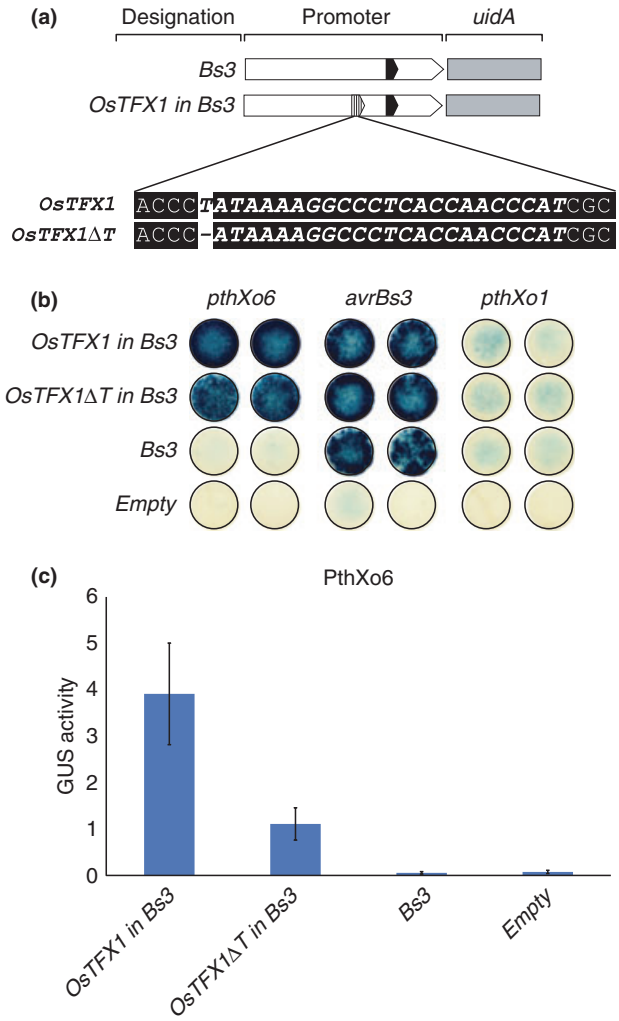

Fig. 2 The transcription activator-like effector (TALe) PthXo6 transcriptionally activates promoters containing the UPT_{PthXo6} box of the rice *OsTFX1* promoter. (a) Graphical display of promoter::*uidA* reporter constructs. The white arrow represents the pepper *Bs3* promoter. A gray box represents the β-glucuronidase (GUS)-encoding *uidA* reporter gene. The UPT_{AvrBs3} and UPT_{PthXo6} boxes are displayed as black and hatched arrows, respectively, with their nucleotide sequences depicted below. Bold italic letters represent the core UPT_{PthXo6} box (*OsTFX1*). A dash represents the deleted 5′ terminal T nucleotide of the mutated UPT_{PthXo6} box (*OsTFX1ΔT*). (b) PthXo6 targets specifically the UPT_{PthXo6} but not the UPT_{AvrBs3} box. A fragment of the *OsTFX1* promoter containing the UPT_{PthXo6} box was placed into the context of pepper *Bs3* promoter (*OsTFX1* in *Bs3*). '*OsTFX1ΔT* in *Bs3*' denotes a *OsTFX1* promoter fragment with a mutated UPT_{PthXo6} box that lacks the 5′ terminal T nucleotide of the core box. The different reporter constructs were delivered into *Nicotiana benthamiana* leaves via *Agrobacterium tumefaciens* with either an empty T-DNA vector (empty) or *35S*-promoter-driven TALe genes *avrBs3*, *pthXo6* or *pthXo1*. (c) Deletion of the 5′ terminal T nucleotide of the UPT_{PthXo6} box significantly reduces its PthXo6-dependent inducibility. GUS activity (pmol 4-MU min^{-1} μg^{-1} protein) was determined 27 h after *A. tumefaciens*-mediated co-delivery of the depicted reporter constructs in combination with a *35S*-promoter-driven *pthXo6* gene. Error bars denote standard deviations.

