Applied Microbiology and Biotechnology (2018) 102:1823–1836
https://doi.org/10.1007/s00253-017-8721-7

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Effective removal of a range of Ti/Ri plasmids using a pBBR1-type vector having a *repABC* operon and a *lux* reporter system

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Received: 9 October 2017 / Revised: 9 December 2017 / Accepted: 15 December 2017 / Published online: 9 January 2018

Abstract

Ti and Ri plasmids of pathogenic *Agrobacterium* strains are stably maintained by the function of a *repABC* operon and have been classified into four incompatibility groups, namely, incRh1, incRh2, incRh3, and incRh4. Removal of these plasmids from their bacterial cells is an important step in determining strain-specific virulence characteristics and to construct strains useful for transformation. Here, we developed two powerful tools to improve this process. We first established a reporter system to detect the presence and absence of Ti/Ri plasmids in cells by using an acetosyringone (AS)-inducible promoter of the *Ti2* small RNA and *luxAB* from *Vibrio harveyi*. This system distinguished a Ti/Ri plasmid-free cell colony among plasmid-harboring cell colonies by causing the latter colonies to emit light in response to AS. We then constructed new "Ti/Ri eviction plasmids," each of which carries a *repABC* from one of four Ti/Ri plasmids that belonged to incRh1, incRh2, incRh3, and incRh4 groups in the suicidal plasmid pK18*mobsacB* and in a broad-host-range pBBR1 vector. Introduction of the new eviction plasmids into *Agrobacterium* cells harboring the corresponding Ti/Ri plasmids led to Ti/Ri plasmid-free cells in every incRh group. The Ti/Ri eviction was more effective by plasmids with the pBBR1 backbone than by those with the pK18*mobsacB* backbone. Furthermore, the highly stable cryptic plasmid pAtC58 in *A. tumefaciens* C58 was effectively evicted by the introduction of a pBBR1 vector containing the *repABC* of pAtC58. These results indicate that the set of pBBR1-*repABC* plasmids is a powerful tool for the removal of stable rhizobial plasmids.

Keywords Ti plasmid · Plasmid curing · *Agrobacterium* · Plasmid incompatibility · Luciferase reporter

Introduction

Pathogenic *Agrobacterium* strains induce tumor or hairy root formation by introducing a DNA fragment of their Ti/Ri plasmids, called T-DNA, into plant genomes. This unique DNA transfer system has been utilized for the transformation of various plants and fungi (Tzfira and Citovsky 2006; Lacroix et al. 2006). The *Agrobacterium*-mediated transformation (AMT) was performed exclusively using a few popular strains such as *Agrobacterium tumefaciens* C58 and LBA4404 strains as donor cells. However, when strains such as *Ensifer meliloti* and *Mesorhizobium loti* are given a Ti plasmid, they can also transfer T-DNA to model plants with efficiencies high enough for practical use (Broothaerts et al. 2005; Wendt et al. 2011). Hairy roots induced by T-DNA transfer from *A. rhizogenes* have been utilized as genetically stable cultured cells to produce valuable plant secondary metabolites (Ono and Tian 2011).

Plant transformation by *Agrobacterium* and related species is growing in significance. Examining, comparing, and evaluating the transformation characteristics of various strains of *Agrobacterium*, *Rhizobium*, and related species are necessary to enhance the transformation efficiency and extend the range of target plant species, including those that are recalcitrant to AMT by commonly used strains. Genes associated with or affecting T-DNA transfer are located on not only Ti/Ri plasmids but also chromosomal DNA and accessory plasmids (Suzuki et al. 2009; Slater et al. 2008; Nair et al. 2003). A combination between Ti/Ri plasmids and their host's chromosomal background also significantly affects their pathogenicity (Hood et al. 1986; Kiyokawa et al. 2009; Kovács and Pueppke 1993). Examining the contributions of each Ti/Ri

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plasmid, host chromosomal backgrounds, and their combination to virulence is essential to entirely evaluate their pathogenicity. Curing Ti/Ri and other cryptic plasmids from host strains is an essential step in this process.

Ti/Ri plasmids are classified based on their incompatibility properties, which are practically determined by a *repABC* locus (Otten et al. 2008; Pappas and Cevallos 2011; Christie and Gordon 2014). Generally, plasmids belonging to the same incompatibility group cannot be comaintained stably in a cell without external selective pressure (Austinand Nordström 1990). The *repABC* locus is responsible for stable inheritance of Ti/Ri and many cryptic plasmids in *Rhizobiaceae*, such as pAtC58 in *A. tumefaciens* C58. The *repABC* locus consists of partitioning and replication genes (*repABC*), a small counter-transcribed RNA (ctRNA), and a centromere-like sequence (*parS*) (Cevallos et al. 2008; Pinto et al. 2012). All components work to cause incompatibility (Ramírez-Romero et al. 2001; Pappas and Winans 2003; Cervantes-Rivera et al. 2011; Soberón et al. 2004; Venkova-Canova et al. 2004; Chai and Winans 2005; Cervantes-Rivera et al. 2010; Pérez-Oseguera and Cevallos 2013). Ti/Ri plasmids have been classified into four incompatibility groups, incRh1, incRh2, incRh3, and incRh4 (Nester and Kosuge 1981; Otten et al. 2008). pTiC58 and pTiA6 in the *A. tumefaciens* C58 and A6 strains, respectively, belong to incRh1 (Hooykaas et al. 1980; Knauf and Nester 1982), pTiBo542 in *A. tumefaciens* Bo542 belongs to incRh2 (Hood et al. 1986), pRiA4b in *A. rhizogenes* A4 belongs to incRh3 (White and Nester 1980), and pTiS4 in *A. vitis* belongs to incRh4 (Szegedi et al. 1996). However, the incompatibility traits of many Ti/Ri plasmids have not been analyzed yet.

Ti/Ri plasmids were cured from bacterial cells by culturing at a high temperature or introducing a small incompatibility plasmid, called an "eviction plasmid" (Hamilton and Fall 1971; Van Larebeke et al. 1974; Uraji et al. 2002). The introduction of the eviction plasmid is considered a less deleterious method for host bacterial cells. The eviction plasmid contains an antibiotic resistance gene, a counter-selection marker, and a *repABC* operon belonging to the same incompatibility group as the target Ti/Ri plasmid. After the introduction of the eviction plasmid into Ti/Ri-containing cells, the Ti/Ri plasmids are either lost from the host or retained as a plasmid amalgamated with the eviction plasmid (Yamamoto et al. 2007). In our previous report, we improved an eviction plasmid by adding an antitoxin gene to disable the stabilization of toxin-antitoxin (TA) genes present on the target Ti/Ri plasmid (Yamamoto and Suzuki 2012). However, this eviction plasmid is applicable to curing only an incRh1-group plasmid. In this study, we introduced the *repABC* segments of incRh2, incRh3, and incRh4 Ti/Ri plasmids into two types of vector plasmids, pK18*mobsacB* (suicidal) and pBBR1 (replicable in multiple copies in *Agrobacterium*). This new series of eviction plasmids successfully cured Ti/Ri plasmids belonging to their corresponding incompatibility groups in most agrobacterial strains. In addition, the pBBR1-type eviction plasmids cured even very stable *repABC* plasmids, including pAtC58 in the *A. tumefaciens* C58 strain.

