The last half-repeat of transcription activator-like effector (TALE) is dispensable and thereby TALE-based technology can be simplified

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SUMMARY

To activate the expression of host genes that contribute to pathogen growth, pathogenic *Xanthomonas* bacteria inject their transcription activator-like effectors (TALEs) into plant cells and the TALEs bind to target gene promoters by the central repeat region consisting of near-perfect 34-amino-acid repeats (34-aa repeats). Based on the recognition codes between the 34-aa repeats and the targeted nucleotides, TALE-based technologies, such as designer TALEs (dTALEs) and TALE nucleases (TALENs), have been developed. Amazingly, every natural TALE invariantly has a truncated last half-repeat (LHR) at the end of the 34-aa repeats. Consequently, all the reported dTALEs and TALENs also harbour their LHRs. Here, we show that the LHRs in dTALEs are dispensable for the function of gene activation by both transient expression assays in *Nicotiana benthamiana* and gene-specific targeting in the rice genome, indicating that TALEs might originate from a single progenitor. In the light of this finding, we demonstrate that dTALEs can be constructed through two simple steps. Moreover, the activation strengths of dTALEs lacking the LHR are comparable with those of dTALEs harbouring the LHR. Our results provide new insights into the origin of natural TALEs, and will facilitate the simplification of the design and assembly of TALE-based tools, such as dTALEs and TALENs, in the near future.

Keywords: dTALE, last half-repeat, TAL effector, TALENs, unit assembly.

INTRODUCTION

Transcription activator-like effectors (TALEs) are a class of proteins identified in many plant-pathogenic *Xanthomonas* spp. (Boch and Bonas, 2010; Bonas *et al.*, 1989), including *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal agent of bacterial blight, which is a disease devastating rice production worldwide (Hopkins *et al.*,

1992). The pathogens inject their TALEs through the type III secretion system (TTSS) into plant cells primarily to activate the expression of host genes that contribute to pathogen growth (Boch and Bonas, 2010; Yang *et al.*, 2006). Natural TALEs have a conserved architecture: an N-terminus required for TTSS secretion, a central repeat region consisting of a varying number of near-perfect 34-amino-acid repeats (34-aa repeats) and a C-terminus containing nuclear localization signals (NLSs) and an acidic transcription activation domain (AD) (Boch and Bonas, 2010). TALEs activate host gene expression by their binding to promoters of targeted genes. The number and order of the 34-aa repeats determine the specificity between a TALE and its target DNA sequence (Boch and Bonas, 2010). Amino acid polymorphism among the 34-aa repeats exists and is concentrated at residues 12 and 13 of each repeat, referred to as the repeat-variable diresidue (RVD), which mediates specific nucleotide recognition (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). The recognition codes between RVDs of TALEs and the nucleotides of the target sites have been deciphered, in which RVDs NI, HD, NG and NN prevalently recognize nucleotides 'A', 'C', 'T' and 'G', respectively (Boch *et al.*, 2009; Moscou and Bogdanove, 2009).

Based on the DNA recognition codes of natural TALEs, the central repeat region or DNA-binding domain of a TALE can be designed and modularly assembled with RVD-defined 34-aa repeats for precise and predicted gene activation (Morbitzer *et al.*, 2010). Moreover, chimeric proteins called TALE nucleases (TALENs) have been developed by replacing the AD of a TALE with the nonspecific DNA nuclease FokI for site-specific genome editing (Christian *et al.*, 2010; Li *et al.*, 2011). As DNA-binding domains targeting virtually any user-selected DNA sequence in a genome can be designed and modularly assembled, custom designer TALEs (dTALEs) and TALENs have rapidly emerged as powerful tools for precise gene activation (Li *et al.*, 2013; Mak *et al.*, 2013; Richter and Boch, 2013) and genome editing (Li *et al.*, 2012; Miller *et al.*, 2011), respectively. Site-specific genome editing has been demonstrated in various organisms, including yeast, insects, plants, animals and human cell lines (Joung and Sander, 2013).

