Title

Development of a reinforced Ti-eviction plasmid useful for construction of Ti plasmid-free *Agrobacterium* strains.

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Abstract

For curing stable Ti plasmids from *Agrobacterium* strains, we developed a new Ti-eviction plasmid. The plasmid contains two antitoxin genes, which originated from two toxin-antitoxin systems in Ti plasmids. The plasmid facilitated production of Ti-less cells in seven tested strains, and was removed easily by simple counter-selection from the cells.

Keywords

Agrobacterium; plasmid removal; Ti plasmid; Toxin-antitoxin system

Abbreviations

TA system, Toxin-antitoxin system; AMT, *Agrobacterium*-mediated transformation

Pathogenic *Agrobacterium* strains induce crown gall or hairy root disease on wounded sites of host plants by introducing the T-DNA region of a pathogenic plasmid (Ti or Ri plasmid) into the host cell chromosomes (Suzuki et al., 2009). *Agrobacterium*-mediated transformation (AMT) method has been widely utilized as gene introduction technology for plants and fungi by exploiting the unique pathogenic property.

Many pathogenic strains of *Agrobacterium* species have been isolated from various dicots and exhibit various pathogenic characteristics such as the range of host plants and tumor morphology (Otten et al., 2008; Sawada and Ieki, 1992). These characteristics differ among the strains depending on their chromosomal backgrounds, the pathogenic plasmids and their combinations even in a same species (De Cleene and De Ley, 1976; Conner and Dommisse, 1992; Hood et al., 1986; Kiyokawa et al., 2009; Kovács and Pueppke, 1993 Porter and Flores, 1991). Even though a large number of diverse pathogenic strains have been isolated and stored so far, several *Agrobacterium tumefaciens* strains and less number of *Agrobacterium rhizogenes* strains have been used as the vector for DNA transfer (Sevón and Oksman-Caldentey, 2002; Stougaard, 1995). To expand the target range and elevate the efficiency of AMT, it is necessary to improve the methodology to convert the diverse pathogenic strains to useful strains applicable for DNA delivery.

Ti-curing manipulation from pathogenic *Agrobacterium* strains is an unavoidable step to construct a large number of AMT strains, which should have an arm-less Ti plasmid instead of an original resident Ti plasmid, as well as to evaluate a chromosomal background contribution on strain-dependent pathogenic characteristics, because Ti-less cells can accept another Ti plasmid easily in terms of high efficiency and no concatemer formation between resident and newcomer plasmids. However, Ti plasmids

are stably maintained in cells by *repABC* replication genes (Cevallos et al., 2008). Furthermore, some Ti plasmids contain a Toxin-antitoxin (TA) system, which consists of toxin and antitoxin genes (Yamamoto et al., 2007; Yamamoto et al., 2009). In general, the toxin gene encodes a robust toxic protein, while the antitoxin gene product is either a labile RNA or a short-lived protein, which can neutralize the cognate toxin (Kobayashi, 2004). Thus, it is difficult to drive out the TA system-containing plasmids, because the remnant toxin will kill or inhibit the growth of the plasmid-less cell. So far, in *Agrobacterium* species, TA systems *tiorf24-tiorf25* and *ietAS* were found in pTi-SAKURA (Suzuki et al., 2000) of MAFF301001 strain and in pTiC58 of C58 strain (ATCC 33970) (Goodner et al., 2001), respectively (Yamamoto et al., 2007; Yamamoto et al., 2009).

To cure Ti plasmids from the original strains without damages such as mutation or gene rearrangement, an incompatible plasmid introduction method has been developed (Uraji et al., 2002). In this method, firstly, a Ti-eviction plasmid, which is mobilizable from *Escherichia coli* to *Agrobacterium* and incompatible with Ti plasmids, is introduced into the Ti-containing cell. Usually, either one of two incompatible plasmids in a cell is lost after several cell divisions (Austin and Nordström, 1990). Therefore, we can obtain Ti-free cells harboring the Ti-eviction plasmid as transconjugants. Finally, the Ti-eviction plasmid is removed from the transconjugants by a simple counter-selection. The convenient method has achieved success in many strains. However, it is very hard or actually impossible to remove some stable Ti plasmids even by the method (Yamamoto et al., 2009).

In order to enhance the ability of the Ti-eviction plasmid in the method, we have inserted the two TA systems, *tiorf24-tiorf25* and *ietAS*, into the Ti-eviction plasmid

(Yamamoto et al., 2009). The TA-containing Ti-eviction plasmid pK18SCat removed Ti plasmids efficiently even in strains recalcitrant against the conventional eviction plasmid. Unfortunately, however, we could not remove the Ti-eviction plasmid from the Ti-less transconjugant cells even by the counter-selection using *sacB* gene as shown later in Table 2.

For this reason, we designed a new Ti-eviction plasmid pK18msr24A, which is easily removable but still highly able to evict Ti plasmids (Fig. 1). The plasmid contains the two antitoxin genes, *tiorf24* and *ietA*, but neither of the corresponding toxin genes *tiorf25* nor *ietS*. If Ti plasmids in the stubborn strains contain a TA system similar to one of the two TA systems, pK18msr24A permits the Ti to quit from the cells by providing the antitoxin, which can lower the toxin pressure. In turn, pK18msr24A in the resultant Ti-less cells has no toxin gene, and thereby it is easy to leave it from the cells.