Materials and methods

Bacterial strains, media, and culture conditions

The bacterial strains used in this study are summarized in Table 1. *Escherichia coli* HB101 (37 °C) and *A. tumefaciens* C58 (ATCC® 33970™) and its derivative strains (28 °C) were cultured in LB medium. *A. rhizogenes* A4 (ATCC® 43057™) and *A. vitis* S4 (ATCC® BAA-846™) were cultured at 28 °C in IFO medium 702 (Tanaka et al. 2009). AB minimal medium (Chilton et al. 1974) was used for the selection of *A. vitis* S4 in conjugation experiments. The AB-C solution, used for the suspension and dilution of agrobacterial cells, is an AB minimal medium that lacks a carbon source. An AB induction medium (Wise et al. 2006) was used to induce expression of a virulence gene reporter. Antibiotics were added to media as needed at the following final concentrations: ampicillin (50 mg/L), gentamicin (30 mg/L), kanamycin (50 mg/L), nalidixic acid (30 mg/L), rifampicin (30 mg/L), and spectinomycin (100 mg/L for *E. coli*, 600 mg/L for *A. tumefaciens* C58).

Plasmid construction

Plasmids and oligonucleotides used in the present study are listed in Tables 2 and 3, respectively.

To construct a reporter plasmid to detect Ti/Ri-free agrobacterial cells, we chose the promoter regions of *virE* and *Ti2* genes to function as activated VirG-dependent promoters. The 315-bp region directly upstream of the *virE* operon was PCR-amplified using two oligonucleotides, PvirE_GFPUV_Fw and PvirEGUS_Rv, as primers and pTi-SAKURA as template DNA. The *virE* promoter fragment was digested by *Hin*dIII and *Xba*I and ligated with pGFPuv that had been cut by the same restriction enzymes. The resultant plasmid, which contains the *virE* promoter segment upstream of the *GFPuv* gene and in the same direction, was named pPvirEGFPuv. This plasmid was digested with *Eco*RI, and the resulting small *Eco*RI fragment, containing GFPuv with the *virE* promoter, was ligated with a large pSRKGm *Eco*RI fragment, which includes *rep*, *mob*, and a gentamicin resistance gene. The resulting plasmid was named pSRKGmPvirEGFPuv. The 227-bp region directly upstream of the *Ti2* gene was amplified by PCR using the oligonucleotides Ti2Fw and Ti2Rv as primers and pTiC58 as template DNA. The *Ti2* promoter fragment was digested by *Xba*I and inserted at a unique *Xba*I site in pGFPuv. The resultant plasmid, which contains the *Ti2* promoter

Table 1 Bacterial strains used in the present study

Strain	Relevant characteristics	Reference or source
A. tumefaciens C58rif	Pathogenic tumor-inducing strain harboring pTiC58; Rif^r	Goodner et al. (2001)
A. tumefaciens C58C1	Ti-less strain of C58rif; Rif^r	Our collection
A. tumefaciens EHA105	C58C1 harboring pTiEHA105; Rif^r	Hood et al. (1993)
A. rhizogenes A4	Pathogenic root-inducing strain harboring pRiA4b; Nal^r	Moore et al. (1979)
A. vitis S4	Pathogenic tumor-inducing strain harboring pTiS4	Szegedi et al. (1988)
E. coli HB101	Host strain for plasmid construction and donor/helper strain for conjugation; *mtl*-1 Sm^r	Our collection

upstream of *GFPuv* and in the same direction, was named pPTi2GFPuv. pPTi2GFPuv was digested with *Eco*RI, and the resultant small *Eco*RI fragment, which contained the *Ti2* promoter and *GFPuv*, was ligated with the pSRKGm *Eco*RI large fragment as was performed in the pSRKGmPvirEGFPuv construction; the resultant plasmid was named pSRKGmPTi2GFPuv. Since the *Ti2* gene is a small RNA, a ribosome binding site (RBS) must be added upstream of the *GFPuv* start codon to express protein. To use PCR to amplify the *GFPuv* gene with an RBS sequence that is functional in agrobacterial cells, we designed oligonucleotides AtRBSgfpFw and GFPuvRv to function as primers. AtRBSgfpFw contains an *Xba*I restriction site and an RBS (5′-GGAGGA-3′) at the 5′ end and a 28-bp homologous sequence identical to the region downstream of the start codon of the *GFPuv*. GFPuvRv is homologous with the 23 nucleotides downstream of the stop codon of *GFPuv*. The RBS-*GFPuv* fragment, which includes *Xba*I and *Sac*I restriction sites immediately upstream of the RBS and close to the end of the *GFPuv* gene, respectively, was amplified by PCR using these primers and template pGFPuv. An *Xba*I-*Sac*I fragment derived from the RBS-*GFPuv* segment was ligated with the *Xba*I-*Sac*I large fragment of pSRKGmPTi2GFPuv. The large fragment lacks the region in pSRKGmPTi2GFPuv from the *Xba*I restriction site immediately downstream of the *Ti2* promoter to the *Sac*I restriction sites close to the end of the *GFPuv* gene. The resultant plasmid, which contains the *Ti2* promoter and RBS followed by the *GFPuv* gene, was named pSRKGt2GFPuv. The *Xba*I-*Sac*I RBS-*GFPuv* fragment was ligated to the *Xba*I-*Sac*I large fragment of pSRKGmPvirEGFPuv, similar to the pSRKGt2GFPuv construction, and the resultant plasmid was named pSRKGveGFPuv.

We replaced the *GFPuv* gene in the GFP reporter plasmid with several types of *lux* gene to construct a luciferase reporter plasmid to detect Ti/Ri-free cells. First, we constructed a pSRK plasmid in which the *Ti2* promoter and the RBS preceded an *Nde*I restriction site (5′-CATATG-3′). Because the ATG sequence in the *Nde*I site can be substituted with a start codon of the insertion gene, we can construct a translational fusion by inserting a *lux* gene into the unique *Nde*I site on the vector. A P_{Ti2}-RBS-*Nde*I site-containing fragment was prepared by PCR amplification using the oligonucleotides Ti2rbsATGfw and Ti2rbsATGrv as primers and pSRKGt2GFPuv as template DNA. The primer Ti2rbsATGrv includes an *Nde*I restriction site at its 5′-end. The amplified fragment was fused with the pSRKGm *Eco*RI fragment by an In-Fusion® cloning system (Clontech Laboratories, Inc., CA), and the resultant plasmid was then named pSRKGmT2RN. Two luciferase gene fragments originating from *Photorhabdus luminescens*, *luxCDABE*, and *luxAB*, were amplified by PCR using pairs of primers, luxNdeInfFw/Rv and luxABfwInf/RvInX, respectively, and pXen-13 was used as the template DNA. A luciferase gene originating from *Vibrio harveyi* was prepared by PCR using the oligonucleotides luxABfwInf and luxABRvInO as primers and pENTR(P4P1R)SDluxAB(#6) as the template DNA. Each *lux* gene fragment was inserted into a unique *Nde*I restriction site in pSRKGmT2RN by an In-Fusion® cloning system. Three resultant plasmids, one containing *P. luminescens luxCDABE*, one *P. luminescens luxAB*, and one *V. harveyi luxAB*, were named pSRKGluxCDABEp, pSRKGluxABp, and pSRKGluxAB, respectively. An IncQ-type reporter plasmid, pHRPEHGmlux, was constructed by fusing a pHRPEH vector fragment with the P_{Ti2}-*luxAB* reporter cassette from pSRKGluxAB. The vector and insert fragments were prepared by PCR amplification using two pairs of primers, pHRPEH_Ba_fw/rv and PvLux_pHRPfw/rv, respectively, on the parent template plasmids.

pSRKKmsacB and pSRKSpcsacB were constructed by fusing *Bam*HI-digested pSRKKm and pSRKSpc, respectively, with a PCR-amplified *sacB*-containing fragment using an In-Fusion® cloning system. The *sacB* fragment was amplified by PCR using InpSRBsacFw and InpSRBsacRv as primers and pK18msr as the template DNA.

pK18msr24A_A4 was constructed by fusing a pK18msr24A fragment lacking a *repABC* region of pTiSAKURA with a *repABC* segment of pRiA4b. A *repABC*-lacking pK18msr24A fragment was prepared by PCR amplification using two primers, 24AInvFw and 24AInvRv, and pK18msr24A template DNA. The pRiA4b *repABC* fragment was obtained by PCR amplification from pRiA4b DNA using the oligonucleotide primers pRA4rInFw and pRA4rInRv. The *repABC* and the vector fragment were fused by an In-Fusion® cloning system.