Mysteriously, the last repeats in all natural TALEs are invariantly truncated with only amino acids 19–20, which are termed the last half-repeat (LHR) (Boch and Bonas, 2010). This may imply either a

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single origin in evolution or a necessity for biological functions of TALEs, but, as yet, no supporting experimental evidence is available. Owing to the presence of the LHR in all natural TALEs, each of the reported dTALEs and TALENs harbours an LHR at the ends of assembled DNA-binding domains. The LHRs in dTALEs and TALENs are achieved by either four versions of pre-synthesized LHRs (with RVDs recognizing 'A', 'C', 'T' or 'G') in the assembly of the repeat arrays (RAs) (Cermak *et al.*, 2011) or four versions of backbone vectors with pre-embedded LHRs (Miller *et al.*, 2011; Reyon *et al.*, 2012; Zhang *et al.*, 2011). This increases the complexity and costs in the construction of dTALEs and TALENs. Here, we report that the LHRs in dTALEs are dispensable for their functions in gene activation, which provides significantly important insights into the origin of natural TALEs. In the light of this finding, we demonstrate that the assembly of a dTALE can be simplified.

RESULTS

dTALEs lacking the LHR activate β -glucuronidase (GUS) expression in *Nicotiana benthamiana*

We first tested whether a dTALE lacking the LHR had gene activation function by transient expression assays in *N. benthamiana* leaves, using GUS as a reporter. Six dTALEs were designed and assembled (Fig. 1a). dTE1 and dTE2 are the wild-type dTALEs with the LHR, used as positive controls, dTE1-0 and dTE2-0 are dTALEs in which the LHR was deleted, and the LHRs in dTE1-1 and dTE2-1 were replaced with the corresponding full 34-aa repeats (Fig. 1a). dTE1, dTE1-0 and dTE1-1 target the effector-binding element (EBE) EBE1 (5'-TCTCGTGCTTCAGCTC-3') and the other three dTALEs target EBE2 (5'-TATTACCCGACGACAT-3') (Fig. 1b). EBE1 and EBE2 were chosen from the hygromycin resistance gene *HptII* (GenBank: AF354045.1).

Agrobacterium strains harbouring the dTALE-expressing constructs and GUS reporter constructs (Fig. 1b) were infiltrated separately or in combination into *N. benthamiana* leaves. Transient expression assays showed that the LHR-lacking dTALEs dTE1-0 and dTE1-1 (Fig. 1a) strongly activated the expression of the *GUS* gene when they were co-infiltrated with the GUS reporter construct Rep1, as did the positive control dTE1 (Fig. 1c: 1–3). Meanwhile, a GUS staining signal was undetectable for Rep1 or dTE1-0 alone (Fig. 1c: 4, 8), indicating that there was no GUS expression leaking in Rep1 and no GUS expression background in the *Agrobacterium* strains. Notably, dTE1, dTE1-0 and dTE1-1 did not activate the expression of the *GUS* gene in Rep2 (Fig. 1c: 9–11), indicating that they specifically bound to EBE1, but not to EBE2 (Fig. 1b). In contrast, the LHR-lacking dTALEs dTE2-0 and dTE2-1 specifically activated the expression of the *GUS* gene in Rep2 (Fig. 1c: 14–15), but not in Rep1 (Fig. 1c: 6–7), further indicating specific binding of the dTALEs to their EBEs. Furthermore, the activation strengths of the LHR-lacking dTALEs were similar to that

of the positive controls targeting the same EBE (Fig. 1c: 1–3 or 13–15). This was further confirmed by GUS quantitative assays (Fig. 1d).

The aforementioned results clearly show that LHR-lacking dTALEs specifically bind to their corresponding EBEs and activate the expression of their target genes (Fig. 1c). In other words, the LHR of a TALE is dispensable for its function in gene activation.

LHR-lacking dTALEs specifically activate *xa27* expression in the rice genome and trigger resistance to bacterial blight