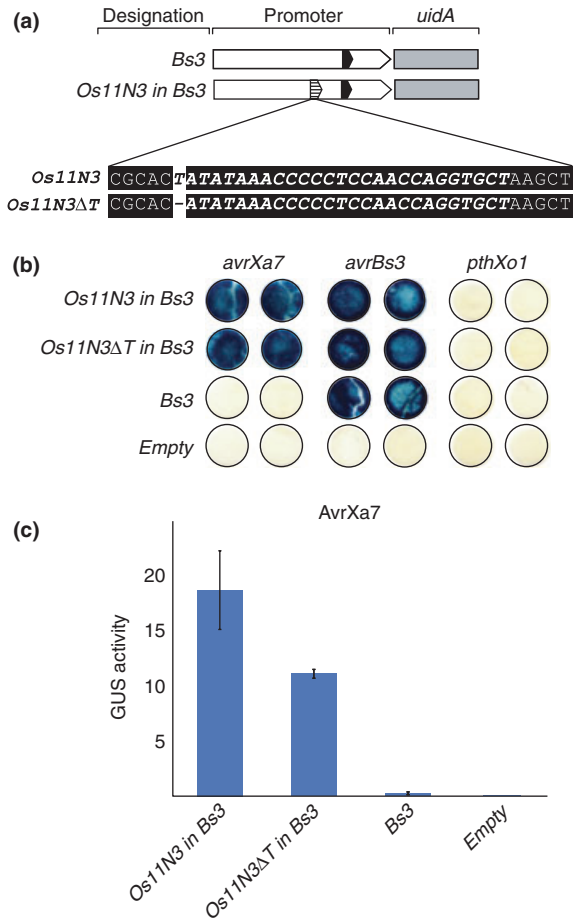

Fig. 3 The transcription activator-like effector (TALe) AvrXa7 transcriptionally activates promoters containing the UPT_{AvrXa7} box of the rice *Os11N3* promoter. (a) Graphical display of promoter::*uidA* reporter constructs. The white arrow represents the pepper *Bs3* promoter. The UPT_{AvrBs3} and UPT_{AvrXa7} boxes are displayed as black and hatched boxes, respectively. A gray box represents the *uidA* reporter gene, encoding the β-glucuronidase (GUS) protein. The letters below the boxes represent the nucleotides of the *Os11N3* promoter that were inserted into the *Bs3* promoter. Bold italic letters represent the core UPT_{AvrXa7} box (*Os11N3*). The dash represents the deleted nucleotide of the mutated UPT_{AvrXa7} box (*Os11N3ΔT*). (b) AvrXa7 targets specifically the UPT_{AvrXa7} but not the UPT_{AvrBs3} box. A fragment of the *Os11N3* promoter containing the UPT_{AvrXa7} box was placed into the context of pepper *Bs3* promoter (*Os11N3* in *Bs3*). '*Os11N3ΔT in Bs3*' denotes an *Os11N3* promoter fragment with the mutated UPT_{AvrXa7} box. The different reporter constructs were delivered into *Nicotiana benthamiana* leaves via *Agrobacterium tumefaciens* in combination with either an empty T-DNA vector (empty) or the *35S*-promoter-driven TALe genes *avrBs3*, *avrXa7* or *pthXo1*. (c) The deletion of the 5′ terminal T nucleotide of the UPT_{AvrXa7} box significantly reduces its AvrXa7-dependent inducibility. GUS activity in *N. benthamiana* is taken as a measure of the AvrXa7-dependent inducibility of the given promoter. GUS activity (pmol 4-MU min^{-1} μg^{-1} protein) was determined 48 h after *A. tumefaciens*-mediated co-delivery of the depicted reporter constructs in combination with a *35S*-promoter-driven *avrXa7* gene. Error bars denote standard deviations.

