Front. Biosci. (Elite Ed) **2026**; 18(1): 39889
https://doi.org/10.31083/FBE39889

Original Research

Small Helper Ti-plasmid Coexisting With the *A281virF* Gene Encoding an F-Box–Like Protein Improves the Efficiency of T-DNA Transfer From *Agrobacterium* Cells to Plant Cells

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Academic Editor: Baohong Zhang
Submitted: 22 April 2025 Revised: 18 July 2025 Accepted: 24 July 2025 Published: 2 February 2026

Abstract

Background: Transient transformation is a convenient and less time-consuming method for investigating gene functions compared with production of stable transformants. Most systems for *Agrobacterium*-mediated plant transformation, regardless of transient or stable transformation, utilize a combination of *Agrobacterium* strains carrying a helper Ti-plasmid and a binary vector. However, the helper Ti-plasmids which are mega-plasmids with sizes of 200–250 kbp and are difficult to manipulate directly with conventional molecular cloning techniques due to their large sizes. **Methods**: A small helper Ti-plasmid, pCU307D with a size of 46 kbp was constructed from pTiEHA101 which is commonly used for plant genetic engineering. They consist of the virulence (vir) region, the mutated replication origin from pTiEHA101, the gentamicin resistance marker and the replication origin for *E. coli* cells. The abilities of T-DNA transfer were examined by transient expression in *Arabidopsis* cultured cells. **Results**: Efficiencies to transfer T-DNA to plant cells by EHA101 and C58C1 carrying pCU307D were similar, although pCU307D had 2.8-fold higher copy numbers than EHA101. The construction processes of pCU307D eliminated the *A281virF* gene encoding F-box-like protein which was located outside of the vir region. The *A281virF* gene was cloned into a binary vector and it was introduced into C58C1 cells with pCU307D which was named as CU307DF. T-DNA transfer efficiencies by EHA101 or CU307DF were examined by GUS activities from transiently transformed T-DNA with the CaMV35S::NLS-GUS cassette in *Arabidopsis* cells. Co-culture with CU307DF carrying the GUS gene conferred 3.2-fold higher GUS activities, compared to that with its parental strain EHA101. **Conclusion**: A new *Agrobacterium* strain CU307DF has higher capacity for T-DNA transfer, compared with its parental strain EHA101. pCU307D is as small as 46 kbp and can propagate in *E. coli*, and that may enable to add further modification to it for further improvement of T-DNA transfer by CU307DF.

Keywords: *Agrobacterium*; Ti-plasmid; T-DNA transfer; *Arabidopsis*; transient expression

1. Introduction

Transgenic plant production is a powerful tool for analyzing gene functions. However, the expression levels in stable transformants depend on the regions of the genome where the transgenes of interest are integrated; therefore, the results complicate the interpretation of transgene expression effects when comparing the phenotypes of transgenic plants [1]. In contrast, transient transformation is a convenient and less time-consuming method for investigating gene functions [2]. The effects caused by transgenes can be compared because they are analyzed before integration into the genome to prevent position-related effects. Currently, agro-infiltration is the most commonly used method for transient expression in plant cells, even though some alternative methods including protoplast transfection, biolistic bombardment, and plant virus infection have been established. Transient expression of exogenous genes in plant cells is also a crucial step for plant genome editing, since it enables to omit a time-consuming step, production of stable transformants. In general genome editing systems, a sequence-specific nuclease such as CRISPR/Cas9 which is transiently expressed creates double-stranded DNA breaks (DSBs) in plant genome and short deletion adjacent to the DSB site is introduced during the process of DSB repairing [3]. However, the efficiencies of gene editing using transient expression of sequence-specific nucleases are not high enough to effortlessly find genome-edited individuals without any selective maker. Raising T-DNA transfer efficiencies is a crucial challenge to improve *Agrobacterium*-mediated genome editing.

Most systems for *Agrobacterium*-mediated plant transformation, regardless of transient or stable transformation, utilize a combination of *Agrobacterium* strains carrying a helper Ti-plasmid and a binary vector [4]. In the systems, the helper Ti-plasmids are disarmed by deletion of T-DNA regions from native Ti-plasmids, on the other hand, binary vectors in which desired genes to be delivered to plant cells as T-strands are inserted between the left border and the right border sequences of T-DNA. In general, the efficiencies of T-DNA transfer depend on the capacity of the helper Ti-plasmids which are mega-plasmids with the sizes of 200–250 kbp and are difficult to manipulate directly with conventional molecular cloning tech-

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niques due to their large sizes. Helper plasmids have been constructed by deleting most parts of the T-DNA region and their resulting sizes are 150–200 kbp. One of the smallest helper Ti-plasmid is pTiLBA4404 (Genbank accession: NZ_KY000037) is still 139,561 bp.

The *virF* gene was originally identified as a gene in the virulence region of nopaline type Ti-plasmid, pTiC58 which was not essential for virulence on most plant species, although it was critical on tomato and tobacco [5]. The *virF* gene encodes F-box protein involved in ubiquitin pathway. The gene product is translocated to plant cells on infection of *Agrobacterium* cells and affects on plant defense system [6–8]. Other type of Ti-plasmids also have genes encoding virF-like F-box proteins [8,9].