Xa27 is a representative of so-called executor-type *R* genes, which function on activation by cognate TALEs from pathogens (Gu *et al.*, 2005; Strauß *et al.*, 2012). The resistant allele (*Xa27*) in rice line IRBB27 and the susceptible allele (*xa27*) in IR24 contain identical coding sequences, but only the resistant allele expresses *Xa27* on infection by *Xoo* strains expressing AvrXa27, a natural TALE protein (Gu *et al.*, 2005). Expression of *Xa27* depends on the interaction between its promoter and AvrXa27. The *xa27* allele is unresponsive to natural AvrXa27 in gene induction owing to the lack of the EBE of AvrXa27 in the *xa27* promoter. Recently, it has been shown that the *xa27* allele can be activated and consequently triggers resistance to the bacterium expressing a corresponding dTALE (Li *et al.*, 2013). Therefore, we assembled dTE3 and dTE3-0 based on the 24-bp EBE3 (Fig. 2a), located within the promoter of the *xa27* allele in rice IR24, to further confirm the gene activation function of dTALEs lacking the LHR. dTE3, as a positive control, harbours an LHR, whereas dTE3-0 lacks the LHR (Fig. 2b). The wild-type *Xoo* strain PXO99^A and its transformants harbouring constructs expressing dTE3 or dTE3-0 were used for inoculation in leaves of PXO99^A-susceptible IR24. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) showed that both LHR-harboring dTE3 and LHR-lacking dTE3-0 strongly activated the expression of the susceptible allele *xa27*, but the wild-type PXO99^A and water (mock) inoculation did not (Fig. 2c). dTE3-0 induced more than 12 times greater transcription than the wild-type PXO99^A (Fig. 2c). With regard to the disease phenotype, wild-type PXO99^A caused a serious susceptible reaction in IR24, consistent with previous studies (Gu *et al.*, 2005), but strains expressing dTE3 or dTE3-0 elicited a typical hypersensitive response [Figs 2d and S3 (Supporting Information)]. The presence of the dTALEs in PXO99^A also restricted the bacterial growth in IR24 leaves by about 50–60-fold of population per inoculation site compared with the wild-type PXO99^A (Fig. 3). These observations indicate that LHR-lacking dTE3-0 indeed activates the expression of the susceptible allele *xa27*, and results in bacterial blight resistance in rice IR24 (Fig. 2d). More importantly, the activation strength of dTE3-0 is comparable with that of dTE3 (Fig. 2c,d), again indicating that the deletion of LHR in a dTALE does not affect its gene activation function.

Fig. 1 Design of designer transcription activator-like effectors (dTALs) and activation assays in transient expression systems. (a) The architecture of dTALs targeting effector-binding element 1 (EBE1) and EBE2. dTAL1 was taken as an example to show the full architecture. Grey boxes represent the N- and C-termini. Green, red, yellow and blue ovals represent repeats with repeat-variable diresidues (RVDs) NI, HD, NG and NN, respectively. The red triangle represents the acidic activation domain (AD). Black bars represent the nuclear localization signals (NLSs). Amino acid numbers in different regions are indicated. The amino acids of the last repeat in different dTALs are particularly listed. RVDs NI, HD, NG and NN recognize nucleotides 'A', 'C', 'T' and 'G', respectively. (b) Schematic representations of the β -glucuronidase (GUS) reporters Rep1 and Rep2. The minimal (99-bp) *BjChi1* promoter (grey arrow) (Wu *et al.*, 2009) was preceded with the EBE1 (yellow) or EBE2 (light blue) sequences and cloned upstream and in frame with the *uidA* (GUS) gene (blue arrows) in binary vector pCambia1305. (c) Activation assay of the dTALs. *Agrobacterium* strains delivering dTAL-expressing constructs and the GUS reporter constructs were individually or co-infiltrated into *Nicotiana benthamiana* leaves. The infiltrated leaves were collected 48 h post-infiltration (hpi), stained with X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) and finally cleared in ethanol. (d) Quantitative assay of GUS activity induced by dTALs. *Agrobacterium* strains delivering dTAL-expressing constructs, *CaMV* 35S::LUCint construct (Wu *et al.*, 2009) and the GUS reporter constructs were co-infiltrated into *N. benthamiana* leaves. The infiltrated leaves were collected at 48 hpi and used for GUS quantitative assay performed following our previously described method (Gao *et al.*, 2014). The GUS reporter constructs alone were used as the negative controls. Values are the mean $\pm$ SD ($n = 3$).

