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Technical Advance

An efficient method to clone TAL effector genes from *Xanthomonas oryzae* using Gibson assembly

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SUMMARY

Transcription Activator-Like effectors (TALes) represent the largest family of type III effectors among pathogenic bacteria and play a critical role in the process of infection. Strains of *Xanthomonas oryzae* pv. *oryzae* (Xoo) and some strains of other *Xanthomonas* pathogens contain large numbers of TALe genes. Previous techniques to clone individual or a complement of TALe genes through conventional strategies are inefficient and time-consuming due to multiple genes (up to 29 copies) in a given genome, and technically challenging due to the repetitive sequences (up to 33 nearly identical 102-nucleotide repeats) of individual TALe genes. Thus, only a limited number of TALe genes have been molecularly cloned and characterized, and the functions of most TALe genes remain unknown. Here, we present an easy and efficient cloning technique to clone TALe genes selectively through *in vitro* homologous recombination and single-strand annealing, and demonstrate the feasibility of this approach with four different Xoo strains. Based on the Gibson assembly strategy, two complementary vectors with scaffolds that can preferentially capture all TALe genes from a pool of genomic fragments were designed. Both vector systems enabled cloning of a full complement of TALe genes from each of four Xoo strains and functional analysis of individual TALes in rice in approximately 1 month compared to 3 months by previously used methods. The results demonstrate a robust tool to advance TALe biology and a potential for broad usage of this approach to clone multiple copies of highly competitive DNA elements in any genome of interest.

Keywords: *Xanthomonas*, TAL effectors, Gibson assembly, rice.

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INTRODUCTION

Phytopathogenic bacteria of the genus *Xanthomonas* cause a variety of plant diseases, including those inflicting severe losses on economically important crop plants worldwide (Leyns *et al.*, 1984). In rice, *Xanthomonas oryzae* pv. *oryzae* (Xoo) invades xylem to cause bacterial blight of vascular disease, while the closely related pathogen *X. oryzae* pv. *oryzicola* (Xoc) is a mesophyll colonizer and causes a disease known as bacterial leaf streak (Niño-Liu *et al.*, 2006). Individual strains of both Xoo and Xoc contain multiple (e.g. up to 29 in a typical Xoc strain) genes of the Transcription Activator-Like effector (TALe) family, a few of which are known virulence or/and avirulence effectors depending on the genetic context of host plants (White, 2016). TALes depend on a type III secretion system for their translocation to host cells (Zhang *et al.*, 2015). Typically, once internalized into nuclei of rice cells, TALes act upon the effector-binding elements (EBEs) in the promoters of host genes (Boch *et al.*, 2010). The general hypothesis is that TALes have evolved to target specific host genes, whose subsequent ectopic expression facilitates infection, and, indeed, a number of TALe-targeted genes have been shown to enhance host susceptibility (Bart *et al.*, 2012; Chakrabarty *et al.*, 1997; Cohn *et al.*, 2014; Cox *et al.*, 2017; Hu *et al.*, 2014; Schwartz *et al.*, 2017; Sugio *et al.*, 2007; Yang *et al.*, 2006; Yu *et al.*, 2011). However, relatively few TALe targets have been shown to be disease susceptibility (S) genes given the large number of TALe genes discovered. Plants have also evolved to turn TALe function against bacterial invasion, and a few TALes trigger host resistance response due to TALe-mediated expression of the so-called executor resistance (R) genes, which, in rice, includes *Xa27*, *Xa10* and *Xa23* (Gu *et al.*, 2005; Tian *et al.*, 2014; Wang *et al.*, 2015). TALes are also recognized by members of the common NBS LRR family of R genes (e.g. *Xa1*) (Ji *et al.*, 2016). A group of TALe variants or iTALes (interfering TALes or truncated

TALes) from Xoo and Xoc can suppress NBS LRR-mediated TALe recognition and resistance (Ji *et al.*, 2016; Read *et al.*, 2016). However, additional mechanisms by which a large number of TALes are involved in host–pathogen interaction remain largely unexplored, and a simple and efficient way to clone and characterize those TALe genes is required.

TALe genes encode three major functional domains. The N-terminal domain contains the signal for bacterial type III secretion, followed by the central tandem repetitive region, which specifies the target nucleotide sequence of host genes, and the C-terminal domain containing nuclear localization signals and transcription activation domain, the latter characteristic of features of eukaryotic transcription factors (Boch *et al.*, 2010). The specificity of interaction between the TALes and their host target DNA is determined by the combination of the number of central repeats and composition of two amino acids at the 12th and 13th positions of repeats of TALes known as the repeat variable diamino (RVD) acid unit (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). Coding sequences of TALes and the *Sph*I or *Bam*HI restriction sites flanking the central or almost whole region across different Xanthomonas subspecies or pathovars are highly conserved. Often, the native TALe genes are not cloned, rather the repeat domains are cloned using the conserved *Sph*I or *Bam*HI sites of both ends of TALe genes. The cloned *Sph*I or *Bam*HI fragment fused with a common scaffold of sequences for N-terminal and C-terminal domains of TALe bestows specificity of the new TALe gene (Hopkins *et al.*, 1992; Yang and White, 2004). The characterization of TALe genes from various *Xanthomonas* strains has primarily involved cloning by library construction followed by hybridization or PCR detection based on a conserved TALe sequence (De Feyter *et al.*, 1993; Leach *et al.*, 1992; Tran *et al.*, 2018b; Yang and White, 2004; Yu *et al.*, 2015). Cloning and screening for the right TALe genes are technically challenging due to the existence of many copies of TALe genes in a genome of *Xanthomonas* and excessive near-identical tandem repeats in individual TALe genes. Sequencing through the whole central repetitive region of TALe genes is difficult. The whole cloning process is also time-consuming and inefficient, taking more than 3 months to clone and test a TALe gene from a given *Xanthomonas* strain.

