SHORT COMMUNICATION

The Tn7-Based Genomic Integration Is Dependent on an *attTn7* Box in the *glms* Gene and Is Site-Specific With Monocopy in *Ralstonia solanacearum* Species Complex

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The Tn7-based genomic integration system enables direct insertion of foreign gene elements into the chromosome downstream of *glms* in many bacteria species. The *glms* gene is greatly conserved in *Ralstonia solanacearum* species complex (RSSC), while its downstream regions are mostly different in the RSSC. Here, we provided genetic evidence to validate that this Tn7 integration is dependent on a conserved 30-bp motif in the *glms*, called an *attTn7* box, and artificial *attTn7* boxes elsewhere are competent for the Tn7 integration, which is further confirmed to be simultaneous downstream of both original and artificial *attTn7* boxes, using PCR. With the whole-genome resequencing on 500 Tn7-colonies, the Tn7 integration was confirmed to be site-specific at 25 bp downstream of *glms* with monocopy as a chromosome of the RSSC. Characteristic of a monocopy in a chromosome enables the Tn7-based complementation to fully restore phenotypes of mutants to those of parent strains that are advantageous rather than those based on plasmids with low-copy numbers. The Tn7-based genomic integration system provides a generally applicable and versatile genetic tool for studies of complementation, pathogenesis, overexpression, and in-vivo promoter activity assays with monocopy in the RSSC.

Keywords: *attTn7* box, monocopy, *Ralstonia solanacearum* species complex, site-specific, Tn7-based genomic integration

The Tn7-based genomic integration system enables direct insertion of foreign gene elements into bacterial chromosomes in a site- and orientation-specific manner that provides a genetic tool for studies of complementation, pathogenesis, overexpression, and in-vivo promoter activity assays with monocopy in many gram-negative bacteria species (Choi and Schweizer 2006a and b; Choi et al. 2005, 2008). The Tn7-transposition minimally requires the Tn7 left (Tn7L) and Tn7 right (Tn7R) ends, called mini-Tn7, and transposition of the Tn7 or mini-Tn7 into a chromosome usually occurs at a Tn7 attachment (*attTn7*) site in the presence of helper plasmids encoding a site-specific transposition pathway (Peters and Craig 2001; Waddell and Craig 1989). The *attTn7* site is located immediately downstream of *glms* genes (Fig. 1A), which encode an essential glucosamine-6-phosphate synthetase and are highly conserved in many bacterial species (Fig. 1B) (Choi et al. 2005; Craig 1991; Peters and Craig 2001).

This Tn7-based genomic integration system works efficiently in *Ralstonia solanacearum*, which is a soil-borne gram-negative plant vascular bacterium and causes destructive bacterial wilt on abundant plant species worldwide (Yao and Allen 2007; Zhang et al. 2011). This bacterium is extremely heterogeneous and is currently referred as a *Ralstonia solanacearum* species complex (RSSC) including its closely related species of *R. syzygii*, *R. picketti*, and banana blood disease bacterium (Fegan and Prior 2005; Genin and Denny 2012). With genome searching, a single *glms* gene is detected in the RSSC that exhibits more than 95% nucleotide identity to *R. solanacearum* strains and 90 to 95% nucleotide identity to strains of its closely related species in the RSSC, while nucleotide sequences of *glms* downstream regions are mostly different, even in *R. solanacearum* strains (Zhang et al. 2019a and b). The Tn7 integration is 25 bp downstream of the *glms* gene in the RSSC (Yao and Allen 2007; Zhang et al. 2011), indicating that the Tn7 integration is possibly dependent on a putative 30-bp *attTn7*-associated motif in the *glms*, hereafter designated as the *attTn7* box. The *attTn7* box is conserved in *glms* genes of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Xanthomonas* species (Choi and Schweizer 2006a;