the 5′ terminal T nucleotide of the UPT_{AvrBs3} or UPT_{Hax3} box resulted in reduced inducibility by the matching TALe (Boch *et al.*, 2009; Römer *et al.*, 2009b). To study the functional importance of the 5′ terminal T nucleotide of the UPT_{PthXo6}, UPT_{AvrXa7} and UPT_{PthXo1} boxes, we created T deletion mutants (ΔT) of the corresponding *Bs3*-promoter-embedded *UPT* boxes and cloned these in front of an *uidA* reporter gene. The *UPT* box ΔT mutants were delivered into *N. benthamiana* leaves via agroinfiltration in combination with the *35S* promoter-driven TALe genes *pthXo6*, *avrXa7*, *pthXo1* or *avrBs3*. Qualitative GUS assays showed that promoters containing the ΔT mutants of the UPT_{PthXo6}, UPT_{AvrXa7} or UPT_{PthXo1} boxes were still induced by their matching TALes (*OsTFX1ΔT* in *Bs3* (Fig. 2b), *Os11N3ΔT* in *Bs3* (Fig. 3b) and *Xa13ΔT in Bs3* (Fig. 4b)). However, quantitative GUS assays demonstrated that the three tested ΔT mutants in all cases produced a significantly reduced GUS activity in comparison to the wild-type *UPT* boxes (Figs 2c, 3c, 4c). Thus the 5′ terminal T nucleotide is important to the function of the UPT_{PthXo6}, UPT_{AvrXa7} and UPT_{PthXo1} boxes.

Rice *Xa13* and *xa13* alleles differ in the predicted UPT_{PthXo1} box

Molecular analysis of a collection of rice *xa13* and *Xa13* rice genotypes uncovered that the *pthXo1* expressing *Xoo* strain PXO99 transcriptionally activates only *Xa13* but not *xa13* genotypes (Chu *et al.*, 2006; Yuan *et al.*, 2009). We anticipated that *Xa13* and *xa13* genotypes are likely to differ in their UPT_{PthXo1} box region. Sequence analysis revealed that the PthXo1-inducible *Xa13* alleles from rice cvs IR24, IR64, Nipponbare, Minghui and 93-11 were sequence identical within the UPT_{PthXo1} box (Figs S3, S4). By contrast, all studied *xa13* alleles differed from the *Xa13* alleles within the UPT_{PthXo1} box. In several *xa13* alleles, the integrity of the UPT_{PthXo1} box was lost as a result of nucleotide insertions or deletions. For example, the *xa13* alleles from rice cv AC 19-1-1 and Kalimekri 77-5 have lost 3′ terminal nucleotides of the UPT_{PthXo1} box as a result of a 34 bp deletion with respect to the IR24 *Xa13* allele (Fig. S4). We also identified five *xa13* genotypes (Tepa1, BJ1, Chinsurah 11484, Chinsurah 11760 and Chinsurah 50930) that showed only a G → T substitution in the second box nucleotide with respect to the UPT_{PthXo1} box from IR24 (Fig. S4a). According to the TALe code the second nucleotide of the UPT_{PthXo1} box is bound by the first PthXo1 repeat unit, which contains an NN-type RVD. Experimental studies with an *in vitro* constructed TALe consisting of NN-type RVDs only have shown that NN recognizes preferentially G (Boch *et al.*, 2009). To clarify how polymorphisms in the second nucleotide of the UPT_{PthXo1} box influence PthXo1-mediated promoter activation, we replaced the G nucleotide of the *Xa13* allele by A, C or T