In this study, we constructed a new helper Ti-plasmid with a size of only 46 kbp derived from a hyper-virulent Ti-plasmid, pEHA101. It is consisted of the virulence region, the mutated *repABC* gene to increase its copy numbers from pEHA101. In addition, a plasmid carrying the putative *virF* gene from pEHA101 which had been removed during the process to construct the new helper Ti-plasmid improved the efficiencies of T-DNA transfer by *Agrobacterium* cells to plant cells.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

Arabidopsis thaliana Wassilewskija plants were used to produce calli. Root segments from *Arabidopsis* plants were incubated on the callus inducing medium (CIM) for four weeks to form calli. The obtained calli were then ground into small calli (approximately 1 mm in diameter) in a sterile plastic bag with the bottom of a glass bottle and suspended in 10 mL of MS medium; then, one mL of this callus solution was spread onto the CIM. The calli were subcultured every two-week. The calli were grown at 22 °C under continuous light conditions.

The Murashige-Skoog (MS) medium contained MS salts (FUJIFILM Wako Pure Chemical Corp., Japan), Gamborg's B5 vitamins (Nacalai Tesque, Japan) 0.05% MES (FUJIFILM Wako Pure Chemical Corp., Japan), 1% sucrose, and CIM contained Gamborg's B5 salts (FUJIFILM Wako Pure Chemical Corp., Japan), 0.05% MES, 2% glucose, Gamborg's B5 vitamins, 2 µM 2,4-D, and 0.45% gellan gum. The shoot inducing medium (SIM) contained MS medium, 12.5 µM 2-ip, and 0.45% gellan gum.

2.2 Construction of Plasmids

The sequences of all primers used in this study are shown in Table 1.

The VL arm (121681-124216) located downstream of the virulence region in pTiEHA101 (Genbank accession: NZ_KY000035) was amplified by PCR from pTiEHA101 using primers, RI-Sbf-L3 F and BamHI-L3 R, then digested with *Eco*RI and *Bam*HI. The *Eco*RI-*Bam*HI fragment was inserted between the *Eco*RI and *Bam*HI sites in p15AGm (Genbank accession: LC792546), resulting in p15AGm::VL. Then the cos site which had been amplified by PCR from bacteriophage λ using primers, SbfI-cos F and HindIII-cos R followed by digestion with *Eco*RI and *Hind*III was inserted between *Hind*III and *Eco*RI sites in p15AGm-VL, resulted in p15AGm::VL/cos. The VR arm (85498-87041) located upstream of the virulence region in pTiEHA101was amplified from pTiEHA101 using primers, R3-Bg F and R3-Pst R and cloned into pMD20 T-vector (Takara Bio Inc.), giving pMD::VR. In order to insert the cos site to pMD::VR, the cos sequence was amplified using primers, SbfI-cos F and HindIII-cos R followed by digestion with *Sbf*I and *Hind*III was cloned between *Sbf*I and *Hind*III in pUC19. The VR region excised with *Eco*RI and *Xba*I from pMD::VR was inserted between *Eco*RI and *Xba*I sites in pUC19 with the cos sequence, resulted in pMD::VR/cos. The repABC sequence with the mutation Y311H in repB (repH) was generated by fusion PCR. The 5′ part of repH was amplified by PCR using primers BamHI-repABC F and Y311H R from pTiEHA101 and the 3′ part was using primers, Y311H F and SpeI-repABC R. At the second PCR, the 5′ part and the 3′ part were mixed and subjected as templates the fused fragment (repH) was amplified using primers BamHI-repABC F and SpeI-repABC R. The repH fragment was digested with *Bam*HI and *Spe*I and inserted between *Bgl*II and *Xba*I in pMD::VR/cos, resulted in pMD::VR/repH/cos. Wild type repABC gene was also amplified from pTiEHA101 using the primers, BamHI-repABC F and SpeI-repABC R. After digestion with *Bam*HI and *Spe*I, the wild type repABC was also inserted between *Bgl*II and *Xba*I in pMD::VR/cos to generate pMD::VR/repABC/cos. To remove the VR region from pMD::VR/repABC/cos and pMD::VR/repH/cos, the *Bam*HI-*Age*I region including the VR arm and the N-terminal part of repABC or repABC in pMD::VR/repABC/cos or pMD::VR/repH/cos were replaced with the PCR fragments amplified from pMD::VR/repABC/cos, BamHI-repABC F and SpeI-repABC R followed by *Bam*HI and *Age*I digestion, resulting in pMD::repABC or pMD::repH, respectively. p15AGm::VL and pMD::VR/cos were sequentially transformed into EHA101 and integrated into pTiEHA101 by homologous recombination, resulting in pEHA101/VLcos/VRcos. pEHA101/VLcos/VRcos was purified from the *Agrobacterium* cells and was subjected to λ packaging using Lambda-inn *in vitro* packaging kit (Code No. 317-0741, Nippon Gene Co., Ltd., Japan), then the reacted solution was infected to *E. coli* strain DH5α. The resulting plasmid, pTiBoVIR was purified from the *E. coli* cells and transformed into C58C1 carrying pMD::VR/repH/cos and they were integrated by homologous recombination, resulting in pInt-repH/vir/cos. pInt-repH/vir/cos was digested with *Sbf*I, then self-ligated and transformed into DH5α, giving pCU307D. The expression cassette of NLS-GUS was excised with *Hind*III and *Eco*RI

Table 1. Sequences of primers.