To improve the efficiency of cloning TALe genes from complex genomes, in the present study we adapted the Gibson assembly strategy using the conserved nature of TALe genes. The Gibson assembly method is a robust molecular cloning technology alternative to restriction/ligation subcloning (Gibson *et al.*, 2009). The method depends on an exonuclease to excise the 5′ nucleotides of double-stranded DNA (dsDNA) to produce a 3′ single-strand overhang, which allows complementarity or annealing to the single strand overhang of an adjacent fragment also caused by the exonuclease. A DNA polymerase extends the nucleotides at the 3′ overhangs, and a DNA ligase is used to seal the nicks. The three reactions can be executed in a single tube using a programmed protocol in a thermocycler, and the reaction can be transferred directly into host cells for replication of the plasmids (Gibson *et al.*, 2009). The strategy was applied to selectively clone the full complements of TALe genes from four strains of Xoo, representing the Asian and African lineages of the pathogen. The resulting TALe genes were then assessed for their abilities to restore virulence to a TALe-defective mutant of Xoo.

RESULTS

Similarity of TAL effector genes in *Xanthomonas*

Gibson assembly requires the overlapping sequences of approximately 20 bp between two adjacent DNA fragments, and the process is summarized in Fig. 1. The TALe gene sequences were retrieved from genome data of 33 Xoo, ten Xoc, two *X. citri* pv. *vignicola* and one *X. citri* pv. *malvacearum* strains in the NCBI (National Center for Biotechnology Information) database (Supplementary Table S1). The TALe gene content of 22 Asian Xoo strains and 11 African Xoo strains ranges from 10 to 20 and 9, respectively, in each strain. Xoc strains contain a range of 20–29 TALe genes. Most TALe genes contain two *Bam*HI restriction sites (GGATCC), one at the ATG start codon and another approximately 150 bp upstream of the stop codon, and two *Sph*I sites (GCATGC), one 33 bp upstream of the first repeat and the second one located about 450 bp downstream of the last repeat. Sequences around these restriction sites are also very conserved, almost 99% identical. However, some rare TALe genes contain single nucleotide polymorphism (SNP) within one of the *Sph*I or *Bam*HI recognition sequences. Only 40 out of 733 TALe genes (5.4%) contained a 1-bp variation in one of the two *Bam*HI restriction sites (GGATCC to GGATCT). Fifty-seven of 733 TALe genes (7.8%) contain a SNP within one of the *Sph*I restriction sequences (Supplementary Table S1). The loss of either *Bam*HI or *Sph*I sites prevents cloning of the *Bam*HI or *Sph*I fragment of corresponding TALe gene in a library made from either *Bam*HI or *Sph*I DNA fragments. The results indicate that the majority (>92%) of TALe genes can be cloned through capture of either *Bam*HI or *Sph*I fragments of full-length genes (Supplementary Table S1), and rare TALe gene variants can be retrieved using other restriction enzyme combinations.

Strategy to selectively clone TALe genes

We next developed a system to selectively isolate the genomic fragments, e.g. *Sph*I or *Bam*HI digested fragments, of TALe genes from a genome. The technique involves *in vitro* linking of a TALe fragment and a vector fragment with short (~20 bp) 5′ and 3′ end sequences matching to both ends of TALe gene fragments by annealing of the 3′ complementary overhangs produced by T5 exonuclease. The single-stranded overhangs of the vector fragment function as

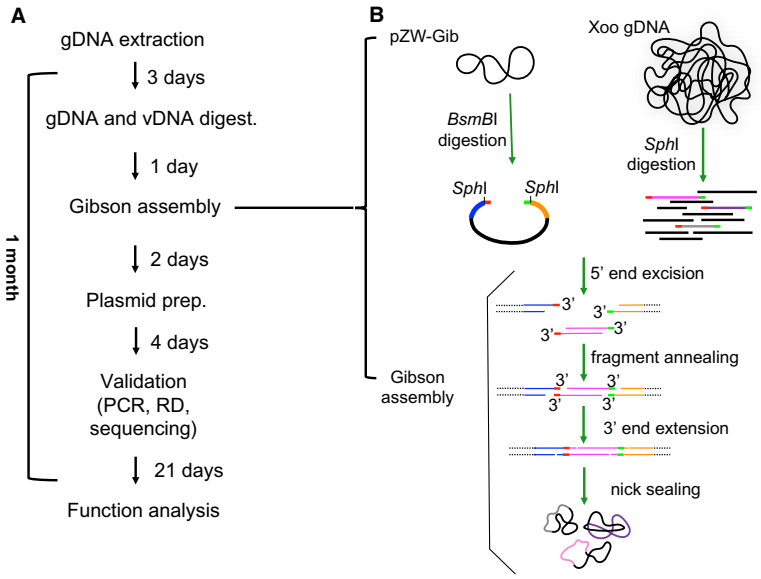

Fig. 1 Flow chart and schematics of selectively isolating TALe genes using the Gibson assembly method. (A) Steps and timing of cloning and analysis of TALe genes from a given *Xanthomonas oryzae* pv. *oryzae* (Xoo) genome. (B) The selective cloning of *Sph*I fragments of TALe genes. *Bsm*BI digestion yields a cloning vector containing a homologous end (hook, coloured end) at each side beyond the *Sph*I site; *Sph*I digestion of genomic DNA of Xoo releases TALe fragments with both ends containing short sequences complementary to the hooks of cloning vector pZW-Gib. Gibson assembly results in plasmids containing individual TALe genes. gDNA, genomic DNA; vDNA, vector DNA, RD, restriction enzume digestion.