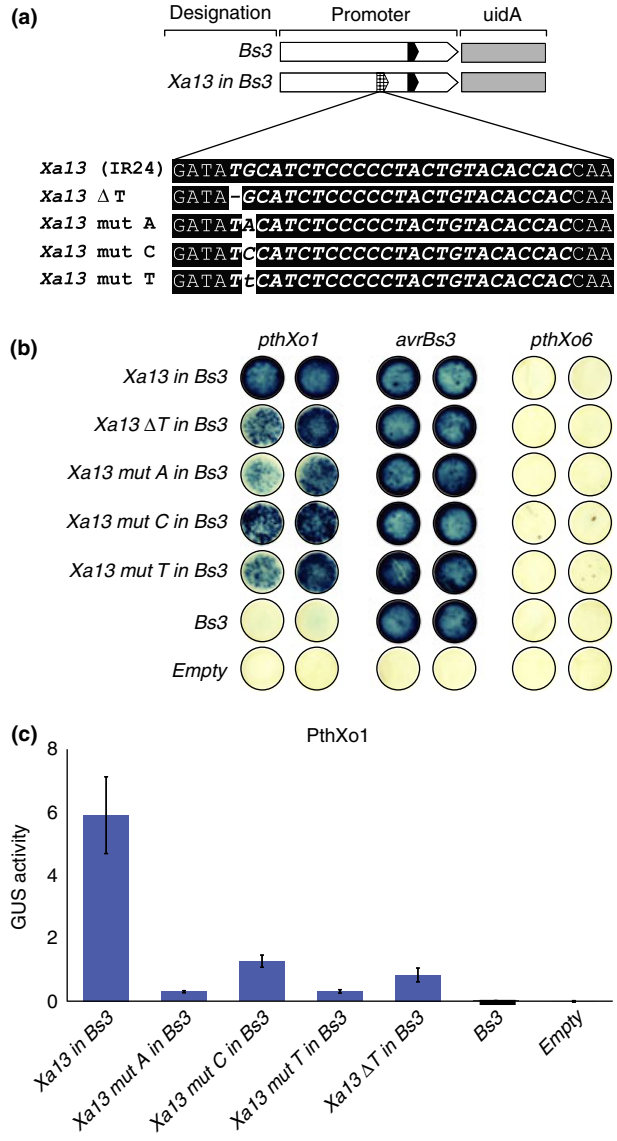

Fig. 4 The transcription activator-like effector (TALe) PthXo1 transcriptionally activates promoters containing the UPT_{PthXo1} box of the rice *Xa13* promoter. (a) Graphical display of promoter::*uidA* reporter constructs. The white arrow represents the pepper *Bs3* promoter. The UPT_{AvrBs3} and UPT_{PthXo1} boxes are displayed as black and hatched boxes, respectively. A gray box represents the *uidA* reporter gene, encoding the β-glucuronidase (GUS) protein. Letters below the boxes represent the nucleotides of the *Xa13* promoter that were inserted into the *Bs3* promoter. Bold italic letters represent the core UPT_{PthXo1} box of the *Xa13* promoter from rice cv IR24. A dash represents the deleted 5′ terminal T nucleotide of the mutated UPT_{PthXo1} (*Xa13*Δ*T*). Black letters on white background represent mutations with respect to the *Xa13* allele of the rice cv IR24. The G → T exchange that is present in several *xa13* alleles is displayed in lower case. (b) PthXo1 targets specifically the UPT_{PthXo1} but not the UPT_{AvrBs3} box. A fragment of the *Xa13* promoter containing the UPT_{PthXo1} box was placed into the context of pepper *Bs3* promoter (*Xa13* in *Bs3*). '*Xa13 in Bs3*' and derivatives with mutations in the UPT_{PthXo1} box were delivered into *Nicotiana benthamiana* leaves via *Agrobacterium tumefaciens* in combination with either an empty T-DNA vector (empty) or the *35S*-promoter-driven TALe genes *pthXo6*, *avrBs3* or *pthXo1*. (c) Mutations within the first and the second nucleotide of the UPT_{PthXo1} box significantly reduce the PthXo1-dependent inducibility. GUS activity (pmol 4-MU min^{-1} μg^{-1} protein) in *N. benthamiana* is taken as a measure of the PthXo1-dependent inducibility of the given promoter. GUS activity was determined 27 h after *A. tumefaciens*-mediated co-delivery of the depicted reporter constructs in combination with a *35S*-promoter-driven *pthXo1* gene. Error bars denote standard deviations.

nucleotides and tested the activity of these boxes in the context of the *Bs3* promoter (Fig. 4c). Quantitative GUS assays showed that G → A, G → C or G → T exchanges of the second box nucleotide resulted in significantly reduced PthXo1 inducibility in comparison to the nonmutated IR24 UPT_{PthXo1} box (Fig. 4c). Thus these experimental findings provide further support for the TALe code.