Table 2 Plasmids used in the present study

Plasmid	Relevant characteristics	Reference or source
pTiC58	Ti plasmid of *A. tumefaciens* C58; incRh1	Goodner et al. (2001)
pTi-SAKURA	Ti plasmid of *A. tumefaciens* MAFF301001; incRh1	Suzuki et al. (2000)
pTiEHA105	Disarmed-pTiBo542 plasmid of *A. tumefaciens* EHA105; incRh2	Hood et al. (1993)
pRiA4b	Ri plasmid of *A. rhizogenes* A4; incRh3	Jouanin et al. (1986)
pTiS4	Ti plasmid of *A. vitis* S4; incRh4	Gérard et al. (1992)
pAtC58	Cryptic megaplasmid (approx. 543 kbp) of *A. tumefaciens* C58	Goodner et al. (2001)
pENTR(P4P1R)SDluxAB(#6)	Source of a *luxAB* derived from *V. harveyi*; Km^r	Baldwin et al. (1984)
pXen-13	Source of a *luxCDABE* operon derived from *P. luminescens*; Ap^r	Caliper Life Sciences, Inc., USA
pGFPuv	Source of *GFPuv* gene; Ap^r	Clontech Laboratories, Inc., USA
pK18*mobsacB*	Mobilizable cloning vector; *pUC-oriV mob sacB* Km^r	Schäfer et al. (1994)
pK18msr	Mobilizable cloning vector; *pUC-oriV mob sacB repABC* of pTi-SAKURA Km^r	Yamamoto et al. (2009)
pK18msr24A	pK18msr with two antitoxin genes (*tiorf24* and *ietA*) of toxin-antitoxin system; Km^r	Yamamoto and Suzuki (2012)
pK18msr24A_B1	pK18msr24A with a *repABC1* locus of pTiBo542 instead of *repABC* of pTi-SAKURA; Km^r	Yamamoto et al. (2017)
pK18msr24A_A4	pK18msr24A with a *repABC* locus of pRiA4b instead of *repABC* of pTi-SAKURA; Km^r	The present study
pK18msr24A_S1	pK18msr24A with a *repABC1* locus of pTiS4 instead of *repABC* of pTi-SAKURA; Km^r	Yamamoto et al. (2017)
pPTi2GFPuv	pGFPuv with a *Ti2* promoter region (RBS-less) immediately upstream of a *GFPuv* gene; Ap^r	The present study
pPvirEGFPuv	pGFPuv with a *virE* promoter region (RBS-less) immediately upstream of a *GFPuv* gene; Ap^r	The present study
pRH210	Conjugation helper plasmid containing *tra* genes of RK2; *pUC-oriV* Ap^r	Nishikawa et al. (1990)
pSRKGm	pBBR expression vector; *pBBR1ori* Gm^r	Khan et al. (2008)
pSRKGluxAB	pSRKGm with P_{Ti2}-$luxAB_{V.\ harveyi}$; Gm^r	The present study
pSRKGluxABp	pSRKGm with P_{Ti2}-$luxAB_{P.\ luminescens}$; Gm^r	The present study
pSRKGluxCDABEp	pSRKGm with P_{Ti2}-$luxCDABE_{P.\ luminescens}$; Gm^r	The present study
pSRKGmPTi2GFPuv	pSRKGm with P_{Ti2}-*GFPuv* (RBS-less); Gm^r	The present study
pSRKGmPvirEGFPuv	pSRKGm with P_{virE}-*GFPuv* (RBS-less); Gm^r	The present study
pSRKGmT2RN	pSRKGm with a *Ti2* promoter, RBS and *Nde*I restriction site; Gm^r	The present study
pSRKGt2GFPuv	pSRKGm with P_{Ti2}-*GFPuv*; Gm^r	The present study
pSRKGveGFPuv	pSRKGm with P_{virE}-*GFPuv*; Gm^r	The present study
pSRKKm	pBBR expression vector; *pBBR1ori* Km^r	Khan et al. (2008)
pSRKKmsacB	pSRKKm with the *sacB* gene; Km^r	The present study
pSRKKSrh1	pSRKKmsacB with the *repABC* locus of pTiC58; Km^r	The present study
pSRKKSrh2	pSRKKmsacB with the *repABC1* locus of pTiBo542; Km^r	The present study
pSRKKSrh3	pSRKKmsacB with the *repABC* locus of pRiA4b; Km^r	The present study
pSRKKSrh4	pSRKKmsacB with the *repABC1* locus of pTiS4; Km^r	The present study
pSRKSpc	pBBR expression vector; *pBBR1ori* Spc^r	Our collection
pSRKSpcsacB	pSRKSpc with the *sacB* gene; Spc^r	The present study
pSRKSpcsacBpAtrep1	pSRKSpcsacB with the *repABC* locus of pAtC58; Spc^r	The present study
pHRPEH	IncQ plasmid pHRP309 derivative lacking a *lacZ* gene; Gm^r	Our collection
pHRPEHGmlux	pHRPEH with theP_{Ti2}-*luxAB* cassette from pSRKGluxAB; Gm^r	The present study

For the construction of pBBR1-type Ti/Ri eviction plasmids, the linearized pSRKKmsacB vector and each *repABC* fragment were fused using an NEBuilder® DNA assembly kit (NEB, UK). The pSRKKmsacB fragment was amplified from the plasmid template using oligonucleotides pSRK_lacCter_inv_fw and pSRK_lacCter_inv_rv as primers. The *repABC* fragments from Ti/Ri plasmids of each incompatibility group were prepared by PCR amplification using oligonucleotide primers and template

DNA as follows: C58repABCfw and C58repABCrv were used to amplify the incRh1 *repABC* from pTiC58 and construct pSRKKSrh1; Rh2_rep1_infSRK_fw and Rh2_rep1_infSRK_rv were used to amplify the incRh2 *repABC1* from pTiEHA105 and construct pSRKKSrh2; Rh3_rep_infSRK_fw and Rh3_rep_infSRK_rv were used to amplify the incRh3 *repABC* from pRiA4b and construct pSRKKSrh3; Rh4_rep1_infSRK_fw and Rh4_rep1_infSRK_rv were used to amplify the incRh4 *repABC1* from pTiS4 and construct pSRKKSrh4. pSRKSpcsacBpAtrep1 was constructed by fusion of pSRKSpcsacB with an *repABC* fragment of pAtC58. The vector fragment was amplified from its parent plasmid by PCR using the oligonucleotide primers pSRK_lacCter_inv_fw and pSRK_lacCter_inv_rv, and the insert *repABC* fragment was amplified using pAtrepABCfw and pATrepABCrv.

The PCR reactions described previously were performed using KOD -Plus- Neo (Toyobo, Osaka). The primers used for the amplification of fragments used for fusion reactions had 15 bp that were homologous with the ends of their counterpart fragments added to their 5′-ends, as described in Table 3.

Determining promoter activity by measuring the fluorescence intensity of GFPuv

Overnight-cultured cells harboring the GFPuv reporter plasmid were washed with saline and suspended with 5 mL of AB induction medium in test tubes at a concentration of $OD_{600} = 0.2$. The induction medium contained gentamicin to retain the reporter plasmid, with or without 100 μM acetosyringone (AS). The cultures were shaken at 28 °C for 24 h. The cells were then collected and suspended in distilled water at a concentration of $OD_{600} = 0.5$, and the intensity of the suspension fluorescence at 505 nm (excited by 400 nm) was measured by an FP-777 spectrofluorometer (Jusco, Tokyo).