sequence-specific hooks to catch the corresponding overhangs of TALe fragments selectively from a pool of the genomic fragments. To make a cloning vector for selective cloning of the *Sph*I fragments of TALe genes, the previously cloned TALe gene *pthXo1*, which encodes the major virulence effector for the Asian Xoo strain PXO99^{A} (Yang and White, 2004), was used as the backbone of the TALe gene vector without the central *Sph*I repetitive region. In addition, a counter selective gene was introduced to avoid undigested cloning vector or vector insert. First, a DNA fragment (gBlock) was synthesized that contained the *ccdB* gene, flanked by a sequence (5′-GCATGCATGGCGCAATGCACTGACGGGTGCAGAGACG-3′) 23 nucleotides (nt) downstream of the first *Sph*I (underlined) and a sequence (5′-CGTCTCAACGCCGGATCAGGCGTC TTTGCATGC-3′) 20 nucleotides upstream of the second *Sph*I of *pthXo1*. The two *ccdB* flanking sequences between *Sph*I and *Bsm*BI (double underlined) sites are highly conserved among the natural TALe genes (Supplementary Fig. S1A). The *ccdB* gene encodes a toxic protein (CcdB) to cause cell death in certain *Escherichia coli* strains (Bernard and Couturier, 1992). Inclusion of *ccdB* ensures loss of any clones that retains the region of *ccdB* when transferred to a strain lacking the antidote *ccdA* gene, in this case *E. coil* XL1-Blue. Digestion of vector with *Bsm*BI releases two ends (23 and 20 nt) that are homologous to the two ends of the *Sph*I fragment of the TALe gene. The gBlock was cloned into pZW-pthXo1 at *Sph*I sites by replacing the central *Sph*I region of *pthXo1*, resulting in pZW-Gib. The backbone of TALe in pZW-Gib contains the conserved ~800-bp region upstream and the ~400-bp region downstream of the *Sph*I recognition sites of TALe genes (Fig. 2A). Insertion of the *Sph*I central repeat region of any TALe gene through Gibson assembly should result in functional TALe genes.

To test the system, pZW-Gib was digested with *Bsm*BI to remove the *ccdB* gene and used to clone the *Sph*I fragment of the alternate major virulence TALe gene *avrXa7*, which was originally cloned from the Xoo strain PXO86 (Fig. 2A). The pZW-Gib-avrXa7 was introduced via subcloning into pHM1, a Xoo suitable plasmid, and transferred to ME2, a mutant derived from PXO99^{A} with *pthXo1* inactivated (Yang and White, 2004). Finally, the function of *avrXa7* for virulence, as assembled in pZW-Gib, was tested in the rice cultivars Kitaake and Zhenshan 97. The results showed that the Gibson-cloned *avrXa7* restored the virulence in ME2 (*avrXa7*) in terms of lesion lengths and disease symptoms compared to ME2 (Fig. 2B,C).

Isolation and functional test of TALe genes from PXO61

The PXO61 genome of Xoo (NCBI accession, CP033187.1) has over 3000 *Sph*I sites and 18 TALe genes (Supplementary Fig. S3A). All TALe genes contain two *Sph*I sites except one, *Tal5b*, which contains only one *Sph*I restriction site with the second one missing due to a deletion in the 3′ region. *Tal5b* belongs to a group of iTALe genes that contribute virulence to Xoo by suppressing *Xa1*-mediated disease resistance (Ji *et al.*, 2016). After ligation of the *Sph*I fragments of genomic DNA derived from PXO61 into the *Bsm*BI-digested pZW-Gib using the Gibson