Primer name	Sequence (5′-3′)
Atu0972F	CGGAATACAAGGCGCTGGAAACG
Atu0972R	GTCGACAGTGAGGGTATGGGAATATTGC
AvrII-A281virF R	AGAGAGCCTAGGCGTTGAAATCGACGGTATCCCGA
BamHI-L3 R	AGAGAGGATCCGTCTTCGATGAATTGCGTGATCTG
BamHI-repABC F	AGAGAGGATCCGCATCATAATCTCCGCATGAACAG
HindIII-cos R	GAGAGAAGCTTCGTTGACATGAGGTTGCCCCGTAT
PlacIq R	AGAAAATACCGCATCAGGCGCTCTTC
R3-Bg F	GCGATCATGTTGAATGCACCTGATCCTATACC
R3-Pst R	GCTACCACCAGGACGAATCGACATTCATC
repB CF	TTTCGCTCGGGTATTCGAAGCTGTCAC
repB CR	GCGGCCTTTTTGTCGATCATTAACGTCAGG
RI-Sbf-L3 F	AGAGAGAATTCCTGCAGGGACCTTCGTTGAGCAGA
SbfI-cos F	GAGACCTGCAGGATACTCGCACCGAAAATGTCAGC
SpeI-repABC R	AGAGAGACTAGTCTCTTGTCACGGTTAGGAGGCAC
virG qF	TAGGTCGTGAAGATGGGCTTGAGAT
virG qR	TTGCTCCTAGCTCGAGTGCAACAAC
XbaI-trfA F	GAGTTCTAGAATACCAAGTACGAGAAGGACGGCCA
XhoI-A281virF F	GAGAGAACTCGAGTGACCCGGGAATTTGCACCCTT
Y311H F	CGCTCCCAGAAATCCACGCTTCGTTCAAAAGGTCG
Y311H R	CTTTTGAACGAAGCGTGGATTTCTGGGAGCGCCGA

from pBI-NLS/GUS (Genbank accession: PV369457) and inserted between *Hind*III and *Eco*RI in pTK222 (Genbank accession:PV754030), resulting in pTES::NLS-GUS. In order to construct pRK2-A281virF, the *A281virF* gene was amplified by PCR using primers, XhoI-A281virF F and AvrII-A281virF R from pTiEHA101 and then digested with *Xho*I and *Avr*II. The vector backbone was amplified by PCR using primers, XbaI-trfA F and PlacIq R from pT5exRKH (Genbank accession: LC792545), then digested with *Xba*I and *Xho*I. pRK2-A281virF was generated by ligating these fragments.

2.3 Determination of Plasmid Copy Numbers by Quantitative PCR (qPCR)

Twenty ng of total DNA extracted from EHA101 (C58C1 carrying pTiEHA101), C58C1 carrying pMD::repABC, C58C1 carrying pMD::Y311H and C58C1 carrying pCU307D was used as a template for the PCR. PCR was performed on the CFX96™ Real-Time PCR Detection System (BIO-RAD, USA) with Premix Ex Taq™ (Probe qPCR) (RR390A, Takara, JAPAN) according to the manufacturer's instruction. The gene-specific primers were designed to produce 126 (primers; virG qF and virG qR), 117 (primers; repB CF and repB CR) and 146 (primers: Atu0972F and Atu0972R) bp DNA fragments from the genes of *virG*, *repB* in Ti-plasmids and *Agrobacterium Atu0972* gene used as an internal control, respectively. The sequences of the primers are shown in Table 1. Relative copy numbers were calculated by normalization with those of the *Atu0972* gene.

2.4 Transient Transformation

Arabidopsis calli were freshly broken into fine pieces as described in 2.1. *Agrobacterium* cells were co-cultured with the crushed calli for 48 hours, then the calli were washed with sterilized water three times. The washed calli were incubated on CIM with 100 µg/mL of carbenicillin for a further 24 hours to allow transient expression in plant cells.

2.5 GUS Staining and MUG Assay

The expression assay of GUS reporter gene was done by GUS histochemical assay and MUG assay in *Arabidopsis* cells. For GUS histochemical assay, the transiently transformed *Arabidopsis* cells were incubated at 37 °C for 4 hours in the GUS staining solution (50 mM Na_3PO_4 pH 7.2, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.1% Triton X-100, 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 2mM $MgCl_2$). After incubation, the buffer was removed and added with a stop buffer containing 50 mM MES and 50 mM KCl. Microscopic analysis was done to determine differences between *Agrobacterium* strains. For the MUG assay, the *Arabidopsis* calli were added with 1x Tris Buffered Saline (TBS) and 0.1% Triton x-100. These were squeezed using the disposable homogenizer. This was centrifuge at 15,000 rpm for 10 minutes and the supernatant was transferred into a new tube. After, 5 µL of the extract was incubated at 37 °C for 1 hour in 50 µL 4-methylumbelliferyl β-D-Glucuronide (MUG) assay buffer (50 mM Sodium phosphate pH 7.0, 1 mM MUG, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton x-100). The reaction was stopped by adding 945 µL of 0.2 M

Fig. 1. Shoot regeneration from *Arabidopsis* calli. Routinely subcultured calli on day 14 after the last subculture were transferred to shoot inducing medium (SIM). Images show identical calli on the SIM. (A) Calli on day 0 after transfer to SIM. (B,C) Calli on day 14 after transfer to SIM. Plate with the calli on the indicated day after transfer to the SIM. Each picture was taken from the table side (B) or the other side (C). (D) Area with black frame in (B). Bar = 2 mm.

Na_2CO_3. Fluorescence was determined using 25 µL of the solution in microplate reader with excitation at 365 nm and emission at 455 nm.