The assembly of dTALs is simplified into two steps

It is complex to assemble dTALs as shown in Fig. 5, and this is mainly caused by the preparation of different versions of the repeat array receptor (RAR) plasmids to achieve the LHR. In the light of the aforementioned finding that the LHR is dispensable, we designed and constructed a universal RAR plasmid pSK-RAR-U (Methods S1, see Supporting Information) that can accept any custom RA assembled by the unit assembly method (Huang *et al.*, 2011). Consequently, dTALs can be constructed through two simple steps: the assembly of the RA and the cloning of the RA into the universal RAR plasmid pSK-RAR-U (Fig. S4, see Supporting

Information). Using this strategy, we cloned the RAs TN-KJ1-1 and TN-KJ2-1 into the universal RAR plasmid pSK-RAR-U, resulting in dTALs dTAL1-0Plus and dTAL2-0Plus (Table 1). Activation assays in *N. benthamiana* showed that dTAL1-0Plus and dTAL2-0Plus functioned well and were comparable in gene activation strength with dTAL1 and dTAL2, respectively (Fig. 4).

DISCUSSION

One of the conserved structural features of natural TALs is the retained LHR at the end of the central 34-aa repeats in every TAL protein. This may be explained by the fact that the LHR is vital for

Fig. 2 Designer transcription activator-like effectors (dTALs) activate the expression of susceptible allele *xa27* and induce disease resistance in rice. (a) The 24-bp nucleotide sequence within the promoter of the *xa27* gene was chosen as the effector-binding element 3 (EBE3) (boxed) for the design of dTE3 and dTE3-0. The numbers above the sequence indicate the nucleotide positions related to the translation start codon of *xa27*. (b) The architectures of dTE3 [with the last half-repeat (LHR)] and dTE3-0 (without the LHR). Amino acid (aa) numbers in the N- and C-terminal regions (grey boxes), the 34-aa repeat region [green, red, yellow and blue ovals represent repeats with repeat-variable diresidues (RVDs) NI, HD, NG and NN, respectively] and the acidic activation domain (AD, red triangle) are indicated. NLS, nuclear localization signals (black bars). The amino acids of the last repeats in the two dTALs are particularly listed. (c) Gene induction of *xa27* in IR24. RNA from leaves treated with water, PXO99^A and dTE3- or dTE3-0-containing PXO99^A was used for quantitative reverse transcription-polymerase chain reaction (RT-PCR) with gene-specific primers. Rice ubiquitin gene was used as an internal control. (d) Disease reactions of IR24 to PXO99^A and dTE3- or dTE3-0-containing PXO99^A strains. Bacterial suspensions were inoculated into rice leaves using the leaf clipping method. Photographs were taken at 14 days post-inoculation.

Fig. 3 Growth ability of PXO99^A, dTE3- and dTE3-0-containing PXO99^A strains infiltrated in IR24 leaves. Populations were expressed as colony forming units (cfu) per inoculation spot at 1–4 days post-inoculation. Significant differences detected between PXO99^A and dTE3 or dTE3-0 are indicated by *** $P < 0.01$. Bars indicate standard deviation (SD).

the function of TALEs by mediating the correct structure of the protein, but, as yet, no experimental evidence is available. In the present study, we assembled LHR-lacking dTALs (Fig. 5) to test the necessity of the LHR. The transient expression assays with four dTALs lacking the LHR revealed that the LHRs in the dTALs were dispensable for gene activation (Fig. 1). We further confirmed this finding using LHR-lacking dTE3-0 which targets the susceptible allele (*xa27*) in the rice genome (Fig. 2a,b). In the experiments, both dTE3-0 and the positive control dTE3 (harbouring the LHR) strongly activated the expression of *xa27* and, consequently, induced bacterial blight resistance in rice (Fig. 2c,d). As the presence of dTALs did not affect the growth of *Xoo* strain PXO99^A (Fig. S5, see Supporting Information), the bacterial blight resist-

Table 1 The relationships among repeat array (RA), repeat array receptor (RAR) and designer transcription activator-like effector (dTAL)-expressing plasmids.

RA plasmid	RAR plasmid	dTAL-expressing plasmids
pTN-KJ1-1	pSK-RAR-C	pdTE1
pTN-KJ1	pSK-RAR-OT	pdTE1-0
pTN-KJ1-1	pSK-RAR-OC	pdTE1-1
pTN-KJ2-1	pSK-RAR-T	pdTE2
pTN-KJ2	pSK-RAR-0A	pdTE2-0
pTN-KJ2-1	pSK-RAR-OT	pdTE2-1
pTN-KJ24-1	pSK-RAR-G	pdTE3
pTN-KJ24	pSK-RAR-0A	pdTE3-0
pTN-KJ1-1	pSK-RAR-U	pdTE1-0Plus
pTN-KJ2-1	pSK-RAR-U	pdTE2-0Plus

ance phenotypes were indeed triggered by the dTALs. Furthermore, both transient expression assays in *N. benthamiana* leaves and site-specific targeting in the rice genome showed comparable activation strengths of the LHR-lacking dTALs with those of the corresponding LHR-harboring dTALs (Figs 1c,d and 2c,d). These results unambiguously indicate that the LHR in a TALE is dispensable.