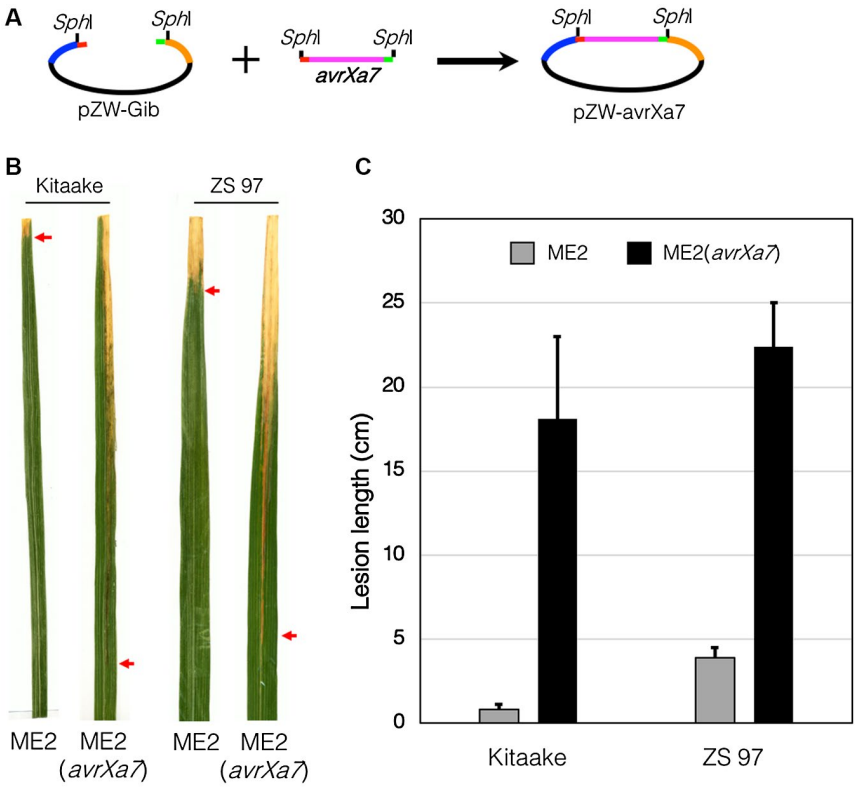

Fig. 2 Validation of pZW-Gib for cloning of a functional TALe gene. (A) The *Sph*I fragment of *avrXa7* was cloned into pZW-Gib, resulting in pZW-avrXa7. (B) Rice leaves of cultivars Kitaake and Zhenshan 97 (ZS 97) showing disease symptoms caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). (C) Lesion lengths in rice leaves caused by Xoo strain ME2 and its transformant ME2(*avrXa7*). Ten fully expanded young leaves were inoculated for lesion length measurements 14 days post-inoculation. The experiment was repeated twice independently with similar results.

assembly protocol, bacterial colonies were picked randomly to screen for the presence of *Sph*I fragments of TALe genes (Fig. 3A,B). Twenty-two out of 26 clones were positive for TALe genes based on PCR with specific primers, restriction enzyme digestion and Sanger sequencing (Fig. 3B). To clone the whole complement of TALe genes ($n = 17$ except *Tal5b*) of PXO61, a total of 45 clones were selected for sequencing based on the sizes of *Sph*I fragments of clones and the predicted number of TALe genes in the PXO61 genome. Seventeen of the cloned TALe genes matched the corresponding annotated genes in the PXO61 genome (Fig. 4). The individual TALe genes in pZW-Gib were inserted into the broad host range vector pHM1, and the constructs transferred to ME2 for functional analysis. One clone contained the previously identified major TALe gene *pthXo3* (also known as *Tal6C*), which conferred disease susceptibility in rice Kitaake and Zhenshan 97 (Figs 5 and S4C), and another clone contained *Tal7* (now *pthXo2B*), encoding a virulence TALe targeting the S gene *SWEET13* and confers disease susceptibility only to Kitaake and not Zhenshan 97 (Figs 5 and S4C). The remaining 15 clones conferred no observable phenotype (Figs 5 and S4C).

Selective cloning and function test of TALe genes from the African strain AXO1947

AXO1947 has been sequenced previously and has nine TALe genes and no iTALe gene (Huguet-Tapia *et al.*, 2016). To further demonstrate the feasibility of pZW-Gib to selectively clone TALe genes, a sub-library of TALe genes was constructed by ligating the *Sph*I fragments of genomic DNA from AXO1947 into the *Bsm*BI-digested pZW-Gib. All nine TALe genes were retrieved that matched the annotated TALes in AXO1947 (Supplementary Fig. S4B). The nine TALe genes were individually subcloned into pHM1 and mobilized into ME2 for virulence tests. Only the gene corresponding to a homolog of the previously identified major TALe *TalC*, which targets the S gene *SWEET14*, conferred virulence to ME2 in Kitaake and Zhenshan 97 (Supplementary Fig. 4D).

Improved vector for cloning TALe genes

Certain TALes, e.g. iTal3a and iTal3b in PXO99^{A}, require their unique N-terminal and C-terminal domains for functionality. Two deletions within the N-termini of iTal3a and iTal3b coding

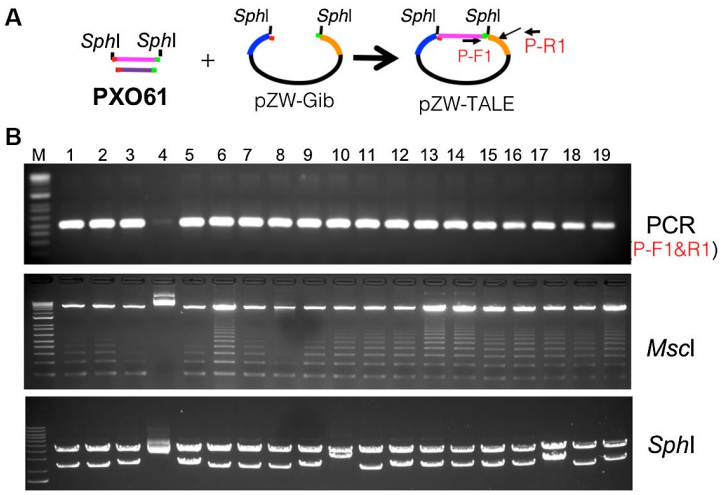

Fig. 3 Validation of TALe clones through PCR, restriction enzyme digestions. (A) Schematic of selective isolation of *Sph*I fragments TALe genes from genomic DNA of PXO61. (B) Validation of TALe clones through PCR with primers P-F1 and P-R1 of individual clones as indicated above lanes of upper gel image, digestion by *Msc*I which cuts each of central repeats (the DNA band patterns resulted from partial digestion) and digestion by *Sph*I.