3. Results

3.1 Regeneration Capacity of Arabidopsis Cultured Tissues

We used the *Arabidopsis* ecotype Wassilewskija (WS) for our tissue culture experiments, as Czakó *et al*. [10] have reported that cultured WS cells have the highest efficiency of regenerating shoots among the examined ecotypes. It is known that long-term incubation of *Arabidopsis* calli on CIM containing 2,4-D inhibits shoot regeneration in SIM containing cytokinin [11]. Our previous study demonstrated that a high cell density of cultured cells caused by extended incubation on CIM had an inhibitory effect on shoot regeneration [12]. To confirm the shoot-regenerating ability of calli that had been maintained for a long time, calli derived from *Arabidopsis* WS root segments by culturing on CIM were subcultured every two weeks for 26 months. Ground calli (see 2.1) were then grown on CIM for two weeks and then transferred onto SIM (Fig. 1). A large num-

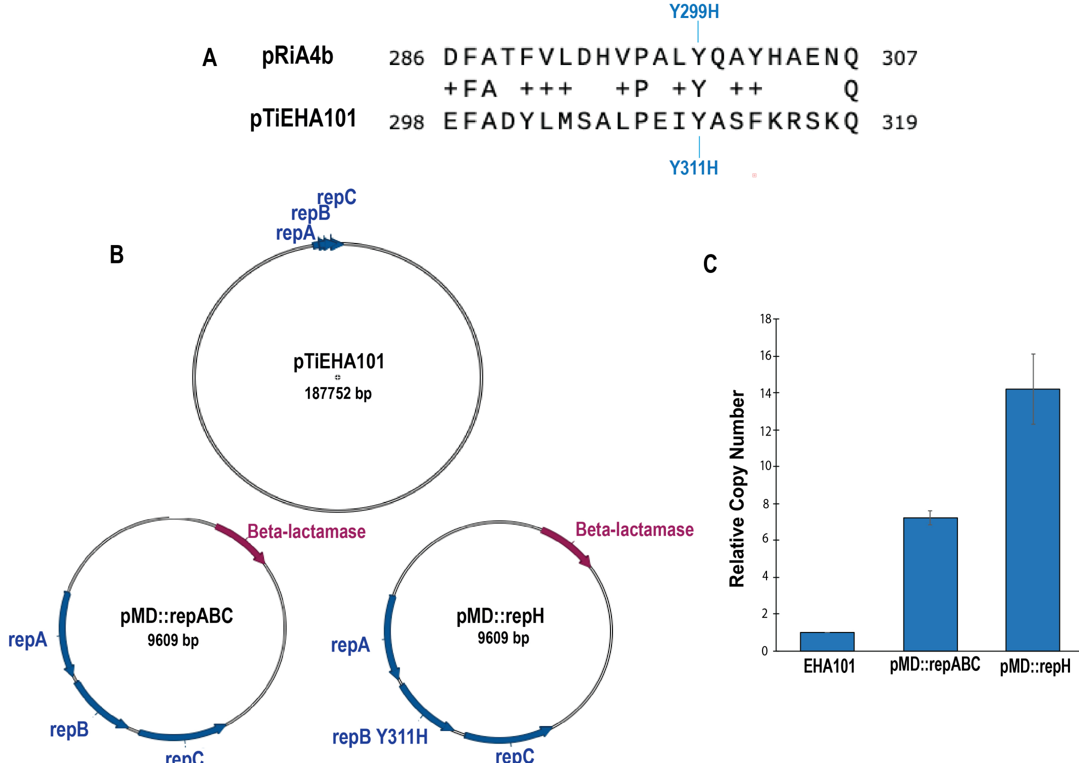


Fig. 2. Relative copy numbers of pTiEHA101-derivatives in *Agrobacterium* cells. (A) Alignment of wildtype RepB protein sequences in pRiA4b and pTiEHA101 corresponding to mutation sites Y299H and Y311H. (B) Schematic structures of plasmids used in the experiments. EHA101; pTiEHA101, pMD::repABC; a small-sized plasmid with wild type repABC, pMD::repH; a small-sized plasmid with repH. (C) The relative copy numbers were determined by qPCR. The vertical axis shows the plasmid copy number. Relative copy numbers are shown as normalized values to those of EHA101. Experiments were individually performed three times. Error bars represent standard error of the mean.

ber of shoots were formed on calli that had grown larger. More shoots formed on parts of the calli in contact with the medium (Fig. 1C) compared to those on aerial parts. These results clearly showed that calli that had been subcultured for a long period retained their ability to regenerate shoots on SIM. We chose *Arabidopsis* calli as plant materials for transient expression experiments since they can be able to be used for model experiments for genome editing.

3.2 Introduction of a Mutation Which Increases Copy Numbers to the Replication Origin of pTiEHA101

In order to improve the efficiencies of T-DNA transfer by *Agrobacterium*, we attempted to increase the copy numbers of Ti-plasmids in *Agrobacterium* cells. Ti-plasmids are megaplasmids with sizes of 200–250 kbp and have low copy numbers. The replication origins of Ti-plasmids are the repABC operons which are commonly found in alfaproteobacteria plasmids [13,14]. EHA101 is called the hypervirulence strain [15] and we chose this strain as the initial material to manipulate. It had been reported that a mutation, Y299H in the *repB* gene in pRiA4b increased the copy numbers of the plasmids and the alignment of amino acid sequences from several *repABC* revealed that the tyrosine residue was conserved among a variety of repB proteins [16]. We introduced the same amino acid change, tyrosine to histidine in the *repB* gene in pTiEHA101 (originated from pTiBo542) (Fig. 2A). Fig. 2B,C shows the comparison of copy numbers in pTiEHA101/C58C1 (common strain name, EHA101), pMD::repABC/C58C1 (wild type repABC gene) and pMD::repH (repH: repABC with the mutation Y311H), determined by qPCR. The copy numbers of pMD::repABC were approximately 7.2-fold higher than those of pTiEHA101, probably owing to the difference of their sizes. The size of pMD::repABC is 6.8 kbp, while that of pTiEHA101 is 189 kbp, suggesting plasmids with smaller sizes give more copy numbers compared to those with larger sizes and the identical replication origin. In addition, introduction of the mutation, Y311H gave approximately twice copy numbers, compared with wild-type repABC and 14.2-fold, and 7.2-fold, compared with pTiEHA101 and pMD::repABC, respectively. The increased ratios caused by the Y311H mutation were much lower (approximately 2.0-fold) than the Y299H mutation (approximately 10-fold) in pRi repABC [16] when plasmids with the same sizes were examined. The effects on copy