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Jittawuttipoka et al. 2009; Waddell and Craig 1989). Multiple *glms* genes and *attTn7* boxes can be detected in some bacterial species, such as *Burkholderia* and *Xanthomonas* species (Choi et al. 2006, 2008; Jittawuttipoka et al. 2009). Moreover, some non-*glms*-linked *attTn7* boxes are also found in *Proteus mirabilis* (Choi and Schweizer 2006b), indicating that Tn7 integration

is possibly occurred at different sites due to different properties of *glms* genes and *attTn7* boxes in different bacterial strains. Although abundant PCR results confirms location of the Tn7 integration at 25-bp downstream of the *glms* stop codon in some RSSC strains (Yao and Allen 2007; Zhang et al. 2011), it remains uncertain whether this Tn7 integration is dependent on

Fig. 1. Tn7 integration in the *Ralstonia solanacearum* species complex (RSSC) and alignment of *attTn7* box in different bacterial species. **A**, Schematic representation of pUC18-mini-Tn7T-Gm based Tn7 integration in the RSSC (Choi et al. 2005; Zhang et al. 2011). The Tn7 element, including Tn7R, Tn7L, Gm resistance cassette (Gm^r), and foreign genes cloned in a multiple cloning site was integrated into chromosome of the RSSC, with helping plasmid pTNS2 encoding a transposase. **B**, Alignment of nucleotide sequences of the *attTn7* box and its 36-bp downstream regions, between GMI1000, a typical *R. solanacearum* strain, *Escherichia coli* (Eco) (Waddell and Craig 1989), *Pseudomonas aeruginosa* (Pae) (Choi and Schweizer 2006b), *Xanthomonas campestris* pv. *campestris* (Xcc) (da Silva et al. 2002), *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Lee et al. 2005), *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (Thieme et al. 2005), and *Xanthomonas axonopodis* pv. *citri* (Xcv) (da Silva et al. 2002). **C**, Alignment of nucleotide sequences of the *attTn7* box and its 36-bp downstream region in five representative phylotypes of *R. solanacearum* strains GMI1000 (ph-I), CFBP2957 (ph-IIA), IBSBF1503 (ph-IIB), CMR15 (ph-III), and PSI07 (ph-IV). The boxed region refers to the 30-bp *attTn7* box motif in the *glms*, and three nucleotide sequences in red refer to the stop codon of *glms*. Asterisks represent sequence identity in all species and arrowheads indicate Tn7 integration sites. **D**, Alignment of nucleotide sequences of the *attTn7* box with a bit score in 221 RSSC strains that, to date, have been available in the National Center for Biotechnology Information database. The beginning 30-nucleotide sequences refer to the *attTn7* box and the last three nucleotide sequences refer to the stop codon of *glms*.

the *attTn7* box in *glms* and whether there are some non-*glms*-linked integrations in the RSSC.

Pioneering studies characterize a putative *attTn7* box in *glms* genes of some bacterial species (Choi and Schweizer 2006a and b; Jittawuttipoka et al. 2009; Waddell and Craig 1989). With alignment of nucleotide sequences of *glms* and its downstream regions of about 26 bp, this conserved *attTn7* box can be also identified in the *glms* of GMI1000, a typical RSSC strain, exhibiting over 67% nucleotide identity to those in *E. coli*, *P. aeruginosa*, and *Xanthomonas* species (Fig. 1B). The RSSC is heterogeneous and classified into five phylotypes, ph-I, ph-IIA, ph-IIB, ph-III, and ph-IV (Genin and Denny 2012). We performed alignment of nucleotide sequences of the *attTn7* box and its downstream regions of 36 bp in five representative phylotypes of *R. solanacearum* strains, GMI1000 (ph-I), CFBP2957 (ph-IIA), IBSBF1503 (ph-IIB), CMR15 (ph-III), and PSI07 (ph-IV), which share more than 90% nucleotide identities in regions including the *attTn7* box and its downstream 10 bp to the stop codon of *glms* (totally 40 bp), but greatly varied in *glms* downstream regions (Fig. 1C). Genome sequences of 221 RSSC strains have, to date, been available in the National Center for Biotechnology Information database, and we compared nucleotide sequences of the *attTn7* box (30 bp) and its downstream

regions of 10 bp to the stop codon of *glms* with a bit score. Most nucleotides of their *attTn7* boxes are identical (Fig. 1D), confirming that the *attTn7* box is extremely conserved in the RSSC. In consideration of the fact that nucleotide sequences of *glms* downstream regions are mostly different in the RSSC, while the Tn7 integration is applicable in several different RSSC strains, this extremely conserved *attTn7* box in the *glms* might play some critical role on the Tn7 integration in the RSSC.