The species *Xoo* mainly comprises two distinct groups, causing economically significant diseases of rice in Asia and Africa, respectively (Gonzalez *et al.*, 2007). It is well known that multiple TALE homologues exist in *Xoo* strains from Asia (Hopkins *et al.*, 1992). African *Xoo* strains belong to another genetic group because they have smaller numbers of TALEs (Gonzalez *et al.*, 2007). Intriguingly, no TALE homologues have been identified in American *Xoo* strains (Triplett *et al.*, 2011), forming a group different from

Fig. 4 Activation assay of dTE1-0Plus (a) and dTE2-0Plus (b). *Agrobacterium* strains delivering designer transcription activator-like effector (dTALE)-expressing constructs and the β -glucuronidase (GUS) reporter constructs were individually or co-infiltrated into *Nicotiana benthamiana* leaves as indicated. The infiltrated leaves were collected at 48 h post-infiltration (hpi), stained with X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) and finally cleared in ethanol. The *Agrobacterium* strain (EHA105) and the pCambia1305 vector (pC1305) were used as negative and positive controls, respectively.

the Asian and African strains. The distribution pattern of TALEs within these species implies that TALE genes first appeared in some strains during the geographical spread of *Xoo* strains.

However, although TALEs exist predominantly in *Xanthomonas*, homologues of TALE genes have also been identified in *Ralstonia solanacearum* (Cunnac *et al.*, 2004; Mukaihara *et al.*, 2004; Salanoubat *et al.*, 2002). *Brg11* is the first member of TALE genes identified in *R. solanacearum* (Cunnac *et al.*, 2004). *Brg11* contains 17.5 35-aa repeats, instead of the typical 34-aa repeats of TALEs in *Xanthomonas* (Cunnac *et al.*, 2004). Moreover, the first half of each *Brg11* amino acid repeat is identical to the AvrBs3 type, whereas the second half is not (Cunnac *et al.*, 2004). Hpx17, a TALE identified in another *R. solanacearum* strain, contains only 2.5 35-aa repeats (Mukaihara *et al.*, 2004). Sequence analysis revealed that Hpx17 evolved from *Brg11* by a deletion of 15 35-aa repeats (Mukaihara *et al.*, 2004). It has been proposed that TALE genes in *R. solanacearum* possibly originated from a horizontal gene transfer event, because the TALE genes are preferentially distributed among *R. solanacearum* strains of the Asiaticum, but not Americanum division (Heuer *et al.*, 2007). Based on the fact that some strains in *X. campestris* pv. *vesicatoria* (*Xcv*) lack *avrBs3* homologues, Boch and Bonas (2010) also proposed that TALE genes evolved by horizontal gene transfer of mobile cassettes. Direct evidence supporting the horizontal transfer of TALE genes comes from *pthB* in *X. citri* pv. *citri*, in which the plasmid-residing *pthB* gene transferred horizontally from pathogenic *X. citri* pv. *citri* strain B69 into a nonpathogenic strain, making the latter acquire pathogenicity causing citrus canker disease (El Yacoubi *et al.*, 2007). Taking together the possibility of TALE gene horizontal transfer and our findings in this study, we propose that the LHR probably originated from a single progenitor that harboured an LHR. The ancestral TALE gene might have been inherited in two pathways: one involving vertical inheritance from generation to generation, in which gene duplication, recombination and translocation events happened within the genome, resulting in multiple

copies in a single genome of some strains; another involving the horizontal transfer of the ancestral TALE gene and its derivatives from one strain to another, and from one species to another.