PthXo1 and PthXo6 bind in EMSA to matching *UPT* boxes

Previous studies have shown that the TALes AvrBs3, AvrBs3Δrep16 and AvrXa27 bind specifically to their matching *UPT* boxes (Römer *et al.*, 2009a,b). Here we carried out EMSA to clarify if PthXo1 and PthXo6 would also bind specifically to their matching *UPT* boxes. EMSA showed that a His::PthXo1 fusion protein binds to a biotin-labeled *Xa13* (cv IR24) promoter fragment containing the UPT_{PthXo1} box and, to a lesser extent, to the corresponding promoter region of the *xa13* allele (cv IRBB13) (Fig. 5). Importantly, binding of His::PthXo1 to biotin-labeled *Xa13* promoter fragments could be readily out-competed by nonlabeled *Xa13* promoter fragments, whereas even a 100-fold excess of nonlabeled *xa13* promoter fragments could not out-compete the binding (Fig. 5). Similarly, His::PthXo6 binds in EMSA to a biotin-labeled *OsTFX1* promoter fragment containing the UPT_{PthXo6} box and, to a much lesser extent, to a mutated *OsTFX1* promoter fragment (*OsTFX1*Δ*T*) that lacks the 5′ terminal T nucleotide of the UPT_{PthXo6} box (Fig. 6). Competition assays with biotin-labeled *OsTFX1*-derived promoter fragments and unlabeled *OsTFX1* and *OsTFX1*Δ*T* promoter fragments further confirmed that His::PthXo6 has high affinity to the UPT_{PthXo6} box and only a very low affinity to a UPT_{PthXo6} box mutant variant that lacks the 5′ terminal T nucleotide (Fig. 6). Together these findings indicate that PthXo1 and PthXo6 bind specifically to their matching *UPT* boxes.

Discussion

The TALe code and its limitations

We have demonstrated that the rice promoters *Xa13*, *OsTFX1* and *Os11N3* are activated by the *Xoo* TALes