In order to assess whether the Tn7 integration is dependent on the *attTn7* box in *glms*, we first attempted to generate mutants with deletion of the *attTn7* box or *glms* from OE1-1, another typical RSSC strain, to confirm the dependence of Tn7 integration on the *attTn7* box, but we failed after several attempts, which might be due to the fact that the *glms* encodes an essential glucosamine-6-phosphate synthetase for bacterial growth. We aimed to generate mutants with artificial *attTn7* boxes from OE1-1 to assess whether artificial *attTn7* boxes are competent for the integration at sites other than the known Tn7 integration site at 25 bp downstream from *glms* (Fig. 2A). Genes of *glms* and *rsc0179* are identical at nucleotide sequences in strains of GMI1000 and OE1-1, while the intergenic region of *glms* and *rsc0179* in OE1-1 is about 32 kbp in length, harbors 21 putative genes (RSOE_08605 to RSOE_08710), and seems to be

Fig. 2. Dependence of Tn7 integration on the *attTn7* box. **A**, Schematic representation of artificial *attTn7* boxes (red boxes) at different positions of the OE1-1 chromosome. Genomes of the *Ralstonia solanacearum* species complex (RSSC) possess a unique feature of two replicons as one chromosome (3.7 Mb) and one megaplasmid (2.1 Mb). The RSSC genome possesses two replicons as one chromosome (3.7 Mb) and one megaplasmid (2.1 Mb). Genes located on the chromosome are named as *rsc* genes, i.e., *glms* (*rsc0179*), and those located on the megaplasmid are named as *rsp* genes, i.e., *rsp1398*. Two genes of RSOE_08665 and *rsp1398*, which are located on the chromosome and megaplasmid, respectively, were here selected for generation of ectopic *attTn7* boxes. Ectopic *attTn7* boxes were generated with pK18mobsacB-based homologous recombination by inserting the 30-bp *attTn7* box and its downstream 7 bp before the stop codon of target genes. RQ6210 = ectopic *attTn7* box in RSOE_08665, and RQ6213 = ectopic *attTn7* box in *rsp1398*. **B**, Detection of Tn7-integration sites using PCR with respective primer pairs for ectopic *attTn7* boxes. Mutant 1 refers to the Tn7 mutant derived from OE1-1 (*glms* downstream), and mutants 2 through 4 (*RSOE_08665* downstream) and 5 through 7 (*rsp1398* downstream) refer to three Tn7 mutants derived from RQ6210 and RQ6213, respectively. M = DNA ladder (DL5000, Takara).

Table 1. Bacterial strains and plasmid used in this study

Strain	Relative characteristics ^a	References
OE1-1	Phylotype I, sequevar 34	Kanda et al. 2003
RQ6210	Ectopic <i>attTn7</i> box in RSOE_08665	This study
RQ6213	Ectopic <i>attTn7</i> box in <i>rsp1398</i>	This study
pUC18-miniTn7T-Gm	Gm ^r on mini-Tn7T; for gene insertion in Gm ^s bacteria	Choi et al. 2005

^a Gm^r and Gm^s = gentamicin resistant and sensitive, respectively.