The dTALEs and TALENs reported so far harbour LHRs. In the construction of these dTALEs and TALENs, four versions of pre-synthesized LHRs (Cermak *et al.*, 2011) or four backbone vectors with LHRs pre-embedded (Miller *et al.*, 2011; Reyon *et al.*, 2012; Zhang *et al.*, 2011) are generally prepared. This increases the complexity and costs of the construction of dTALEs and TALENs, as shown in Fig. 5. Our finding that the LHR of a TALE is dispensable should have a direct impact on the design and assembly of dTALEs and TALENs. As demonstrated in this study by dTE1-0Plus and dTE2-0Plus (Fig. 4), dTALEs can be constructed via two simple steps using the universal RAR plasmid pSK-RAR-U (Fig. S4). Our results and technical advances will simplify the design and assembly of other TALE-based tools, such as TALENs, in the future.

EXPERIMENTAL PROCEDURES

Plant materials, bacterial strains and growth conditions

Nicotiana benthamiana plants were grown in a light incubator under standard conditions (day and night temperatures of 25 and 20 °C, respectively), with 16 h of light (30–40 μ mol/m²) and 50%–60% humidity. Rice (*Oryza sativa* L.) variety IR24 plants were grown in a glasshouse at a temperature of 26–30 °C, relative humidity of 60%–70% and photoperiod of 12 h.

Escherichia coli DH5 α and *Agrobacterium tumefaciens* EHA105 were grown in Luria–Bertani (LB) medium (tryptone, 15 g/L; yeast extract, 5 g/L; sodium chloride, 5 g/L; pH 6.8–7.0) at 37 °C and 28 °C, respectively. The wild-type *Xoo* strain PX099^A and its transformants expressing dTALEs were grown on PSA medium (polypeptone, 10 g/L; sucrose, 10 g/L; glutamic acid, 1 g/L) at 28 °C. Antibiotics used in this study were: ampicillin (Amp), 100 μ g/mL; kanamycin (Km), 50 μ g/mL; spectinomycin (Spe), 100 μ g/mL; rifampicin (Rf), 50 μ g/mL.

Fig. 5 Procedures for the construction of designer transcription activator-like effector (dTALE)-expressing plasmids. Starting with *avrXa23*-containing plasmid pSK-*avrXa23*, the intermediate plasmid pSK-*avrXa23*ΔSphF-XH was prepared in step (1), and six repeat array receptor (RAR) plasmids (pSK-RAR-C, pSK-RAR-G, pSK-RAR-T, pSK-RAR-0A, pSK-RAR-0C and pSK-RAR-0T) were constructed in steps (2) and (3). In step (4), starting with the single repeat unit vectors pA, pT, pC and pG, six repeat arrays (RAs) in the RA plasmids pTN-KJ1, pTN-KJ1-1, pTN-KJ2, pTN-KJ2-1, pTN-KJ24 and pTN-KJ24-1 were assembled by the 'unit assembly' method. In steps (5) and (6), RAs were cloned into the RAR plasmids and the dTALE-expressing plasmids were constructed. LHR, last half-repeat; PCR, polymerase chain reaction;

Construction of dTALE-expressing plasmids

We exploited *AvrXa23* (GenBank: GU732172.1), a natural TALE from *Xoo*, as the scaffold of dTALEs. The major procedures for the construction of the dTALE-expressing plasmids are shown in Fig. 5.

First, DNA of *avrXa23*-containing plasmid pSK-*avrXa23* (Wang *et al.*, 2013) was digested with *SphI* and subsequently ligated, resulting in plasmid pSK-*avrXa23*ΔSphF (*avrXa23* without the *SphI* fragment). The jointed region encoding the N- and C-termini of *AvrXa23* was amplified by PCR with the primers *avrXa23F6* and *avrXa23R6* (Table S1, see Supporting Information), through which *XbaI* and *HindIII* restriction sites were

introduced into the PCR product at the 5' and 3' ends, respectively. The amplified DNA fragment was then recombined into the *SmaI*-linearized vector pBluescript II SK through Jinsite's proprietary CloneEZ® Enzyme PCR Cloning Kit (GenScript Corporation, Middlesex County, NJ, USA), resulting in plasmid pSK-*avrXa23*ΔSphF-XH [Fig. 5, (1)].

Second, overlapping PCRs were performed using the pSK-*avrXa23* plasmid as template to join the N- and C-termini-encoding regions with different LHR-encoding sequences with an incorporated *NheI* restriction site (Methods S1 and Fig. S1, see Supporting Information). The overlapping PCR products were then cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China), resulting in plasmids designated as pEASY-SNSs.