Fig. 3. Schematic diagram of pCU307D construction. Abbreviations indicate the followings: cos, the recognition sequence by lambda terminase; AmpR, the ampicillin/carbenicillin resistance gene; VR, homologous sequence to pTiEHA101 located at 5′ of the vir region; VL, homologous sequence to pTiEHA101 located at 3′ of the vir region; RepH, the replication origin for *Agrobacterium* cell; *Sbf*I, the recognition sequence by *Sbf*I; p15 ori and pUC ori, the replication origins for *E. coli* cells.

numbers by reduced sizes were much greater than those by the Y311H mutation.

3.3 T-DNA Transfer Efficiencies by C58C1 Carrying pCU307D

These results led us to make the size of the helper Ti-plasmid reduce by cloning only the virulence (vir) region. According to information reported previously [15], we designed the strategy to clone the vir region by introducing two cos sites at the ends of the vir region, a selectable marker gene and the p15A replication origin for *E. coli*, then packaging as λ phage particles since its size was close to λ phage genome. As shown Fig. 3, pTiBoVIR (approximately 41 kbp) was successfully constructed. Then the repH operon was also integrated into pTiBoVIR, resulting in pCU307D. The copy numbers of pCU307D in *Agrobacterium* strain, C58C1 were 2.8-fold higher compared with those of pTiEHA101 in C58C1 (EHA101) (Fig. 4). Their T-DNA transfer efficiencies were examined by transient expression of β-glucuronidase (GUS) in *Arabidopsis* calli after co-culture with EHA101 or CU307D (C58C1 with pCU307D) carrying the reporter plasmid, pTES::NLS-GUS. The GUS coding sequence with a nuclear localization sequence at its N-terminal to prevent the products from diffusion to adjacent cells through plasmodesmata was used for transient expression in plant cells (Fig. 5A). GUS activities in *Arabidopsis* cells after co-culture with EHA101 or CU307D carrying pTES::NLS-GUS were similar against our expectation that higher copy numbers of the *vir* genes would confer more efficient T-DNA transfer (Fig. 5B), although these results demonstrated that indeed, only the vir region was sufficient to transfer T-DNA into plant cells.

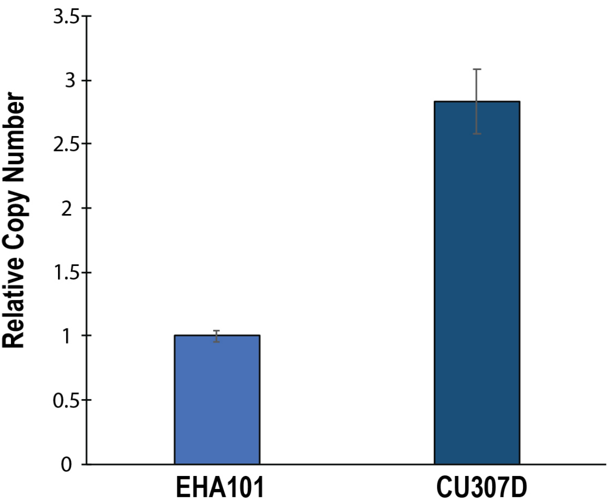
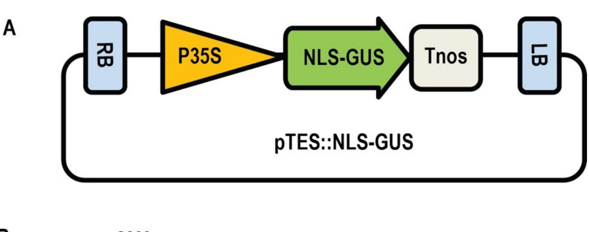


Fig. 4. Determination of relative copy numbers of pCU307D. The relative copy numbers were determined by qPCR. Relative copy numbers were calculated as normalized values to those of EHA101. Experiments were individually performed three times. Error bars represent standard error of the mean.



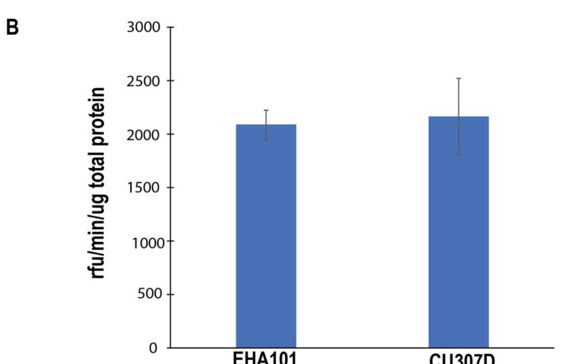


Fig. 5. T-DNA transfer efficiencies by EHA101 and CU307D. (A) A schematic diagram of the GUS expression construct where the T-DNA region is flanked by Right Border (RB) and Left Border (LB). The T-DNA transfer efficiencies were examined by transient expression of β -glucuronidase (GUS) in *Arabidopsis* cells. The construct contains the cauliflower mosaic virus 35S promoter (P35S) driving expression of the β-glucuronidase (GUS) coding sequence fused to a nuclear localization signal (NLS-GUS) followed by the nopaline synthase terminator (Tnos). (B) GUS activity in *Arabidopsis* cells co-cultured with EHA101 and CU307D carrying pTES::NLS-GUS was quantified using the MUG assay. Experiments were individually performed three times. Error bars represent standard error of the mean.