$TALE_{PXO61}$	RVDs
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
TALE1a	NI-NN-NI-HG-HG-HD-NG-HD-HG-HD-HD-HD-NG
TALE1b	NI-NN-N*-NG-NS-NN-NN-NN-NI-NN-NI-NG-HD-HD-NI-NG
TALE1c	HD-HD-NN-NN-NI-NG-HD-S*-HG-HD-NG-N*-NG-HD-HD-N*-NI-NI-NN-HD-HI-ND-HD-NG-NN-HG-N*
TALE1d	HD-HD-HD-NG-N*-NN-HD-HD-N*-NI-NI-NN-HD-HI-ND-HD-NI-HD-NG-NG
TALE2a	NI-NS-HD-NG-NS-NN-HD-N*-NN-NN-NI-NG-HD-NG-HD-HD-HD-NG
TALE2b	NI-HG-NI-NI-NI-NN-HD-NS-NN-NS-NN-HD-NN-NI-HD-NN-NI-NG-HD-NG
TALE2c	NN-HD-NS-NG-HD-NN-N*-NI-HD-NS-HD-NN-HD-NN-HD-NN-NN-NN-NN-NN-NN-HD-NG
TALE3a	NI-N*-NI-HG-NI-NI-NS-HD-NN-HD-NS-NG-SS-HD-NI-NI-NN-NI-NN-NI-NG
TALE3b	NI-NN-NN-NI-NI-NI-HD-NS-HG-NN-NN-NN-NI-NI-NG-HD
TALE4a	NI-N*-NI-NS-NN-NG-NN-NS-N*-NS-NN-NS-N*-HD-HG-HD-NI-HD-HD-NG
TALE4b	NI-HG-NI-NG-HG-HD-NS-NG-HD-NN-NG-HG-NG-HD-HG-HD-HD-NI-NN-NG
TALE4c	NI-H*-NI-NN-NN-NN-NN-NN-HD-NI-NS-HG-HD-NI-N*-NS-NI-NI-HD-HD-N*-NS-NG
TALE5a	NS-HD-NG-NG-NG-NG-HD-HD-HD-HD-NN-HD-NG-HD-NI-HD-NN-N*
TALE6a	NI-NG-NN-NG-NK-NG-NI-NN-NI-NN-NI-NN-NS-NG-NS-NN-NI-N*-NS-NG
TALE6b	NI-HG-NI-NI-HG-HD-NN-HD-HD-HD-NI-NI-NN-NI-HD-HD-HD-HG-NN-NN-HD-NS-NN-HD-NG-NS-N*
TALE6c(PthXo3)	NI-HG-NI-HG-NI-NI-NI-HD-NN-HD-HD-HD-NG-HD-N*-NI-HD-HD-NN-NS-NI-NN-NN-NG-NN-HD-N*-NS-NG
TALE7 (PthXo2B)	NI-HG-NI-NN-NI-NN-HD-NI-HD-HD-NS-NS-HD-NI-NI-HD-NG-HD-HD-HD-NG-NG

Fig. 4 Seventeen of 18 annotated TALe genes from PXO61 were cloned using pZW-Gib vector and Gibson assembly method. The repeat variable diamino (RVD) acid units of individual TALes are shown under numbers (1 to 29) indicating the order of 33–34 amino acid repeats. Asterisks (*) indicate that the amino acid at the 13th position missing.

sequences are required to suppress the *Xa1*-mediated resistance triggered by TALes (Ji *et al.*, 2016). Other TALe genes simply lack one *Sph*I site in their DNA sequences due to DNA polymorphisms. The vector pZW-Gib is not able to capture such sequences from the genomes. Furthermore, pZW-Gib derived TALe clones need to be moved into a broad host range vector, pHM1 in this case, for further characterization of function in Xoo. To overcome such constraints, a cloning vector capable of capturing of the *Bam*HI fragments and avoiding the lack of *Sph*I restriction site of natural TALe genes and direct transfer to Xoo was devised, using pHM1 as the recipient vector for TALe *Bam*HI fragments. Differing from pZW-Gib, pHM1-Gib contains the promoter sequence, the 3′ region downstream of the second *Bam*HI site of *pthXo1* from PXO99^{A}, and the *ccdB* cassette. Additionally, a sequence of ColE1 (origin of replication from pBluescript) was integrated into pHM1-Gib to increase the copy number of plasmids in *E. coli*, which facilitates DNA isolation and manipulation (Supplementary Fig. S1B). For validation, the *Bam*HI fragment of *pthXo2B* was

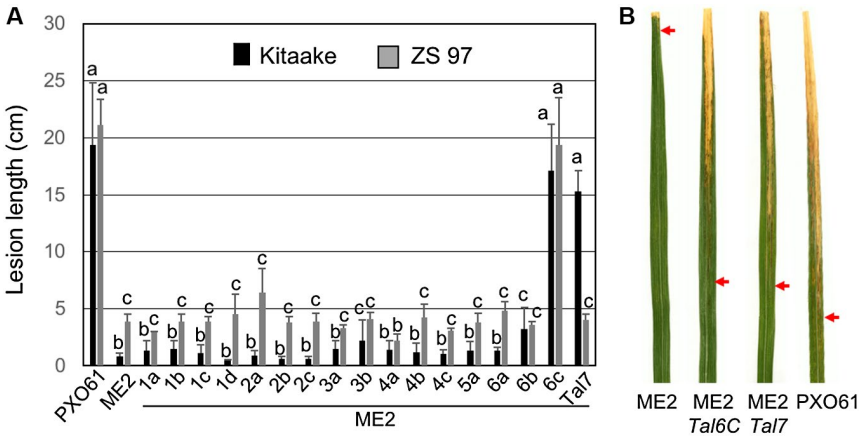

Fig. 5 Virulence contribution of 17 TALes cloned from PXO61. (A) Lesion lengths caused in rice cultivars Kitaake and Zhenshan 97 (ZS 97) by different *Xanthomonas oryzae* pv. *oryzae* (Xoo) strains indicated below the paired columns. Different lower case letters indicate statistically significant differences (means ± SEM, $n = 10$, $P < 0.05$). (B) Blight symptom in Kitaake leaves caused by the Xoo strains as indicated below each leaf. Arrows indicate the edges of lesions.

retrieved from the *Bam*HI digestion of PXO61 genomic DNA in pHM1-Gib through Gibson assembly. The resultant plasmids were directly transferred into ME2, and each transformant tested for the ability to cause disease in Kitaake. The newly assembled *pthXo2B* directed induction of *SWEET13*. To further demonstrate the improved capability of pHM1-Gib, all nine TALe genes from two Africa strains CFBP7321 and CFBP7325 were cloned in the vector. Thirty-nine out of 48 clones in CFBP7325 and 45 out of 48 in CFBP7321, respectively, were positive for TALe-containing sequences (Supplementary Figs S5–S7). A disease assay confirmed the presence of clones for *TalC* and *TalF*, both previously identified major TALe genes, which could direct disease in rice Kitaake (Supplementary Fig. S8).