Luminescence measurement of luciferase reporter

Cells harboring the luciferase reporter plasmid were cultured overnight, washed with saline, and then suspended with AB-C to a concentration of $OD_{600} = 1.0$. Next, 5 μL of the cell suspension was dropped onto an AB induction agar plate containing gentamicin and AS at 100 μM final concentrations. After 24 h of incubation at 28 °C, 100 μL of 3% decanal dissolved in canola oil was added to the lids of plates to function as a substrate of luciferase, except for plates with cells harboring pSRKGluxCDABEp. The luminescence of each spot was imaged using a BU-50LN cooled CCD camera (Bitran, Saitama, Japan), and its intensity was quantified by the ImageJ software v. 1.47. The luminescence intensity was expressed in relative light units (RLU).

Detection of plasmid-free cells

In the Ti/Ri curing experiments, the transconjugant colonies generated by the introduction of an eviction plasmid were subcultured on an AB induction agar plate containing appropriate antibiotics and AS. After the cells were incubated overnight at 28 °C, the light emitted from the luciferase reporter was monitored by using a cooled CCD camera after the addition of 3% decanal as described above. We counted the colonies that produced no luminescence as Ti/Ri-free cell colonies. The loss rates of Ti/Ri plasmids were expressed as the percentage of the colonies without luminescence of all tested colonies. Regarding the removal of pAtC58, the presence or absence of the plasmid in transconjugants induced by introduction of the eviction plasmid was judged by colony direct PCR using primers to amplify the regions upstream and downstream of *repABC* in pAtC58. The primer pairs pAtrep_Rf/Rr and pATrep_Lf/Lr were used for the amplification of the regions upstream and downstream of *repABC*, respectively. Loss of the resident plasmid in the transconjugant was also confirmed by S1 nuclease-pulsed-field gel electrophoresis (PFGE) as described as follows.

Conjugation

Conjugation was performed as described previously (Yamamoto et al. 2017) with some modification. Donor, helper, and recipient cells were cultured in fresh liquid media until the optical density was approximately $OD_{600} = 1.0$. Next, 1 mL of each culture was centrifuged, and the collected cells were rinsed twice with AB-C, followed by resuspension in 40 μL of AB-C. Aliquots (10 mL) of donor, helper, and recipient cell suspensions were mixed and dropped on a nylon membrane placed on agar medium appropriate for the growth of the recipient strains, namely, LB medium for *A. tumefaciens* C58 and EHA105 and IFO medium for *A. rhizogenes* A4 and *A. vitis* S4. After an overnight incubation at 28 °C, the cells were suspended with AB-C and plated on a selective agar medium. When the S4 strain was used as a recipient, an AB minimal medium was used as a selective medium. After 2 to 5 days of incubation at 28 °C, the number of the transconjugant colonies was counted. The transfer efficiency of each conjugation was obtained by dividing the number of transconjugants by the number of input donors.

In the Ti and Ri plasmid eviction experiments, pK18*mobsacB*-type and pBBR1-type eviction plasmids were introduced into the strains containing pSRKGluxAB (Inc pBBR) and pHRPEHGmlux (Inc Q) as reporter plasmids, respectively, to avoid an incompatibility between the eviction and reporter plasmids.

Table 3 Oligonucleotide primers used in the present study

Primer name	Nucleotide sequence (5′-3′)	Amplified region
pRA4rInFw	**TGGTCCGGCGAAACT**GGATCCTACAAGGTAGAATCCGCCT	*repABC* of pRiA4
pRA4rInRv	**GCGATTAAGTTGGGT**AAGCTTTCTGAGCCGCCGATTTTCC	*repABC* of pRiA4
24AInvFw	ACCCAACTTAATCGCCTTGCAGCACATCC	pK18msr24A
24AInvRv	AGTTTCGCCGGACCAATACGAACG	pK18msr24A
pSRK_lacCter_inv_fw	GCGGGCCTCTTCGCTATTACG	pSRKKmsacB/pSRKSpcsacB
pSRK_lacCter_inv_rv	GATCGCCCTTCCCAACAGTTGC	pSRKKmsacB/pSRKSpcsacB
PvirE_GFPUV_Fw	CCCAAGCTTGGGGAATTCTCGACAAGCGAAGAGTCTG	*virE* promoter
PvirEGUS_Rv	GCTCTAGATGCTTTCTAGGCTGCTGC	*virE* promoter
Ti2Fw	GCTCTAGAATTCACATCGCATCGTCTTG	*Ti2* promoter
Ti2Rv	GCTCTAGAGGTTGACACGGCCCCTTCA	*Ti2* promoter
AtRBSgfpFw	CGTCTAGAGGAGGAAGAAAAAATGAGTAAAGGAGAAGA ACTTTTCACTG	RBS-*GFPuv*
GFPuvRv	CCAGACAAGTTGGTAATGGTAGC	RBS-*GFPuv*
Ti2rbsATGfw	**ATTCGAAATTGAATT**ACATCGCATCGTCTTGCCGC	P_{Ti2}-RBS-*Nde*I site
Ti2rbsATGrv	**GCTTGATATCGAATT**CATATGTTTTTCTTCCTCCTCTAGAGG	P_{Ti2}-RBS-*Nde*I site
InpSRBsacFw	**TAGAACTAGTGGATC**CTAGAGGATCGATCCTTTTT	*sacB* gene
InpSRBsacRv	**GCAGCCCGGGGGATC**GTCGGTCATTTCGCTCGGTA	*sacB* gene
luxNdeInfFw	**AGGAAGAAAAACATA**TGACTAAAAAAATTTCATTCATTA	*luxCDABE* $_{P.\ luminescens}$
luxNdeInfRv	**GATATCGAATTCATA**AACCCTCACTAAAGGGAAC	*luxCDABE* $_{P.\ luminescens}$
luxABfwInf	**AGGAAGAAAAACATA**TGAAATTTGGAAACTT	*luxAB*$_{P.\ luminescens,\ V.\ harveyi}$
luxABRvInX	**GATATCGAATTCATA**TTAGGTATATTCCATGTGGT	*luxAB* $_{P.\ luminescens}$
luxABRvInO	**GATATCGAATTCATA**TTACGAGTGGTATTTGACGA	*luxAB* $_{V.\ harveyi}$
C58repABCfw	**TTGGGAAGGGCGATC**TCGTATTGGTCCGGCGAAAC	*repABC* of pTiC58
C58repABCrv	**AGCGAAGAGGCCCGC**CAGCCAAGTCCAAAGGAAAGC	*repABC* of pTiC58
Rh2_rep1_infSRK_fw	**TTGGGAAGGGCGATC**GGAGGCTTTGACTACGCTCGTGTTGG	*repABC1* of pTiEHA105
Rh2_rep1_infSRK_rv	**AGCGAAGAGGCCCGC**CGGCTTTGCTCACGGTGTAGTCTCTTG	*repABC1* of pTiEHA105
Rh3_rep_infSRK_fw	**TTGGGAAGGGCGATC**GGATCCTACAAGGTAGAATCCGCCT	*repABC* of pRiA4b
Rh3_rep_infSRK_rv	**AGCGAAGAGGCCCGC**GCTTTCTGAGCCGCCGATTTTCC	*repABC* of pRiA4b
Rh4_rep1_infSRK_fw	**TTGGGAAGGGCGATC**GCGAGACGGTCAGAATCAGCATG	*repABC1* of pTiS4
Rh4_rep1_infSRK_rv	**AGCGAAGAGGCCCGC**CTTGCGCTTCGCTTTCCACATCG	*repABC1* of pTiS4
pAtrepABCfw	**TTGGGAAGGGCGATC**CGTCGGTGAACTCTACAAATCC	*repABC* of pAtC58
pATrepABCrv	**AGCGAAGAGGCCCGC**TTTCGAGATTGTCACCGGGACC	*repABC* of pAtC58
pAtrep_Rf	TGACGCAAACGATGACCAGG	Region upstream of pAtC58 *repABC*
pAtrep_Rr	AGTGTGCTGCTGTTCCGTG	Region upstream of pAtC58 *repABC*
pATrep_Lf	AGCAATTCGCCCACGATCAG	Region downstream of pAtC58 *repABC*
pATrep_Lr	AGGTTTCGCTTGCCTATGACG	Region downstream of pAtC58 *repABC*
pHRPEH_Ba_fw	GATCCCCGGGTACCGAGCTCGAATTA	pHRPEH
pHRPEH_Ba_rv	GATCCTCTAGAGTCGACCTGCAGTTC	pHRPEH
PvLux_pHRPfw	**CGACTCTAGAGGATC**ACATCGCATCGTCTTGCCGCT	P_{Ti2}-*luxAB*
PvLux_pHRPrv	**CGGTACCCGGGGATC**GGTCGACGGTATCGATAAGCTT	P_{Ti2}-*luxAB*