generated from GMI1000 by an insertion of a 32-kbp DNA fragment at 24 bp after *glms* (Zhang et al. 2019a and b). The RSSC genome possesses a unique feature of two replicons as one chromosome (3.7 Mb) and one megaplasmid (2.1 Mb) in GMI1000 (Salanoubat et al. 2002). Genes located on the chromosome are named as Rsc series, i.e., *glms* (*rsc0178*) and *rsc0179*. Genes located on the megaplasmid are named as Rsp series, i.e., *rsp1398*, encoding a putative shikimate 5-dehydrogenase, and its deletion does not alter growth of OE1-1 in nutrient-rich broth medium (data not shown). Two genes of RSOE_08665 and *rsp1398*, which are located on a chromosome and megaplasmid, respectively, were selected for generation of ectopic *attTn7* boxes in this study. With the pK18mobsacB-based homologous recombination to insert the 30-bp *attTn7* box at 7 bp before the stop codon of RSOE_08665 or *rsp139* (Fig. 2A), mutants of RQ6210 (ectopic *attTn7* box in *RSOE_08665*) and RQ6213 (ectopic *attTn7* box in *rsp1398*) were generated (Table 1). Note that ectopic *attTn7* boxes and their downstream nucleotides to the stop codon are identical to that in the OE1-1 *glms*. Candidate insertions were assessed whether they could occur downstream of ectopic *attTn7* boxes, using PCR with respective primer pairs (Fig. 2A) (primer sequences are listed in Supplementary Table S1). PCR results confirmed that these two artificial *attTn7* boxes are competent for the Tn7 integration (Fig. 2B), while no PCR amplification was detected in strains without ectopic *attTn7* box (data not shown). Nucleotide sequence analysis of these PCR amplifications revealed that these Tn7 integrations occurred 25 bp downstream from artificial *attTn7* boxes in *RSOE_08665* and

rsp1398 (data not shown), confirming that the Tn7 integration is dependent on the 30-bp *attTn7* box in the RSSC, which is consistent with the fact that the Tn7-based complementation is general in some RSSC strains, although nucleotide sequences of their *glms* downstream are mostly different (Yao and Allen 2007; Zhang et al. 2011, 2019a and b). Intriguingly, the Tn7 integration at 25 bp downstream from *glms*, hereafter referred to as the original Tn7 integration, was also detected in these Tn7 colonies derived from RQ6210 and RQ6213 (Fig. 2B). We assessed whether the original and artificial *attTn7* boxes exhibit similar capacity (i.e., integration frequency) for the Tn7 integration. We generated abundant Tn7 colonies by performing electroporation with pUC18-miniTn7T-Gm on RQ6210 and RQ6213 and picked 100 Tn7 colonies from 50 times of electroporation to detect the Tn7 integration sites in these Tn7 colonies. PCR results confirmed that each colony harbors two kinds of Tn7 integration sites, one at *glms* downstream and the other at downstream of artificial *attTn7* boxes (data not shown), indicating that the Tn7 integration is simultaneous downstream of both original and artificial *attTn7* boxes.

We further performed the whole-genome resequencing on abundant Tn7 colonies to investigate whether unknown Tn7 integration sites occurred in the RSSC. We have generated more than 200 Tn7 colonies based plasmid on pUC18-miniTn7T-Gm for studies of complementation and promoter activity assays during the past several years for our previous studies (Zhang et al. 2011, 2019a and b), and we here generated 300 more Tn7 colonies from 150 times of electroporation. Genomic DNA (gDNA)