3.4 Effects of the A281virF Gene Encoding an F-Box Protein on T-DNA Transfer by pCU307D

F-box protein encoded by the *virF* gene in pTiC58 has been reported to enhance transformation efficiencies by *Agrobacterium* strain, C58 [17]. The *virF* gene is located in the vir region of pTiC58, while a similar gene encoding an F-box protein (165435-166301 in Genbank accession: NZ_KY000035) in agropine-type Ti-plasmids is located outside of the vir region [9]. In order to examine the effects of the *A281virF* gene encoding the F-box protein in pTiEHA101 (derived from pTiBo542 in *Agrobacterium* strain, A281) on T-DNA transfer, a plasmid carrying the gene, pRK2-A281virF was introduced into C58C1 with pCU307D, since it had been eliminated during construction of pCU307D from pTiEHA101. Introduction of pRK2-A281virF into C58C1 carrying pCU307D clearly enhanced transient expression of GUS in plant cells to 3.2 higher (Fig. 6) and the rates are similar to those of their copy numbers. We were not able to judge that the enhancement was caused by more efficient infection by *Agrobacterium* cells or larger amounts of T-strand transportation from the pictures of GUS staining. We named C58C1 carrying pCU307D and pRK2-A281virF as CU307DF.

4. Discussion

We investigated several factors which potentially affected on T-DNA transfer by *Agrobacterium* cells. Smaller sizes of helper Ti-plasmids gave positive effects on copy numbers of Ti-plasmids (Fig. 2). A mutation in the *repB* gene also had positive effects on T-DNA transfer (Figs. 2,4). Copy numbers of pCU307D were 2.8-fold higher than those of its parental plasmid, pTiEHA101 (Fig. 4). In general higher copy numbers of plasmids enable higher expression of genes carried by the plasmids. However, efficiencies of T-DNA transfer examined by expression of the reporter gene in *Arabidopsis* cells were similar (Fig. 5). The positive effects of higher copy numbers on T-DNA transfer might be offset by deletion of the *A281virF* gene. In fact, the *A281virF* enhanced T-DNA transfer 2.8-fold in GUS activities when the 35S::NLS-GUS was transiently expressed in plant cells, although molecular mechanisms of enhanced T-DNA transfer by *A281virF* have not been investigated. This enhancement may reflect net effects of copy numbers. Since most genes in pTiBo542 except the *vir* genes and the *repABC* gene had been eliminated in pCU307D, the enhancement of T-DNA transfer may suggest expression levels of the *vir* genes caused by the higher copy numbers. The *virF* gene in pTiC58 encodes an F-box protein which is thought to be involved in degradation of plant defence proteins (reviewed by Magori and Citovsky [17,18]). Although functional analyses of *A281virF* have not reported, structural similarities of these *virF* products may suggest their common mechanisms in T-DNA transfer by *Agrobacterium*.