*The 15-bp homology arm for the linkage reaction is indicated in bold letters

Preparation of the gel plug containing intact agrobacterial DNA for S1 nuclease-PFGE

Gel plug preparation for S1 nuclease-PFGE was conducted following the procedures described by Barton and colleagues with some modification (Barton et al. 1995). Agrobacterial cells were cultured overnight in an appropriate liquid medium. Approximately 5×10^8 cells were collected from the culture and washed with NT solution (1 M NaCl and 10 mM Tris-HCl (pH 7.5)). The cells were resuspended in 0.2 mL of NT and mixed with an equal volume of 1% molten low-melting-point agarose in NT that had been kept at 40 °C. The mixed solution

was immediately dispensed into plug molds and then kept on ice until it solidified. The gel plugs were transferred into EC solution (6 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.1 M EDTA, 0.5% (*v*/*v*) Brij 58, 0.2% (*w*/*v*) deoxycholate, and 0.5% (*w*/*v*) *N*-lauroylsarcosine) containing lysozyme and RNase A at concentrations of 1.25 mg/mL and 1.5 μg/mL, respectively, and incubated with gentle shaking for more than 6 h at 37 °C. After the plugs were rinsed with the ES solution (0.5 M EDTA and 1% (*w*/*v*) *N*-lauroylsarcosine), they were added to ES solution containing 1 mg/mL proteinase K and incubated with mild agitation overnight at 42 °C. The plugs were incubated twice in TE containing 1 mM PMSF (phenylmethylsulfonyl fluoride) with gentle shaking for 1 h at RT, and they were then incubated twice in TE under the same conditions for 30 min. The gel plugs were stored in TE at 4 °C until use.

S1 nuclease reaction and PFGE

A slice of the gel plug, prepared by cutting the plug into three equal pieces with a sterilized blade, was transferred into 200 μL of 1× S1 nuclease buffer (30 mM sodium acetate (pH 4.6), 1 mM zinc acetate, 50 mM NaCl, and 5% (*v*/*v*) glycerol) and incubated with gentle shaking at approximately 5 rpm for 30 min at 37 °C. The gel slice was transferred into a plastic tube containing 100 μL of the S1 nuclease reaction solution, which contains 1 unit of S1 nuclease (Thermo Fisher Scientific, Inc., MA) in 1× S1 nuclease buffer, and then incubated under the same conditions described previously for digestion. After the enzyme solution was discarded, 300 μL of ES solution were added to the tube; this tube was then kept on ice for 30 min to stop the nuclease reaction. The gel slice was transferred to a 0.5× TBE buffer and incubated for over 30 min before electrophoresis. PFGE was performed using a CHEF-DR III system (Bio-Rad laboratories, Inc., CA) at 6 V/cm^2 for 20 h in 0.5× TBE buffer with 1% agarose.

Results

The stability of Ti/Ri plasmids is often enhanced by stability genes such as TA modules, resulting in some plasmids being rarely removed from their host cells (Yamamoto et al. 2007, 2009). To overcome this problem, we developed two methodologies: one to detect rare plasmid-free cells and the other to increase the efficiency of producing the plasmid-free cells.

Construction of a reporter system for the detection of Ti/Ri-free cells

We first constructed reporter plasmids to detect Ti/Ri plasmid-free cells. The reporter plasmids contain a reporter gene downstream of a virulence (*vir*) gene promoter that is activated by a VirG protein provided by the Ti/Ri plasmid in the host cells. When supplied with a *vir* gene-inducing compound such as AS, Ti/Ri-free cells were distinguished from cells with the plasmids because the plasmid-free cells did not express the reporter gene. To obtain a better AS-inducible promoter, which is highly active in the presence of AS but exhibits a low level of leaky expression without AS, we selected two promoter regions of AS-inducible genes, *virE* and *Ti2*. The *virE* genes encode chaperone and effector proteins that are crucial for infection. *Ti2* is a small RNA-coding region located between *atu6154* and *atu6155* in pTiC58 of *A. tumefaciens* C58. Wilms and coworkers reported that the expression of *Ti2* was upregulated over 100-fold by the addition of AS (Wilms et al. 2012). We constructed two reporter plasmids containing the GFPuv gene under the control of the two AS-inducible promoters, P_{virE} and P_{Ti2}, and named them pSRKGVeGFPuv and pSRKGT2GFPuv, respectively (Fig. 1a). The two reporter plasmids were introduced into *A. tumefaciens* C58rif and evaluated for their reporter activity in AB induction media with or without AS. The two *vir* promoters strongly induced the expression of GFPuv in the medium containing AS. In the absence of AS, the basal expression of the P_{Ti2} reporter was less than that of the P_{virE} reporter (Table 4). We next tested three types of luciferase genes as reporters in host *Agrobacterium* cells. The three luciferase gene fragments were *luxCDABE* (a full-length luciferase operon originating from *Photorhabdus luminescens* (previously called *Xenorhabdus luminescens*)) and *luxAB* gene segments that originated from *P. luminescens* and *Vibrio harveyi*; these fragments were inserted in place of GFPuv immediately downstream of P_{Ti2} in the reporter plasmid (Fig. 1b). The *luxAB* segment encodes proteins responsible for oxidative reactions accompanied by light emission, which require a long-chain aldehyde and the reductive flavin mononucleotide (FMN) as substrates. *luxCDE* codes a complex of fatty acid reductases to biosynthesize the long-chain aldehyde substrate. Addition of the substrate for the oxidative reaction that results in light emission is therefore required when only *luxAB* is employed in the reporter plasmids. These reporter plasmids were introduced into *A. tumefaciens* C58rif (carrying a Ti plasmid) and C58C1 (Ti plasmid-less C58rif strain), and their reporter activities were then measured (Fig. 1c). Table 5 shows that the three *lux* reporter plasmids clearly exhibited luminescence in response to AS. Among the three, *V. harveyi luxAB* was the strongest emitter in this strain under the experimental conditions. The P_{Ti2}-*luxAB* cassette in pSRKGluxAB also functioned well in the incQ-type vector plasmid pHRPEHGmlux (data not shown). We therefore chose the P_{Ti2} and *V. harveyi luxAB* as the reporter for further experiments.