DISCUSSION

In the present study, we present two complementary vector systems for cloning TALe gene fragments selectively from a genomic pool of fragments through *in vitro* homologous recombination and single-strand annealing, a protocol known as the Gibson assembly method. The strategy depends on exonuclease (e.g. T5 exonuclease) to generate the 3′ end overhangs by chewing back the 5′ nucleotides of vector fragments and genomic fragments, and the assembly of vector fragments and selectively the TALe gene fragments in a single reaction. Each vector system has been demonstrated as functional by reproducibly applying the vector to multiple Xoo strains. Approximately 87% ($n = 122$) of the clones are positive for TALe gene fragments, and a total of 44 TALe genes out of 45, with *Tal5b* of PXO61 missing, were retrieved from four Xoo genomes. The results indicate both plasmids are highly efficient and time saving compared to the conventional cloning methods for cloning of TALe genes from a given *Xanthomonas* genome. The strategy and even the specific vector system are applicable to other *Xanthomonas* species and pathovars (e.g. Xoc) due to the conserved nucleotide sequences around the *Sph*I and *Bam*HI sites of the TALe genes. Some species with a more divergent TALe sequence, including *X. translucens*, may require customization of the vector annealing regions (Peng *et al.*, 2016).

The prior studies reported the difficult and time-consuming task in cloning a complement of TALe gene fragments from a given genome largely due to a large number of TALe genes that were highly conserved in a given genome and the large number of central tandem repeats of individual TALe genes. The cloning was performed by making genomic libraries followed by Southern hybridization- or PCR-based screening, which is laborious and inefficient. Due to this difficulty, only a few from a large number of sequenced and annotated *Xanthomonas* genomes have been subjected to systematic TALe gene cloning and characterization in contrast to Cernadas *et al.* (2014), Yang and White (2004) and Yu *et al.* (2015). For example, Yu *et al.* pulled out 115 positive clones (representing 18 unique TALe genes) from 3000 individual transformant colonies (*c.* 4%) through screening a library of *Bam*HI fragments of Xoo K74 genome using dot- and Southern blotting (Yu *et al.*, 2015). A similar but improved approach was developed to enrich the *Bam*HI fragments of TALe genes from a given genome by applying two additional restriction enzymes (*Apa*LI and *Sfo*I) to further cut the non-TALe gene fragments of genomic DNA. Ligation and screening by PCR yielded 25.0% and 26.9% positive clones of TALe gene fragments for MAI1 and BAI3 strains of Xoo, respectively (Tran *et al.*, 2018a). Both methods require further subcloning of the TALe *Bam*HI fragments from the pUC-based vector into TALe gene scaffold and *Xanthomonas*-suitable vector for function analysis.

TALes represent the largest type III effector family of pathogenic bacteria, and some TALes have been found to be crucial to help bacteria to infect crop plants (Boch *et al.*, 2010). Such important

TALes represent attractive targets to create disease resistance in host crop plants by interfering with the disease mechanism (Schornack *et al*., 2013). Naturally occurring or gene editing-derived alleles with EBE variations, if disruptive to TALe binding, in the promoters of S genes targeted by TALes confer genetically recessive resistance (Blanvillain-Baufumé *et al*., 2017; Chu *et al*., 2006; Li *et al*., 2012; Liu *et al*., 2011). Multiplex genome editing produced single engineered rice lines that carried multiple mutations in three *SWEET* gene promoters and resulted in broad-spectrum blight resistance (R. Oliva *et al*., unpublished data). Executor R genes along with promoters containing the synthetic EBEs have been used to trap the widely spread cognate TALes for induced resistance (Hummel *et al*., 2012; Römer *et al*., 2009). Deployment of such resistances (dominant, recessive, naturally occurring or artificially made) against TALes creates tremendous selection pressure on the dynamic populations of *Xanthomonas* pathogens. The repetitive and multigenic nature provides a repertoire of TALe genes in a given genome to evolve and potentially overcome the resistances. Broad and durable resistance depends on effectively monitoring the evolving pathogenic populations and quickly identifying newly evolving TALes, monitoring tools including a diagnostic kit comprising seven components based on TALe-mediated disease mechanism (J. Eom *et al*., unpublished data) and the cloning methods described here. Additionally, the Gibson-based vectors developed here have potential for high-throughput profiling of complement TALe genes from multiple *Xanthomonas* strains when bar-coded and combined with next-generation sequencing technologies, including PacBio and Nanopore platforms. Development of a simple, cheap and efficient cloning system will also advance our understanding of TALe biology.