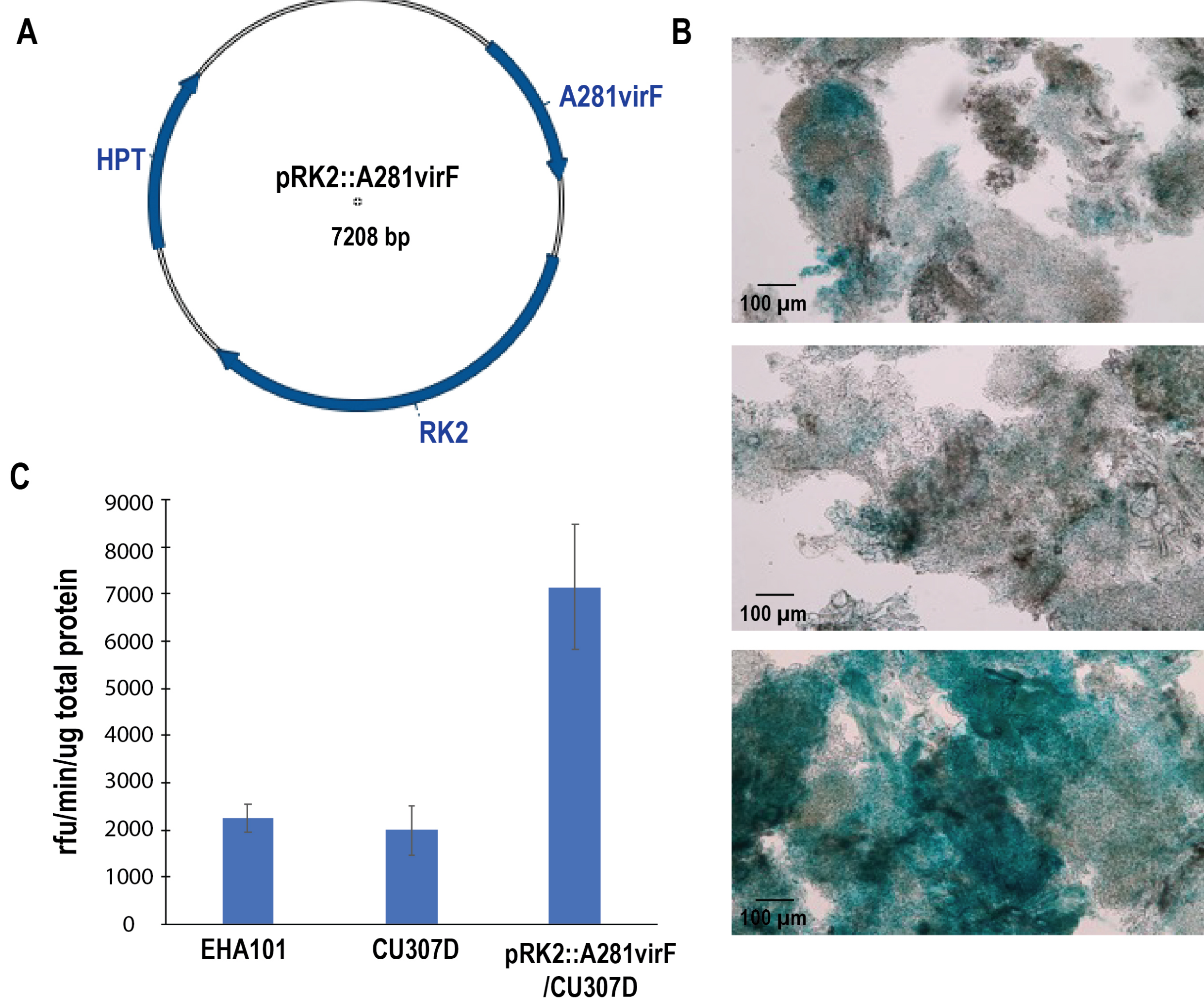


Fig. 6. Effects of the *A281virF* gene on T-DNA transfer efficiencies. Transient expression of GUS was examined after co-culture of *Arabidopsis* cells and *Agrobacterium strains* indicated. All strains carry pTES::NLS-GUS shown Fig. 5A. (A) The *A281virF* gene was cloned into a vector with RK2 (the replication origin for *Agrobacterium*) and HPT (the hygromycin resistance gene). (B) Transient expressions of GUS in *Arabidopsis* cells were visualized by X-gluc staining. Scale bars indicates 100 µm. (C) Quantitative GUS activities in *Arabidopsis* calli. Experiments were individually performed three times. Error bars represent standard error of the mean.

Most binary vectors have the kanamycin resistance gene or the spectinomycin/streptomycin resistance gene as a selective marker. We confirmed CU307DF is sensitive to kanamycin and spectinomycin/streptomycin, therefore, CU307DF is compatible with a variety of binary vectors. We merely developed our transformation system for transient transformation, although it may have disadvantages for stable transformation of plant cells, owing to its high capacity to transfer T-DNA which may cause multiple integration of transgenes. Recently, gene knock-in by transient transfer of the CRISPR/Cas9 system and the donor DNA in protoplast of *Nicotiana tobacum* has been reported [19]. However, production of intact protoplasts requires special skills and are not necessarily applicable to many plant species. If abilities to transfer T-DNAs by *Agrobacterium* cells can be drastically improved, it might be going to be possible to transfer T-DNAs including a sequence encoding a sequence-specific nuclease and a donor DNA with homology surrounding the target sequence by the nuclease, leading to establish strategies for knock-ins in plant cells. Our results may be able to the initial step for the strategies mediated by *Agrobacterium*.

At the initial stage of construction of new helper Ti-plasmids, we attempted the strategy, *Sbf*I-digestion followed by self-ligation, since there was no *Sbf*I site in the vir region in pTiEHA101. However, the attempt was not successful, probably owing to a large amount of non-related DNAs including other parts of pTiEHA101 than the vir region and a megaplasmid, pAtC58 carried by EHA101. Then we realized that the size of the vir region was close to the size which can be accepted by λ packaging. The resulting plasmid, pTiBoVIR has one cos site and one *Sbf*I

site outside the vector backbone for cloning in *E. coli* cells, that enabled integration of the repH operon (Fig. 5). We were able to use both λ packaging and *Sbf*I-digestion/self-ligation options, *Sbf*I-digestion/self-ligation strategy was chosen, owing to cost performance. In addition, pTiBoVIR or pCU307D may be good tools to analyze functional analyses of genes in Ti-plasmids, since original Ti-plasmids are as large as 200–250 kbp and contain large numbers of genes involved in opine synthesis, replication, conjugation, or quorum-sensing, other than the T-DNA transfer machinery [20]. Large Ti-plasmids are difficult to manipulate directly with conventional molecular techniques. By adding other components to host strains, the small helper Ti-plasmid may enable to analyze functions of the specific genes involved in T-DNA transfer. In fact, advantages of utilizing the *A281virF* were demonstrated using our new helper plasmid in this study.

5. Conclusions

A newly produced *Agrobacterium* strain, CU307DF (C58C1 carrying pCU307D and pRK2::A281virF) had enhanced abilities to transfer T-DNA, compared with a conventional hypervirulent strain, EHA101. We consider that CU307DF is a good option as the host strain to deliver constructs for transient expression in plant cells.

Availability of Data and Materials

The data and materials used in this study are available from the corresponding author.

Author Contributions

HB conducted the whole research and wrote the manuscript. NFHA performed all experiments except qPCR and made the figures. MA designed and conducted determination of plasmid copy numbers. NT performed qPCR experiments. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Seeds of *Arabidopsis thaliana* ecotype Wassilewskija was obtained from Arabidopsis Biological Resource Center, Ohio State University 24 years ago.

Acknowledgment

We thank Dr. Ktsunori Suzuki for useful informations about *Agrobacterium* plasmids.

Funding

This research is supported by the Sengoku Private Fund (CU2000).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Matzke AJ, Matzke MA. Position effects and epigenetic silencing of plant transgenes. Current Opinion in Plant Biology. 1998; 1: 142–148. https://doi.org/10.1016/s1369-5266(98)80016-2.