Then, the *SphI* fragments in pEASY-SNSs were released by *SphI* digestion [Fig. 5, (2)] and cloned into pSK-avrXa23ΔSphF-XH, resulting in the RAR plasmids [Fig. 5, (3); Methods S1 and Fig. S1].

Third, the four starting vectors (pA, pC, pG and pT) containing single alternative units, described by Huang *et al.* (2011), were synthesized by CWBIO (<http://www.cwbiotech.com/en/>). The RAs in plasmids pTN-KJ1, pTN-KJ1-1, pTN-KJ2, pTN-KJ2-1, pTN-KJ24 and pTN-KJ24-1 for construction of dTALEs were assembled via the 'unit assembly' method (Huang *et al.*, 2011) [Fig. 5, (4)]. The basic structures of these plasmids are shown in Fig. S2 (see Supporting Information).

During the construction of dTALE-expressing plasmid pdTE1-0, the *SpeI*-*NheI* fragments in RA plasmid pTN-KJ1 (Fig. S2) were isolated and cloned into RAR plasmid pSK-RAR-OT at the *NheI* site, resulting in pdTE1-0. With the same procedures, other dTALE-expressing plasmids were constructed [Fig. 5, (5) and (6)]. The relationships among the RA plasmids, RAR plasmids and dTALE-expressing plasmids are listed in Table 1. All the dTALE-expressing plasmids were verified by sequencing (Sequences S1, see Supporting Information).

Finally, the *XbaI*-*HindIII* fragments of dTALE-expressing plasmids pdTE1, pdTE1-0, pdTE1-1, pdTE2, pdTE2-0 and pdTE2-1 (Fig. 5, Table 1) were released by *XbaI*-*HindIII* digestion and cloned into pCambia1305, in which the *GUS* gene had been deleted. The resulting dTALE-expressing constructs pCambia1305-dTE1, pCambia1305-dTE1-0, pCambia1305-dTE1-1, pCambia1305-dTE2, pCambia1305-dTE2-0 and pCambia1305-dTE2-1 were then individually transformed into *A. tumefaciens* strain EHA105 for transient expression assays in *N. benthamiana* leaves. The dTALE-expressing plasmids pdTE3 and pdTE3-0 (Fig. 5, Table 1) were linearized by *HindIII* digestion and individually cloned into the broad host range vector pHM1 (Hopkins *et al.*, 1992), resulting in dTALE-expressing constructs pHM1-dTE3 and pHM1-dTE3-0, which were then transformed into *Xoo* strain PXO99^A for inoculation into rice leaves.

Construction of reporter vectors Rep1 and Rep2

The *uidA* gene was used to report the activation activity of dTALEs targeting EBE1 and EBE2. The minimal BjC-P promoter (−99 to +17 relative to the transcription start site) (Wu *et al.*, 2009) was amplified by PCR with the primer pair URP1/URP2 (Table S1) and the PCR fragment was cloned into the pEASY-Blunt vector (TransGen Biotech), resulting in intermediate vector pB-KJ1URP. It should be noted that the target site EBE1 of dTE1 was incorporated at 5' upstream of the minimal BjC-P promoter in the PCR fragment (Fig. 1b, Table S1). The PCR fragment was confirmed by sequencing, isolated from pB-KJ1URP by digestion with *HindIII* and *NcoI*, and finally cloned into pCambia1305, resulting in the *GUS* reporter construct Rep1 (Fig. 1b). Using the same procedures, we constructed Rep2, for which the primer pair URP3/URP2 (Table S1) was used to amplify the minimal BjC-P promoter with the incorporated target site EBE2 (Fig. 1b). The two reporter constructs were individually transformed into *A. tumefaciens* strain EHA105 for transient expression assays in *N. benthamiana* leaves.