Construction of the eviction plasmid for curing Ti/Ri plasmids belonging to incRh2, incRh3, and incRh4

In our previous report (Yamamoto and Suzuki 2012), we developed a Ti-eviction plasmid, pK18msr24A, that carried an incRh1 *repABC* locus and two antitoxin genes (*ietA* and

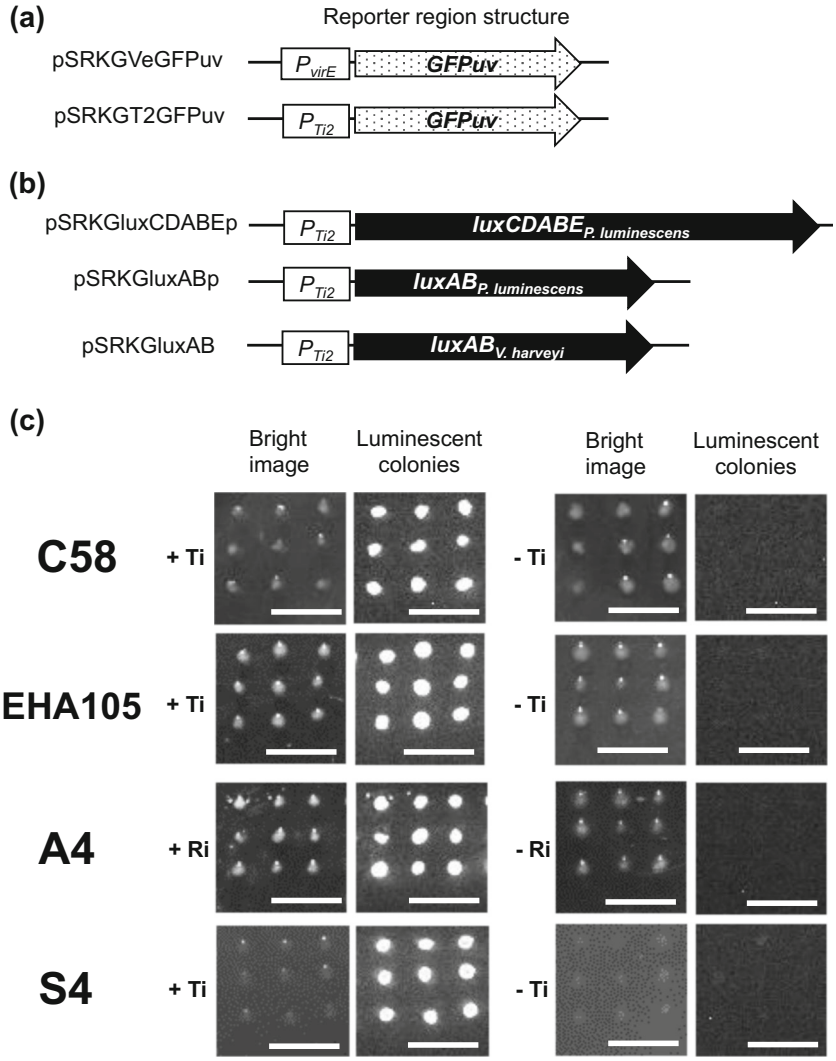

Fig. 1 Structures of plasmids with reporter genes and the detection of Ti/Ri-free *Agrobacterium* cells. Ti/Ri-dependent promoters and reporter genes are indicated as white boxes and thick arrows, respectively (**a**, **b**). Colonies of *Agrobacterium* cells with the *lux* reporter plasmid and with or without Ti/Ri plasmid, grown on AB induction medium (**c**). The colonies were exposed to vaporized decanal and photographed with a CCD camera under white light (bright image) and in a dark room (luminescent colonies), as described in the "Materials and methods" section. White bars indicate 10 mm

tiorf24) from TA modules in a pK18*mobsacB* vector to remove incRh1 Ti plasmids. To broaden the range of target plasmids to evict using plasmid incompatibility, we replaced the incRh1 *repABC* locus in the eviction plasmid with that of other incompatibility group. A new series of eviction plasmids was generated on the pK18*mobsacB* backbone (Fig. 2). The *repABC* segment in pK18msr24A was replaced with *repABC* loci of Ti/Ri plasmids belonging to incompatibility groups other than incRh1, resulting in pK18msr24A_B1 (incRh2 *repABC* of pTiBo542), pK18msr24A_A4 (incRh3 *repABC* of pRiA4b), and pK18msr24A_S1 (incRh4 *repABC* of pTiS4). pTiBo542 and pTiS4 each contain two *repABC* loci. One of the two *repABC* loci (named "*repABC1*" in our previous report, Yamamoto et al. 2017), which is located adjacent to the *trb*/*traI* cluster, is responsible for replication in *Agrobacterium* cells. The *repABC1* loci in the two Ti plasmids were therefore subcloned in the vector plasmids.

A dual *repABC*-type Ti plasmid, pTiBo542, was replicated by an incRh2 *repABC1* but is incompatible with an incRh1 plasmid because of a redundant "*repABC2*"' locus; pTiBo542 can therefore effectively displace a coresident incRh1 plasmid as well as an incRh2 plasmid from a host cell (Yamamoto et al. 2017). Placing a plasmid with two replication loci, one that is compatible and one that is incompatible with target plasmids, could be an effective approach for eviction. We therefore constructed another new eviction plasmid series in similar fashion to pTiBo542; members of this series have a pBBR1 plasmid backbone (Inc pBBR) that is replicated at a medium copy number in *Agrobacterium* cells (Fig. 2). Each *repABC* locus in the four Ti/Ri plasmids was inserted into a pSRKKmsacB vector for the construction of pBBR1-type eviction plasmids.

Powerful eviction by new plasmid series

The reporter plasmids described previously were first introduced into four *Agrobacterium* strains, *A. tumefaciens* C58rif, EHA105, *A. rhizogenes* A4, and *A. vitis* S4. Each of four strains has Ti/Ri plasmids of different incompatibility groups,

Table 4 Induction of *GFPuv* gene expression from P_{virE} or P_{Ti2} by AS

Plasmid	Fluorescence intensity (Ex_{400}/Em_{505})		Induction ratio[a] (+AS/−AS)
	−AS	+AS	
pSRKGm	35	31	0.9
pSRKGVeGFPuv (P_{virE})	131	4732	36.1
pSRKGT2GFPuv(P_{Ti2})	48	4106	85.5

[a] Induction ratio was calculated by dividing the fluorescence intensity of the cells cultured in AB induction medium with AS by that in medium without AS

namely, incRh1 pTiC58 in C58rif, incRh2 pTiEHA105 in EHA105, incRh3 pRiA4b in A4, and incRh4 pTiS4 in S4. To examine the eviction ability of the new eviction plasmids, we introduced eight plasmids in total by conjugation into the strains containing the corresponding incompatible Ti/Ri plasmids (Table 6). The introduction of pK18*mobsacB*-backbone plasmids led to the loss of incRh1 pTiC58 in most transconjugants (99%), because the eviction plasmid contains antitoxin genes derived from pTiC58 and therefore neutralizes the effect of the TA system in the Ti plasmid, as previously reported (Yamamoto et al. 2009). However, other Ti/Ri plasmids belonging to incRh2, incRh3, and incRh4 were stubbornly maintained in most or all transconjugants. In contrast to the pK18*mobsacB*-backbone plasmid, introduction of pBBR1-type eviction plasmids led the efficient loss of the target Ti/Ri plasmids. Table 6 shows that more than 50% of each bacterial host strain lost the target plasmid. All transconjugants lost the pTiEHA105 plasmid in EHA105. pRiA4b and pTiS4 were also displaced frequently in A4 and S4 strains, with 50 and 86% of the transconjugants being lost, respectively. The absence of Ti/Ri plasmids in the candidate Ti/Ri-free clones, which did not emit light when grown on AS-containing AB induction agar, at least five clones in each strain, was confirmed by S1 nuclease-PFGE (Fig. 3a, b).