[2] Tyurin AA, Suhorukova AV, Kabardaeva KV, Goldenkova-Pavlova IV. Transient Gene Expression is an Effective Experimental Tool for the Research into the Fine Mechanisms of Plant Gene Function: Advantages, Limitations, and Solutions. Plants. 2020; 9: 1187. https://doi.org/10.3390/plants9091187.

[3] Kaur M, Manchanda P, Kalia A, Ahmed FK, Nepovimova E, Kuca K, *et al.* Agroinfiltration Mediated Scalable Transient Gene Expression in Genome Edited Crop Plants. International Journal of Molecular Sciences. 2021; 22: 10882. https://doi.org/10.3390/ijms221910882.

[4] Lee LY, Gelvin SB. T-DNA binary vectors and systems. Plant Physiology. 2008; 146: 325–332. https://doi.org/10.1104/pp.107.113001.

[5] Hooykaas PJ, Hofker M, den Dulk-Ras H, Schilperoort RA. A comparison of virulence determinants in an octopine Ti-plasmid, a nopaline Ti-plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium* tumefaciens mutants. Plasmid. 1984; 11: 195–205. https://doi.org/10.1016/0147-619x(84)90026-x.

[6] Zaltsman A, Krichevsky A, Loyter A, Citovsky V. *Agrobacterium* induces expression of a host F-box protein required for tumorigenicity. Cell Host & Microbe. 2010; 7: 197–209. https://doi.org/10.1016/j.chom.2010.02.009.

[7] Lacroix B, Citovsky V. Nopaline-type Ti-plasmid of *Agrobacterium* encodes a VirF-like functional F-box protein. Scientific Reports. 2015; 5: 16610. https://doi.org/10.1038/srep16610.

[8] García-Cano E, Hak H, Magori S, Lazarowitz SG, Citovsky V. The *Agrobacterium* F-Box Protein Effector VirF Destabilizes the *Arabidopsis* GLABROUS1 Enhancer/Binding Protein-Like Transcription Factor VFP4, a Transcriptional Activator of Defense Response Genes. Molecular Plant-Microbe Interactions. 2018; 31: 576–586. https://doi.org/10.1094/MPMI-07-17-0188-FI.

[9] Shao S, Zhang X, van Heusden GPH, Hooykaas PJJ. Complete sequence of the tumor-inducing plasmid pTiChry5 from the hypervirulent *Agrobacterium* tumefaciens strain Chry5. Plasmid. 2018; 96-97: 1–6. https://doi.org/10.1016/j.plasmid.2018.02.001.

[10] Czakó M, Wilson J, Yu X, Márton L. Sustained root culture for generation and vegetative propagation of transgenic *Arabidopsis* thaliana. Plant Cell Reports. 1993; 12: 603–606. https://doi.org/10.1007/BF00232807.

[11] Valvekens D, Van Montagu M, Van Lijsebettens M. *Agrobacterium* tumefaciens-mediated transformation of *Arabidopsis* thaliana root explants by using kanamycin selection. Proceedings of the National Academy of Sciences of the United States of America. 1988; 85: 5536–5540. https://doi.org/10.1073/pnas.85.15.5536.

[12] Matsuo N, Mase H, Makino M, Takahashi H, Banno H. Identification of ENHANCER OF SHOOT REGENERATION 1-upregulated genes during in vitro shoot regeneration. Plant Biotechnology. 2009; 26: 385–393. https://doi.org/10.5511/plantbiotechnology.26.385.

[13] Cevallos MA, Cervantes-Rivera R, Gutiérrez-Ríos RM. The repABC plasmid family. Plasmid. 2008; 60: 19–37. https://doi.org/10.1016/j.plasmid.2008.03.001.

[14] Cervantes-Rivera R, Pedraza-López F, Pérez-Segura G, Cevallos MA. The replication origin of a repABC plasmid. BMC Microbiology. 2011; 11: 158. https://doi.org/10.1186/

1471-2180-11-158.

[15] Hood EE, Helmer GL, Fraley RT, Chilton MD. The hypervirulence of *Agrobacterium* tumefaciens A281 is encoded in a region of pTiBo542 outside of T-DNA. Journal of Bacteriology. 1986; 168: 1291–1301. https://doi.org/10.1128/jb.168.3.1291-1301.1986.

[16] Vaghchhipawala Z, Radke S, Nagy E, Russell ML, Johnson S, Gelvin SB, *et al*. RepB C-terminus mutation of a pRi-repABC binary vector affects plasmid copy number in *Agrobacterium* and transgene copy number in plants. PLoS ONE. 2018; 13: e0200972. https://doi.org/10.1371/journal.pone.0200972.

[17] Magori S, Citovsky V. Hijacking of the Host SCF Ubiquitin Ligase Machinery by Plant Pathogens. Frontiers in Plant Science. 2011; 2: 87. https://doi.org/10.3389/fpls.2011.00087.

[18] Magori S, Citovsky V. The role of the ubiquitin-proteasome system in *Agrobacterium* tumefaciens-mediated genetic transformation of plants. Plant Physiology. 2012; 160: 65–71. https://doi.org/10.1104/pp.112.200949.

[19] Li Y, Huang C, Liu Y, Zeng J, Yu H, Tong Z, *et al*. CRISPR/Cas9-mediated seamless gene replacement in protoplasts expands the resistance spectrum to TMV-U1 strain in regenerated Nicotiana tabacum. Plant Biotechnology Journal. 2023; 21: 2641–2653. https://doi.org/10.1111/pbi.14159.

[20] Hooykaas PJJ. The Ti-plasmid, Driver of *Agrobacterium* Pathogenesis. Phytopathology. 2023; 113: 594–604. https://doi.org/10.1094/PHYTO-11-22-0432-IA.