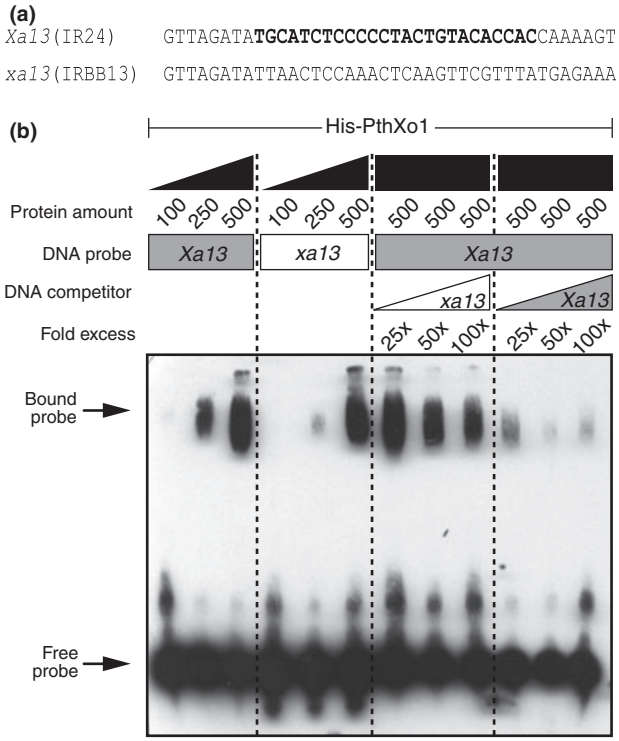

Fig. 5 The transcription activator-like effector (TALe) PthXo1 binds to the UPT_{PthXo1} box of the *Xa13* promoter. (a) Probes derived from *Xa13* (cv IR24) and *xa13* (cv IRBB13) promoters used in electrophoretic mobility shift assays (EMSAs). The predicted UPT_{PthXo1} box of the *Xa13* promoter is shown in bold letters. (b) PthXo1 binds with higher affinity to the *Xa13* promoter (gray boxes) than to the *xa13* promoter (white boxes). EMSA with PthXo1 and *Xa13*- or *xa13*-derived probes or competitor DNA. A molar excess of nonlabeled *Xa13* or *xa13* fragments of 25×, 50× and 100× were used for competition experiments. Protein amounts are in fmol. Positions of the bound and free probes are indicated.

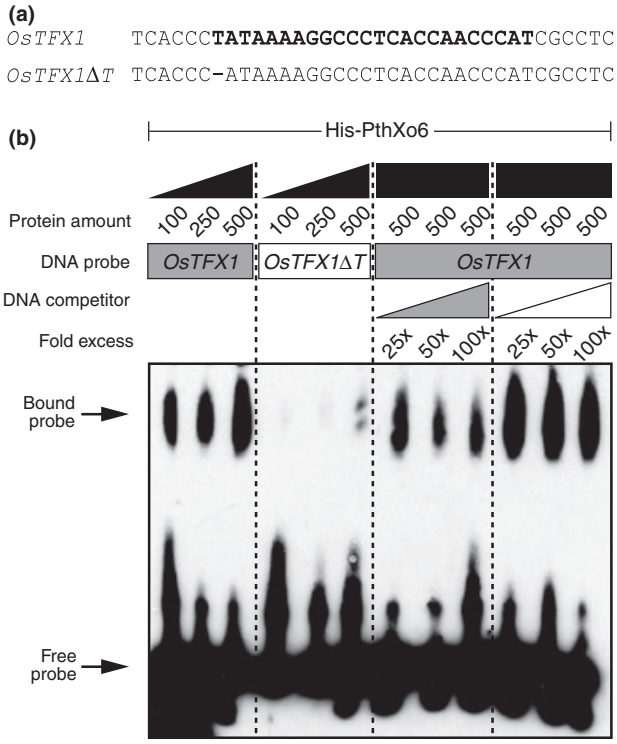

Fig. 6 The transcription activator-like effector (TALe) PthXo6 binds to the UPT_{PthXo6} box of the *OsTFX1* promoter. (a) Probes derived from the *OsTFX1* promoter and a mutant derivative (*OsTFX1ΔT*) were used in electrophoretic mobility shift assays (EMSAs). The predicted UPT_{PthXo6} box of the *OsTFX1* promoter is shown in bold letters. A dash '–' indicates a deletion. (b) A deletion of the first nucleotide of the UPT_{PthXo6} box strongly reduces its affinity to PthXo6. EMSA with PthXo6 and *OsTFX1*-derived probes or competitor DNA. A molar excess of nonlabeled *OsTFX1*- and *OsTFX1ΔT*-fragments of 25×, 50×, and 100× was used for competition experiments. Protein amounts are in fmol. Positions of the bound and free probes are indicated.