Table 5 Luciferase activity of the three types of P_{Ti2}::*lux* constructs expressed in *Agrobacterium* cells

Plasmid	Coexisting Ti plasmid	Reporter activity (RLU)
pSRKGm	None	0
pSRKGm	pTiC58	115
pSRKGluxCDABEp ($luxCDABE_{P.\ luminescens}$)	None	0
pSRKGluxCDABEp ($luxCDABE_{P.\ luminescens}$)	pTiC58	2400
pSRKGluxABp ($luxAB_{P.\ luminescens}$)	None	0
pSRKGluxABp ($luxAB_{P.\ luminescens}$)	pTiC58	2734
pSRKGluxAB ($luxAB_{V.\ harveyi}$)	None	36
pSRKGluxAB ($luxAB_{V.\ harveyi}$)	pTiC58	17,293

Removal of a stable megaplasmid pAtC58 by a pBBR1-type eviction plasmid

The pBBR1-type eviction plasmids enabled us to effectively cure the Ti/Ri plasmids belonging to all four incRh groups. To evaluate a broad applicability of this type of eviction plasmid, we applied it to remove a highly stable megaplasmid, pAtC58 (approx. 543 kbp), from *A. tumefaciens* C58rif. Morton and colleagues (Morton et al. 2013) reported that introduction of only a pAtC58-*repABC*-containing suicidal plasmid was insufficient to remove pAtC58. We constructed a pBBR1 plasmid containing the *repABC* locus of pAtC58 and then introduced it into the C58rif strain by electroporation. Two of fifteen transconjugant clones lost their pAtC58 plasmids (Fig. 3c). The pBBR1 eviction plasmid can be cured from the bacterial cells by culturing on sucrose-containing agar medium due to its *sacB* gene, which confers the hypersensitivity to sucrose. The culture of the pAtC58-less transconjugant was diluted, spread on a 5% sucrose-containing LB plate, and then incubated at 28 °C for 2 days. All 30 colonies selected randomly on the plate exhibited susceptibility to spectinomycin, indicating that all of these clones lost the eviction plasmid containing the spectinomycin resistant gene and suggesting that the pBBR1-type eviction plasmid can be used to remove stable megaplasmids other than Ti/Ri plasmids.

Discussion

In this study, a new reporter plasmid carrying the *Ti2* promoter region and *luxAB* from *V. harveyi* enabled us to effectively quantify Ti/Ri-free clones in a large population. The transcriptional regulatory regions of *virE* and *virB* genes have been utilized as promoters for reporter genes to measure the expression level of virulence-related genes (Hattori et al. 2001; Kiyokawa et al. 2012). We showed that the *Ti2* promoter region is more suitable to report on *vir* induction, because it highly induced the expression of a downstream gene in the presence of AS but maintained a

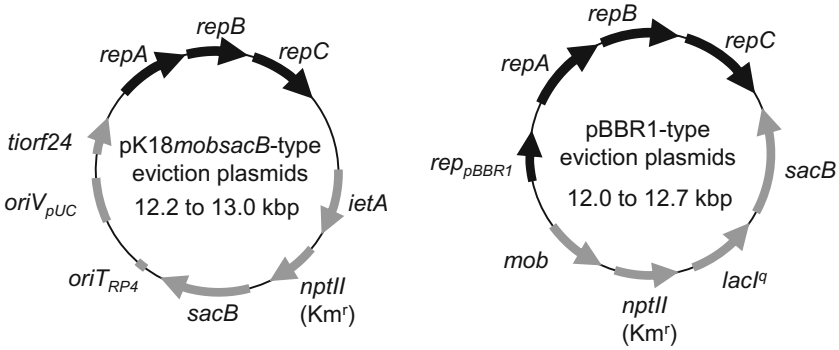

Fig. 2 Structures of the new eviction plasmids. Arrows indicate replication genes that function in *Agrobacterium* (black), other genes (gray), and their directions. Boxes show origins of transfer (*oriT*) and vegetative replication (*oriV*)

low level of basal expression in the absence of the inducer (Table 4). However, the function of a short RNA encoded in *Ti2* has not been determined. Whether the *Ti2* promoter can function similarly to other *vir* gene promoters is unknown; however, this promoter is at least very useful for reporter genes in a *vir* induction medium.

In this study, we compared the performance of *lux* genes derived from two bacteria in *A. tumefaciens*. The *luxAB* of *V. harveyi* emitted approximately 6-fold more light than that of *P. luminescens* (Table 5). The activity of *P. luminescens luxAB* was high enough for most practical use but significantly lower than that of the *luxAB* of *V. harveyi*. Our results suggest that the low activity is not caused by a substrate shortage but by a difference in LuxAB enzymatic characteristics or a relatively small amount of protein products due to differences in codon usage or stability. In terms of enzymatic properties, the two Lux proteins have almost identical aldehyde specificity, but the turnover ratio of the *V. harveyi* Lux, generally defined as the number of molecules of products produced by a single enzyme molecule per second, was approximately 1.5 times higher than that of *P. luminescens* when decanal was used as the substrate (Szittner and Meighen 1990). The high activity of *V. harveyi* Lux observed in this study might be thus based on the specific enzymatic properties in the host *Agrobacterium* cells. Since the Lux activity would differ depending on the host *Agrobacterium* strain (Boivin et al. 1988), the high activity might be not observed in strains other than *A. tumefaciens* C58.

Table 6 Introduction of the eviction plasmids into Ti/Ri-harboring *Agrobacterium* cells and the loss rate of the resident Ti/Ri in resulting conjugants

Eviction plasmid* (replicon)	Recipient strain (Ti/Ri plasmid, inc group)	Transfer efficiency**	Loss rate of Ti/Ri plasmid (%)***
pK18msr24A	*A. tumefaciens* C58	7.1×10^{-3}	99.0
(*repABC*$_{\text{incRh1}}$)	(pTiC58, incRh1)	(3.5×10^{-3})	
pK18msr24A_B1	*A. tumefaciens* EHA105	1.0×10^{-3}	0.0
(*repABC1*$_{\text{incRh2}}$)	(pTiEHA105, incRh2)	(4.2×10^{-4})	
pK18msr24A_A4	*A. rhizogenes* A4	2.0×10^{-5}	1.4
(*repABC*$_{\text{incRh3}}$)	(pRiA4b, incRh3)	(2.8×10^{-5})	
pK18msr24A_S1	*A. vitis* S4	1.4×10^{-4}	0.3
(*repABC1*$_{\text{incRh4}}$)	(pTiS4, incRh4)	(6.6×10^{-5})	
pSRKKSrh1	*A. tumefaciens* C58	9.6×10^{-3}	100.0
(pBBR1, *repABC*$_{\text{incRh1}}$)	(pTiC58, incRh1)	(4.2×10^{-3})	
pSRKKSrh2	*A. tumefaciens* EHA105	1.1×10^{-4}	100.0
(pBBR1, *repABC1*$_{\text{incRh2}}$)	(pTiEHA105, incRh2)	(2.3×10^{-5})	
pSRKKSrh3	*A. rhizogenes* A4	6.3×10^{-5}	50.0
(pBBR1, *repABC*$_{\text{incRh3}}$)	(pRiA4b, incRh3)	(1.0×10^{-4})	
pSRKKSrh4	*A. vitis* S4	2.1×10^{-4}	85.6
(pBBR1, *repABC1*$_{\text{incRh4}}$)	(pTiS4, incRh4)	(1.6×10^{-4})	

*Eviction plasmids were introduced into recipient strains by tri-parental mating as described in the "Materials and methods" section

**Transfer efficiency is expressed as the number of transconjugants divided by the number of input donors. Each value is the average of at least three independent experiments. Standard deviations are shown in the parentheses

***The loss rate of a resident plasmid is the percentage of Ti/Ri-free transconjugants in the total transconjugants. The presence or absence of Ti/Ri plasmids in transconjugants was judged by the presence of light emission after colonies were exposed to decanal vapor