EXPERIMENTAL PROCEDURES

Plant materials, bacterial strains and growth conditions

Rice (*Oryza sativa*) cultivar Kitaake and Zhenshan 97 were used in this study. Plasmids, Xoo and *E. coli* strains used in this study are listed in Supplementary Table S2. Rice plants were grown in a growth chamber at a temperature of 28 °C, relative humidity of 85% and a photoperiod of 12 h. *Escherichia coli* DB3.0 and Trans1-T1 were grown in Luria–Bertani medium at 37 °C. Xoo strains were grown in either nutrient broth (NB: beef extract 3 g/L, peptone 5 g/L) or tryptone sucrose medium (TSA: tryptone 10 g/L, sucrose 10 g/L, glutamic acid 1 g/L) at 28 °C. Antibiotics used in this study include spectinomycin (100 mg/L) and ampicillin (100 mg/L) for bacterial strains containing appropriate plasmids.

DNA manipulation and plasmid construction

DNA manipulation and PCR were conducted according to standard protocols (Ausubel *et al*., 1998). Genomic DNA from Xoo was extracted from bacterial cells grown in NB medium using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany) following the manufacturer's manual. Plasmids were introduced by electroporation into *X. oryzae* and *E. coli* bacterial competent cells as described previously (Yang and White, 2004). Primers for PCR were synthesized by Integrated DNA Technologies (Coralville, IA, USA). PCR was performed using *Taq* DNA polymerase or Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA).

Construction of pZW-Gib

The central repeat region of *pthXo1*was replaced by a gBlock, which contained a *ccdB* sequence, as follows. First the 3′ fragment of a TAL effector gene was PCR-amplified using the primers TalAatII-F and TalFlH3-R along the genomic DNA of PXO99^{A}. The PCR amplicon was cloned into pZW-*pthXo1* at *Aat*II and *Hin*dIII sites through Gibson assembly. The ligation reaction was transferred into *E. coli* Trans1-T1 cells. The positive bacterial colonies were screened by colony PCR using primers TalAatII-F and TALFLH3-R. Candidate clones were sequenced for confirmation of *PthXo1* downstream sequence. A gBlock fragment (Supplementary Fig. S1A) synthesized by Integrated DNA Technologies was cloned into the new pZW-*pthXo1* at *Sph*I sites by Gibson cloning. The ligation reaction was transferred into *E. coil* DB3.0 cells. Candidate clones were sequenced for confirmation of gBlock sequence.

Construct pZW-Gib-avrXa7 via Gibson cloning

The central repeat region of *avrXa7* was obtained by digesting pZW-avrXa7 with *Sph*I and selecting a DNA fragment of right size through electrophoresis in 1% agarose gel. The *Sph*I fragment was cloned into the backbone of pZW-Gib that was derived from *Bsm*BI-digestion and purification from 1% agarose gel. The ligation was transferred into *E. coli* Trans1-T1 cells; positive colonies were first screened via PCR using primer P-F and P-R, *Sph*I digestion and finally sequencing for confirmation of *avrXa7* insertion. Primer sequences are provided in Supplementary Table S3.

Construct TALe gene sublibraries of PXO61 and AXO1947

Genomic DNA of PXO61 and AXO1947 was digested with *Sph*I and the fragments were separated through electrophoresis in 1% agarose gel. DNA fragments of about 2–5 kb were extracted and purified from the agarose gel and subcloned through Gibson assembly into the backbone of pZW-Gib that was *Bsm*BI-digested and gel-purified. The Gibson Assembly Cloning kit (New England Biolabs) was used following the manufacturer's manual. The subsequent process was identical to the construction of pZW-Gib-avrXa7. Finally, a select set of clones was chosen based on the expected sizes of repeats

as determined by *Sph*I digestion and sequenced using primers Tal-*Sph*I-F and P-R.

Transform X. oryzae cells with plasmid DNA

Competent cells of Xoo ME2 were prepared and transformation was performed as previously described (Ji *et al.*, 2016). Briefly, TSA + NB medium (NB 8 g/L, tryptone 10 g/L, sucrose 10 g/L, glutamic acid 1 g/L) was used to grow Xoo cells. An aliquot of 50 μL of bacterial competent cells was mixed with 10 ng (0.5 μL) of plasmid DNA for electroporation using a Bio-Rad electroporation instrument (Bio-Rad, Hercules, CA, USA). An electric field of 15 kV/cm with a resistance of 200 Ω and a capacitance of 25 μF was applied. After pulse delivery, cells were immediately transferred into 1 mL of SOC medium (20 g tryptone, 5 g yeast extract, 4.8 g $MgSO_4$, 3.6 g dextrose, 0.5 g NaCl and 0.2 g KCl per L) in a 2 mL round-bottom polypropylene tube. After incubation at 28 °C with constant shaking for 2–4 h, the electroporated cells were plated onto TSA-NB medium containing the appropriate antibiotics and incubated at 28 °C for 3–5 days. Non-electroporated competent cells were used as a control. Positive clones were picked for further analysis.

Disease assays

Disease assays to test the function of cloned TALe genes were conducted as follows. Briefly, Xoo strains were grown in TSA + NB with appropriate antibiotics at 28 °C. Bacterial cells were collected from culture through low-speed (2300 *g*) centrifugation, washed twice and suspended in sterile water. The suspensions were adjusted to an optical density of 0.5 at 600 nm (OD_{600}) and were used to inoculate into fully expanded leaves of 7–8-week-old rice plants using the leaf-tip clipping method (Kauffman *et al.*, 1973). Lesion lengths were measured about 12–14 days post-inoculation. The disease assays were performed independently at least twice and on at least four plants each time. One-way analysis of variance statistical analysis was performed on all measurements. The Tukey's honestly significant difference test was used for post-analysis of variance pairwise tests for significance, set at 5% ($P < 0.05$).