PthXo1, PthXo6 and AvrXa7, respectively (Fig. 1). Furthermore, we demonstrated that code-predicted *UPT* boxes are functional in the context of the pepper *Bs3* promoter (Figs 2–4) and that TALes interact physically with code-predicted *UPT* boxes (Figs 5, 6). Given that functional *UPT* boxes could be reliably predicted for promoters that are known to be activated by given TALes, the question arises whether functional *UPT* boxes can also be identified from sequenced host genomes. One obvious limitation of the current version of the TALe code is that RVDs with low frequency of occurrence in sequenced TALes (e.g. HI, SS, NQ, NC and NV) have not yet been deciphered, although their specificity should be readily determined. The major limitation of the TALe code, then, is the uncertainty of the functional consequences of mismatches between *UPT* box nucleotides and individual RVDs. In this context it needs to be noted that our previous study on the TALe code (Boch *et al.*, 2009) was focused on *in vitro* generated *UPT* boxes that show no or very few mismatches with respect to the given TALe. By contrast, all identified natural *UPT* boxes in plant promoters and their matching TALes contain many mismatches. For example, the TALe PthXo1 contains three NI-type RVDs that do not match the code-predicted A in the UPT_{PthXo1} box of the PthXo1-inducible *Xa13* promoter (see PthXo1 repeat units 10 (NI → C), 19 (NI → C) and 21 (NI → C); Fig. S2). Whereas some mismatches have little effect on the magnitude of transcription activation, other mismatches have proved to be critical to TALe-mediated promoter activation. One striking example is the PthXo1-inducible *Xa13* gene from the rice cv IR24 and the allelic, non PthXo1-inducible *xa13* gene from the rice cv Tepa1. These *Xa13/xa13* alleles differ only in a G → T substitution of the second nucleotide of the UPT_{PthXo1} box which pairs to a NN-type repeat (Figs S2, S4). Reverse transcription polymerase chain reaction (RT-PCR) analysis of rice leaf tissue that was infected with a *pthXo1*-expressing *Xoo* strain revealed transcriptional activation of the IR24 *Xa13* but not the Tepa1 *xa13* allele (Chu *et al.*, 2006). Similarly, agroinfiltration assays revealed a sig-

nificantly reduced PthXo1-mediated transcriptional activation of the Tepa1 *xa13* allele as compared with induction of the IR24 *Xa13* allele (Fig. 4c; the Tepa1 *xa13* allele corresponds to '*Xa13* mut T in *Bs3*'). This strong effect of a single mismatched NN-type repeat is somewhat unexpected considering that the UPT_{PthXo1} box of the IR24 *Xa13* promoter, which mediates PthXo1-mediated promoter activation, contains seven mismatches compared with the code-predicted UPT_{PthXo1} box (Fig. S2). Thus it seems that correct pairing of the second (NN-type) repeat of PthXo1 is crucial in the context of the PthXo1–*UPT* box interaction than correct pairing of other RVDs.

We postulate that the sum of RVDs that pair to code-predicted nucleotides determines the overall affinity of a TALe to a given *UPT* box, with a minimum number of matching RVDs required to promote TALe-mediated transcriptional activation. This hypothesis is supported by the observation that longer TALes appear to tolerate more mismatches than shorter TALes. For example, AvrXa7 (26 repeat units) and PthXo1 (24 repeats units) transcriptionally activate the rice *Os11N3* and *Xa13* promoter despite the fact that there are eight and seven mismatches in the corresponding UPT_{AvrXa7} (*Os11N3* promoter) and UPT_{PthXo1} (IR24 *Xa13* promoter) boxes, respectively (Fig. S2). By contrast, the *UPT* boxes that are targeted by the shorter TALes AvrHah1 (14 repeats units; activates *Bs3* promoter) (Schornack *et al.*, 2008) and AvrBs3Δrep16 (14 repeats units; activates *Bs3-E* promoter) (Römer *et al.*, 2007, 2009b; Boch *et al.*, 2009; Moscou & Bogdanove, 2009) each contains a single mismatch as compared with the code-predicted *UPT* boxes.