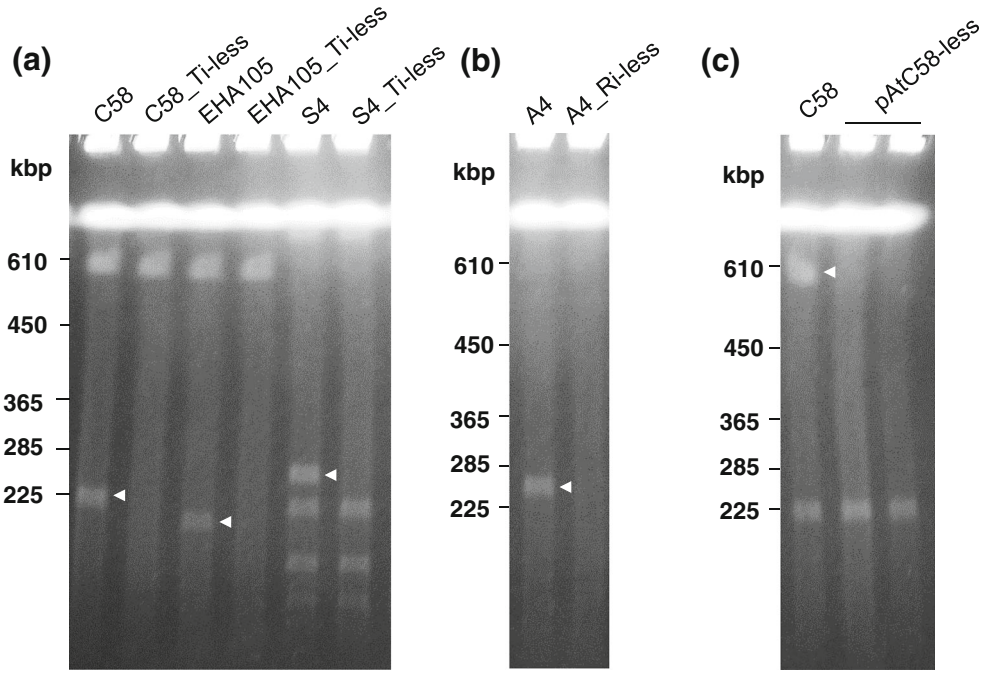

Fig. 3 Plasmid profiles of the transconjugant clones induced by the introduction of eviction plasmids. Intact genomic DNA samples were prepared in agarose gel plugs, briefly digested by S1 nuclease and finally separated by PFGE as described in the "Materials and methods" section. Ti-less (**a**), Ri-less (**b**), and pAtC58-less (**c**) strains were obtained by the introduction of a corresponding eviction plasmid and using sucrose selection to select for the eviction plasmid-free cells. White arrow heads indicate linearized Ti/Ri (**a**, **b**) and pAtC58 (**c**) plasmid DNA

pBBR1-type eviction plasmids, which contain two replication loci (*rep* of pBBR1 and *repABC* of Ti/Ri) that function in *Agrobacterium*, expelled the incompatible resident Ti/Ri plasmids more efficiently than the pK18*mobsacB*-type plasmid, which contained *repABC* alone (Table 6). Introduction of the pBBR1-type eviction plasmid into cells containing compatible Ti plasmids, for example, pSRKKSrh2 (incRh2) introduction into the C58 strain containing pTiC58 (incRh1), did not affect the resident Ti/Ri plasmids (data not shown). The pBBR1-type eviction plasmid could provide the *repABC* components (RepA, RepB, RepC, ctRNA, and *parS*), which exert incompatibility toward the target *repABC* replicon. Therefore, they suggest that the effective eviction by the pBBR1-type eviction plasmid is caused by abundant *repABC* components interfering with the replication of the target Ti and Ri plasmids provided by the eviction plasmid that can stably replicate with a higher copy number than the target plasmid through the pBBR1 *rep* gene.

The IncF bireplicon plasmid pCG86, a bireplicon plasmid containing two functional replication loci, was lost frequently by the introduction of plasmids that carried either one of two replicator genes present in the resident bireplicon plasmid (Maas et al. 1989). In contrast, pTiBo542 carries functional incRh2 *repABC* and nonfunctional incRh1 *repABC* loci for replication, and its inheritance was not influenced by the introduction of an incRh1 plasmid (Yamamoto et al. 2017). The eviction plasmids with two functional replication genes are sufficient to remove plasmids. Studies of the incompatibility determinants of various *repABC* show that overexpression of the *trans*-acting incompatibility factors RepA, RepB, and ctRNA or the presence of high copy numbers of the *cis*-acting incompatibility factor *parS* are sufficient for the removal of a coexistent incompatible replicon. (Ramírez-Romero et al. 2000; Pappas and Winans 2003; Venkova-Canova et al. 2004; Soberón et al. 2004; Pérez-Oseguera and Cevallos 2013). Practically speaking, cloning the *repABC* region into a broad-host-range vector during eviction plasmid construction is easy.

Which of two single-replicon plasmids remain or disappear is probably strongly affected by factors other than the replication gene, e.g., plasmid stability genes (Yamamoto et al. 2009). When stability-enhancing genes such as TA genes prevent plasmid loss, an additional factor(s) must be added to attenuate the stability function. In our previous report (Yamamoto and Suzuki 2012), addition of the antitoxin genes (*tiorf24* and *ietA*) in the eviction plasmid enhanced the Ti eviction rate in 7 out of 10 *Agrobacterium* strains. If there is a very stable plasmid that cannot be cured by the pBBR1-type eviction plasmid, an addition of an antitoxin gene present in the stable target plasmid to the eviction plasmid is a possible strategy.

The pBBR1-type eviction plasmids constructed here effectively expelled the stable megaplasmid pAtC58 (Fig. 3c). The *repABC* replicons are disseminated among Alphaproteobacteria with various ecological niches, such as *Rhizobium*, *Brucella*, and *Paracoccus* (Cevallos et al. 2008). *R. etli* CFN42 and *R. leguminosarum* bv. *viciae* 3841 harbor six *repABC* replicons, suggesting that the replicons belong to six distinct incompatibility groups in these strains (Gonzalez et al. 2006; Young et al. 2006). The abundance of *repABC* replicons, even in single species, implies that quite a few incompatibility groups are represented in the *repABC* replicons. The pBBR1-type eviction

plasmids shown here would be useful for the removal of various *repABC* replicons that belong to known and novel incompatibility groups.

In summary, we developed a new powerful system to exclude a series of Ti and Ri plasmids from *Agrobacterium*/*Rhizobium* strains. First, we showed that our reliable but low cost and less labor-consuming detection by constructing a new reporter plasmid consisting a promoter of the *Ti2* and luciferase genes *luxAB* from *V. harveyi* enables detection of Ti/Ri plasmid-free cell colonies among a number of plasmid-harboring cell colonies readily. Second, we made new series of Ti/Ri eviction plasmids for removal of Ti/Ri plasmids belong to incRh2, incRh3, and incRh4 in addition to incRh1 from host *Agrobacterium* species. Among them, the pBBR1-type eviction plasmids removed efficiently even a very stable plasmid pAtC58 in *A. tumefaciens* C58 strain as well as Ti/Ri plasmids. It suggests that the set of pBBR1-type eviction plasmid is a powerful tool for the removal of stable plasmids in *Rhizobiaceae*.

Acknowledgments This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers 25850003, 24580115, and 15H04479. We thank Dr. E. Szegedi for providing bacterial strains and Dr. T. Oyama (Kyoto University) for the *luxAB* plasmid. Pathogenic strains isolated outside of Japan were imported with permissions issued by the Japanese Plant Quarantine Office and were handled under directions given by the same organization.

Funding This study was funded by the JSPS (25850003, 24580115, and 15H04479).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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