Sequence analysis of TALe genes in sequenced *Xanthomonas* genomes

Genomes of *Xanthomonas* strains were obtained from the NCBI under accession numbers Xoo OS198: CP031461.1, Xoo JL25: CP031457.1, Xoo PX086: CP031463.1, Xoo PX079: CP031462.1, Xoo YC11: CP031464.1, Xoo JL33: CP031459.1, Xoo JP01: CP031460.1, Xoo HuN37: CP031456.1, Xoo JL28: CP031458.1, Xoo BAI3: CP025610.1, Xoo MAI1: CP025609.1, Xoo MAI145: CP019092.1, Xoo MAI134: CP019091.1, Xoo MAI129: CP019090.1, Xoo MAI106: CP019089.1, Xoo MAI99: CP019088.1, Xoo MAI95: CP019087.1, Xoo MAI73: CP019086.1, Xoo MAI68: CP019085.1, Xoo PXO61: CP021789.1, Xoo PXO145: CP013961.1, Xoo AXO1947: CP013666.1, Xoo XF89b: CP011532.1, Xoo PXO602: CP013679.1, Xoo PXO563: CP013678.1, Xoo PXO524: CP013677.1, Xoo PXO282: CP013676.1, Xoo PXO236: CP013675.1, Xoo PXO211: CP013674.1, Xoo PXO71: CP013670.1, Xoo PXO83: CP012947.1, Xoo PXO99A: CP000967.2, Xoo PXO86: CP007166.1, Xoo MAFF 311018: NC_007705.1, Xoo KACC 10331: AE013598.1, Xoc CFBP7331: CP011958.1, Xoc CFBP2286: CP011962.1, Xoc RS105: CP011961.1, Xoc L8: CP011960.1, Xoc CFBP7341: CP011959.1, Xoc BXOR1: CP011957.1, Xoc BLS279: CP011956.1, Xoc B8-12: CP011955.1, Xoc CFBP7342: CP007221.1, Xoc BLS256: CP003057.2, Xcv CFBP7113: CP022270.1, Xcv CFBP7112: CP022269.1, Xcm XcmH1005: CP013004.1.

Analysis of all TALe gene sequences was performed through NCBI blast and further confirmed by using SnapGene software. Sequences used for comparison were 5′-GCA TGCATGGCGCAATGCACTGACGGGTGC-3′ and 5′- ACGCCGGAT CAGGCGTCTTTGCATGC-3′ around the two *Sph*I restriction sites, while the pair of sequences 5′-GGATCCCATTCGTTCGCGCA CGCCAAGTCCTGCCCGCG-3′ and 5′-ACCAGGATCGGGGGCGGCC TCCCGGATCC-3′ were used around the two *Bam*HI sites of TALe genes.

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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:

Fig. S1 DNA sequences of gBlock fragments synthesized to make two Gibson cloning vectors. (A) gBlock1 was used to inserted into pZW-pthXo1 at *Sph*I sites through Gibson cloning. The two sequences shaded in yellow are homologous to the ends of *Sph*I fragments of TALe genes. (B) gBlock2 was used to make pHM1-Gib. The two sequences shaded in yellow are homologous to the ends of *Bam*HI fragments of TALe genes.

Fig. S2 Nine TALe genes from AXO1947 were cloned using the pZW-Gib vector and Gibson assembly method. The RVDs of individual TALes are shown under numbers 1 to 26, indicating the order of 33–34 amino acid repeats. Asterisks (*) indicate that the amino acid at the 13th position missing.

Fig. S3 Virulence contribution of nine TALes cloned from AXO1947. (A) Lesion lengths caused in rice Kitaake and Zhenshan 97 (ZS 97) by different Xoo strains indicated below the paired columns. Different lower letters indicate statistically significant differences (mean SEM, $n = 10$, $P < 0.05$). (B) Blight symptom in Kitaake leaves caused by the Xoo strains as indicated below each leaf. Arrows indicate the edges of lesions.

Fig. S4 TALe genes from PXO61 and AXO1947. (A) TALe genes clustered in the genome of PXO61. (B) TALe genes clustered in the genome of AXO1947. (C, D) Lesion length measurements (mean SEM, $n = 10$) in Kitaake caused by different strains.

Fig. S5 Validation of TALe clones through PCR, restriction enzyme digestions. (A) Schematics of selective isolation of *Bam*HI fragment TALe genes from genomic DNA of CFBP7321. (B) Validation of TALe clones through PCR with primers P-F and P-R of individual clones as indicated above lanes of upper gel image, digestion by *Msc*I which cuts each of central repeats (the DNA band patterns resulted from partial digestion) and digestion by *Bam*HI.

Fig. S6 Validation of TALe clones through PCR, restriction enzyme digestions. (A) Schematics of selective isolation of *Bam*HI fragments TALe genes from genomic DNA of CFBP7325. (B) Validation of TALe clones through PCR with primers P-F1 and P-R1 of individual clones as indicated above lanes of upper gel image, digestion by *Msc*I which cuts each of central repeats (the DNA band patterns resulted from partial digestion) and digestion by *Bam*HI.

Fig. S7 TALe gene distribution in two African Xoo genomes. (A, B) Nine TALe genes in each of two Xoo genomes are syntenic in their locations with the same colors denoting identical TALes at the amino acid level while different colors indicate different TALes at the amino acid level.

Fig. S8 Two TALe genes cloned with the pHM1-Gib system were functional in virulence. The virulences of *TalC* from CFBP7321 and *TalF* from CFBP7325 were tested in Kitaake leaves. Different letters indicate statistically significant differences.

Table S1 Number of TALe genes from different *Xanthomonas* that can be selectively cloned with Gibson assembly.

Table S2 Plasmids and bacterial strains used in this study.

Table S3 Primers used in this study.