Fig. 3. Characterization of Tn7 integration in the *Ralstonia solanacearum* species complex with whole-genome resequencing. **A**, Schematic representation of fused reads from whole-genome resequencing that contain both the Tn7 element and OE1-1 genomic DNA (gDNA). **B**, Mapping of fused reads to the OE1-1 genome. gDNA of 500 Tn7 colonies was isolated with a mixture and was subjected for whole-genome resequencing with an Illumina platform at 400x sequencing depth, which results in about 2 Gb of raw reads. The high-quality sequencing reads were aligned to the reference genome sequence, using BWA software with “bwa mem” mode. After removing PCR-duplication reads, using SAMtools software, the gene coverage reads are calculated by custom perl scripts, and 21 representative fused reads (line 2 to 22, which contain a 1-bp descending Tn7- element) were selected and compared with OE1-1 gDNA (line 1, shaded in green). Nucleotide sequences with red boxes refer to the *attTn7* box and stop codon of OE1-1 *glms*, respectively. Nucleotide sequences shaded in red refer to the junction of OE1-1 DNA and the Tn7 element, and a red arrow points to the Tn7 integration site. **C**, Distribution frequency of reads mapping to different genes. Clean reads with coverage to target genes are calculated by custom perl scripts and are divided by gene length (number of reads per kilobase pair). The Tn7 element refers to a 1,870-bp DNA fragment between Tn7L and Tn7R in pUC18-miniTn7T-Gm, and Gm^R refers to a gentamicin resistance cassette in the Tn7 element, 534 bp in length; *glms*, *hrpB*, and *egl* refer to three representative genes in OE1-1.

of these 500 Tn7 colonies was isolated with a mixture and was entrusted to the Shanghai Biozeron Biotechnology Co., Ltd (Shanghai, China) for whole-genome resequencing with an Illumina platform at 400× sequencing depth, which results in about 2 Gb of raw reads. Tn7 integration sites were then detected by mapping all fused reads containing both the Tn7 element in pUC18- miniTn7T-Gm and gDNA to the OE1-1 chromosome (accession number CP009764.1) and megaplasmid (accession number CP009763.1) (Fig. 3A). The high-quality sequencing reads are aligned to the reference genome sequence using BWA software with “bwa mem” mode. After removing PCR-duplication reads, using SAMtools software, the gene coverage reads are calculated by custom perl scripts. The Tn7 element, minimally containing Tn7L (200 bp), Tn7R (200 bp), and a gentamicin resistance cassette (Gm^r) in pUC18-miniTn7T-Gm, were transposed into gDNA of the RSSC (Figs. 1A and 3A), and reads containing partial Tn7L or Tn7R (more than 20 bp) were extracted from raw reads. All fused reads were mapped to OE1-1 DNA fragments containing the *att*Tn7 box in *glms* (Fig. 3B), confirming that the Tn7 integration is site-specific at 25 bp downstream of *glms* in the RSSC. We further assessed whether integrated Tn7 elements share the same monocopy as the OE1-1 genome by comparing their distribution frequency of mapping reads from the whole-genome resequencing in read numbers per kilobase pair (kbp). For instance, the Gm^r cassette is 534 bp in length and a total of 1,466 reads were mapped to the Gm^r cassette, resulting in a value of 2.745 for the distribution frequency to the Gm^r cassette (Fig. 3C). It is consistent with those of representative genes of *glms*, *hrpB*, and *egl* in OE1-1 (Fig. 3C). Moreover, the DNA fragment between Tn7L and Tn7R in pUC18-miniTn7T-Gm, 1,870 bp in length, exhibits similar distribution frequency as genes of *glms*, *hrpB*, and *egl* in OE1-1 (Fig. 3C), confirming that the integrated Tn7 element, including foreign genes, shares the same monocopy as genomes of the RSSC. Characteristic of the monocopy in genomes, indeed, enables the Tn7-based complementation to fully restore virulence and promoter activities of mutants to those of parent strains in OE1-1 and GMI1000 (Zhang et al. 2011, 2015; 2019a and b), which is more advantageous than those based on plasmids, even with low-copy numbers (Plener et al. 2010; Zhang et al. 2013).

Taken together, we here genetically confirmed that the Tn7-based genomic integration is dependent on the *att*Tn7 box in *glms* and is site-specific with monocopy in the RSSC. This Tn7 integration is generally applicable in the RSSC and provides a versatile tool for genetic studies of complementation, pathogenesis, overexpression, and in-vivo promoter activity assays with monocopy in the RSSC.

AUTHOR-RECOMMENDED INTERNET RESOURCES

BWA software: <http://bio-bwa.sourceforge.net>
SAMtools: <http://samtools.sourceforge.net>

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