GUS staining and GUS quantitative assay

Bacterial cells of *A. tumefaciens* strains harbouring dTALE-expressing constructs or the *GUS* reporter constructs were suspended in MMA solution [10 mM MgCl₂, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (pH 5.5),

100 μM acetosyringone] at an optical density of 1.0 at 600 nm (OD₆₀₀ = 1.0), and individually or co-infiltrated into *N. benthamiana* leaves by a needleless syringe. Leaves were sampled at 2 days post-infiltration (dpi), incubated in X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) staining solution [3 mM X-Gluc, 0.1 M phosphate buffer sodium (pH 7.0), 0.1% Triton X-100, 8 mM β-mercaptoethanol], destained in ethanol and dried. Experiments were performed at least three times. GUS quantitative assays were performed following our previously described method (Gao *et al.*, 2014).

Pathogenicity assessment of *Xoo* strains in rice leaves

Xoo strains were cultured in PSA medium at 28 °C for 48 h. Bacterial suspensions (OD₆₀₀ = 1.0) were used for inoculation on fully expanded rice leaves at the seedling stage. The pathogenicities of *Xoo* strain PXO99^A and its transformants expressing dTALEs were evaluated using the leaf tip clipping method. Each strain was inoculated on five plants and two fully expanded leaves per plant. Disease symptoms were recorded 2 weeks after inoculation by lesion length (centimetre).

For bacterial population counting, *Xoo* strains were inoculated onto IR24 plants using the syringe infiltration method. Three plants were inoculated for each of the *Xoo* strains, and three infiltrated leaf discs from different leaves were harvested as one sample. The leaf discs were ground with a sterilized mortar and pestle in 2 mL of sterile distilled water (SDW), plated with different concentrations to determine the number of colony-forming units (cfu)/cm². Serial dilutions were spotted in triplicate onto PSA plates with appropriate antibiotics. The plates were incubated at 28 °C for 3–4 days until colonies could be counted. The experiments were repeated three times.

Measurement of bacterial growth in PSA medium

Cell cultures of the wild-type *Xoo* strain PXO99^A and the dTE3- or dTE3-0-containing PXO99^A strains in liquid PSA medium were adjusted to OD₆₀₀ = 1.0. Each 5 mL of suspension was then inoculated into 50 mL of fresh PSA medium, with shaking at 230 rpm at 28 °C. The OD of the cell cultures was measured at 600 nm every hour within 17 h.

Quantitative RT-PCR

Rice leaves were infiltrated with bacterial suspensions (OD₆₀₀ = 1.0) of the wild-type *Xoo* strain PXO99^A and the dTE3- or dTE3-0-containing PXO99^A. Water was used as a negative control. Total RNAs from infiltrated leaves were extracted using an RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China), and the RNA concentration and quality were measured using an ND-2000 Nanodrop spectrophotometer (Nanodrop Technologies, Thermo Scientific, Waltham, MA, USA). RNA (1.5 μg) was reverse transcribed into cDNA using a FastQuant RT Kit (Tiangen Biotech) with random primers. cDNA derived from 37.5 ng of total RNA was used for each real-time PCR with the gene-specific primers Xa27F and Xa27R (Table S1). Rice ubiquitin gene was used as an internal control. RT-PCR was performed on a 7500 Real Time PCR system using the SYBR SELECT MASTER MIX (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The average threshold cycle (Ct) was used to determine the fold change of gene expression. The 2^{−ΔΔCt} method was used for relative quantification (Livak and Schmittgen, 2001).

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SUPPORTING INFORMATION

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

- Methods S1** Construction of repeat array receptor (RAR) plasmids.
- Fig. S1** Schematic overview of the positions of the primers used for the construction of repeat array receptor (RAR) plasmids.
- Fig. S2** Schematic structures of starting vectors containing single alternative units and the assembled repeat array (RA) plasmids.
- Fig. S3** Disease reactions of IR24 to PXO99^A and transformed PXO99^A strains expressing dTE3 or dTE3-0.
- Fig. S4** Simplified procedures for the construction of designer transcription activator-like effector (dTALE)-expressing plasmids.
- Fig. S5** Growth ability of PXO99^A, dTE3- and dTE3-0-containing PXO99^A strains in liquid PSA medium (polypeptone, 10 g/L; sucrose, 10 g/L; glutamic acid, 1 g/L).
- Table S1** The oligonucleotide primers used in this study.
- Sequences S1** Open reading frame sequences of engineered genes encoding designer transcription activator-like effectors (dTALEs): dTE1, dTE1-0, dTE1-1, dTE2, dTE2-0, dTE2-1, dTE3, dTE3-0, dTE1-0Plus and dTE2-0Plus.