Although longer TALes seem to target *UPT* boxes with multiple mismatches, it is conceivable that longer TALes also require a minimum number of RVDs that pair to matching nucleotides in order to promote transcriptional activation. Given that the UPT_{PthXo1} box from the IR24 *Xa13* promoter contains seven mismatches as compared with the code-predicted UPT_{PthXo1} box (Fig. S2), one might speculate that any additional mismatch will result in reduced inducibility of the given box. Thus the reduced inducibility of the Tepa1 *xa13* allele (G → T substitution of the second nucleotide of the UPT_{PthXo1} box) might be a consequence of the reduced overall affinity of PthXo1 to the Tepa1 *xa13* allele and does not necessarily imply that correct pairing of this particular RVD is crucial to the TALe–*UPT* box interaction.

In summary, TALes target not only code-predicted *UPT* boxes but also closely related boxes. However, the functional consequences of mismatches between *UPT* box nucleotides and corresponding RVDs remain, to some extent, unpredictable. It remains to be clarified if all RVDs make an equal contribution to the TALe–DNA interaction or if certain RVDs are of particular importance. Obviously a crystal structure of a TALe and its matching *UPT* box will help to give further insights into the molecular basis of this interaction.

The 5′ terminal T of the *UPT* boxes is crucial to transcriptional activation by, and interaction with, its matching TALe

Previous studies uncovered that all functional *UPT* boxes contain a conserved, invariant 5′ terminal T nucleotide (Boch *et al.*, 2009; Moscou & Bogdanove, 2009). Mutational studies of the conserved T in the *UPT* boxes of the TALes AvrBs3 and Hax3 resulted in reduced induction of the corresponding promoter mutant derivatives as compared with the promoters containing the conserved T nucleotide (Boch *et al.*, 2009; Römer *et al.*, 2009b). Analogously, our studies showed that a mutation in the conserved 5′ terminal T nucleotide of the PthXo1, PthXo6 and AvrXa7 *UPT* boxes also resulted in reduced inducibility of the corresponding rice *Xa13*, *OsTFX1* and *Os11N3* promoters (Figs 2c, 3c, 4c). Thus the functional relevance of the conserved 5′ terminal T nucleotide has by now been confirmed for five different TALes, suggesting that the invariant T is crucial to the function of most, or possibly all, *UPT* boxes.

Previous EMSAs on the TALe AvrBs3Δrep16 suggested that the 5′ terminal T nucleotide of the corresponding pepper *Bs3-E* promoter $UPT_{AvrBs3\Delta rep16}$ box makes a significant contribution to the TALe–DNA interaction (Römer *et al.*, 2007, 2009b). However, an EMSA-based comparison of identical DNA fragments that contain or lack the conserved 5′ terminal T nucleotide of a *UPT* box had not yet been carried out. We compared by EMSA the affinities of the wild-type UPT_{PthXo6} box from the rice *OsTFX1* promoter and a corresponding mutant box lacking the conserved T nucleotide (*OsTFX1*Δ*T*), and found a drastically reduced interaction between PthXo6 and the mutant box as compared with the wild-type UPT_{PthXo6} box (Fig. 6). These findings demonstrate that the 5′ terminal T nucleotide of the UPT_{PthXo6} box is crucial to physical interaction between PthXo6 and the UPT_{PthXo6} box. Given that similar findings have been observed for the TALe AvrBs3Δrep16 (Römer *et al.*, 2007, 2009b), it seems likely that, in general, the 5′ terminal T nucleotide of a *UPT* box is crucial to its physical interaction with a corresponding TALe. Future studies will have to clarify which TALe residues pair to the conserved T. Once this question is resolved, we may be able to modify TALes in such a way that pairing to nucleotides other than a 5′ terminal T is possible.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Nucleotide sequence of the promoter fragments that were amplified from genomic DNA to analyze recognition specificity.

Fig. S2 Alignment of the predicted and naturally occurring *UPT* (up-regulated by transcription activator-like effectors) boxes in the different rice promoters.

Fig. S3 FASTA files of rice *Xa13* and *xa13* alleles from different rice genotypes.

Fig. S4 Alignment of the *Xa13/xa13* promoters.

Table S1 Amino acids of repeat unit residues 12 and 13 and predicted target DNA specificities.

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