Firstly, we amplified two antitoxin genes by PCR from Ti plasmids: *tiorf24* from pTi-SAKURA and *ietA* from pTiC58. Each gene keeps its own putative promoter. The *tiorf24*-containing fragment (nucleotide position between 24,297 and 25,118 in GenBank/DDBJ accession no. AB016260.1) and the *ietA*-containing fragment (nucleotide position between 97,533 and 98,938 in GenBank/DDBJ accession no. AE007871.2) were prepared using two origonucleotide primer pairs (5'-gc*tctaga*tattgtctgaaacaccctgc-3' and 5'-gc*tctaga*atctctcgaaccgagatcac-3' for *tiorf24*; 5'-gc*tctaga*gctagcatccaatgcggtcc-3' and 5'-gc*tctaga*tggcagtgtagtcctggatg-3' for *ietA* amplification). Each primer contains a *Xba*I site at their 5' ends. The *tiorf24*-containing fragment was digested with *Xba*I followed by insertion into a unique *Xba*I site of pK18msr (Yamamoto et al., 2007). Next, the *ietA*-containing fragment was cleaved by

*Xba*I and then was introduced into a unique *Nhe*I site of the *tiorf24*-containing pK18msr derivative mentioned above. The resultant plasmid pK18msr24A has a transfer origin ($oriT_{RK2}$), the counter-selection maker gene *sacB*, and the replication genes *repABC*. The *repABC* genes originate from pTi-SAKURA and the plasmid is incompatible with many Ti plasmids. The plasmid construction was carried out using *E. coli* S17-1 λ*pir* as a host strain (Simon et al., 1983), and the DNA manipulations were performed using standard procedures (Sambrook et al., 1989).

We confirmed the predicted functions of the new plasmid using two strains MAFF301001 and C58, which contains *tiorf24-tiorf25* and *ietAS* in each Ti plasmid, respectively. In addition, we also applied the plasmid to eight additional strains (as shown in Table 1), in order to evaluate how much the plasmid is effective among the strains (Sawada and Ieki, 1992). In our previous paper (Yamamoto et al., 2009) and preliminary data, we used the same set of strains and found that many of them are recalcitrant to the Ti-eviction treatment.

pK18msr24A as well as pK18SCat and a *repABC* vector pK18msr were introduced from *E. coli* to each *Agrobacterium* strain by conjugal transfer as described elsewhere (Uraji et al., 2002). The *E. coli* and the *A. tumefaciens* strains (MAFF301001 and C58) were cultured in LB medium (Davis et al., 1980) at 37 °C and 28 °C, respectively. The *A. rhizogenes* strains (Ch-Ag-2, Ch-Ag-10, Pch-Ag-2, Pch-Ag-4, Pl-Ag-1, P-Ag-5, Ro-Ag-10 and Ro-Ag-13) were cultured at 28 °C in IFO medium 702 (0.5% [wt/vol] polypeptone, 0.2% yeast extract, 0.01% $MgSO_4$, pH 7.0) (Tanaka et al., 2009). When necessary, antibiotics were added to the media at the following final concentrations: kanamycin (50 μg/ml), neomycin (50 μg/ml), rifampicin (40 μg/ml) and nalidixic acid (30 μg/ml). In order

to determine a loss frequency of the target Ti plasmid, the resulting transconjugant colonies were checked for the presence or absence of the Ti plasmid. Either colony PCR using *virC* gene specific primers (VCF3: 5'-ggcgggcgygcygaaagraaracyt-3' and VCR3: 5'-aagaacgyggnatgttgcatctyac-3') for amplification of a 0.4-kbp *virC* segment of Ti plasmid (nucleotide position between 194,786 and 195,198 in GenBank/DDBJ accession no. AE007871.2) (Suzaki et al., 2004) or colony hybridization using the 0.4-kbp *virC* gene fragment as a probe were used to detect the Ti plasmid in the clones (Yamamoto et al., 2007).

As shown in Table 1, the transconjugants induced by pK18SCat and pK18msr24A lost their Ti plasmids at higher frequency in seven strains (MAFF301001, C58, Ch-Ag-2, Ch-Ag-10, Pch-Ag-2, Pch-Ag-4 and Pl-Ag-1) than those by the *repABC* vector pK18msr. The result demonstrates that high Ti-eviction ability is retained in pK18msr24A as well as pK18SCat due to the presence of the antitoxin genes, and suggests that many strains (five among eight) harbor a TA system similar with either *ietAS* or *tiorf24-tiorf25* in their Ti plasmids. Furthermore, in Ch-Ag-2, Pch-Ag-2 and Pch-Ag-4, the transconjugant efficiency of pK18msr24A was at least 50-fold higher than that of pK18msr (data not shown). P-Ag-5 lost the Ti plasmid by introduction of the *repABC* vector at the similar efficiency with that of the two Ti-eviction plasmids (Table 1). It is likely that the Ti plasmid in P-Ag-5 has practically no stability-enhancing genes. As for Ro-Ag-10 and Ro-Ag-13, Ti-free cells were hardly obtained by introduction of any of the three Ti-eviction plasmids in this study. It suggests that the Ti plasmids in the two strains contain some strong stability-enhancing factor(s) different from *ietAS* and *tiorf24-tiorf25*.

We then performed the curing treatment of the Ti-eviction plasmid from the host for construction of *Agrobacterium* strains free of the eviction plasmid. First, *Agrobacterium* strains harboring the eviction plasmid were grown to logarithmic phase. Aliquots of the cells were washed and resuspended with 0.9% (wt/vol) NaCl for *A. tumefaciens* and with IFO medium for *A. rhizogenes*. The appropriate volume of the cell suspension was spread onto LB or IFO agar containing 5% sucrose. Because the *sacB* gene on the Ti-eviction plasmid confers sucrose sensitivity to the host cell, the growth of the eviction plasmid-containing cell is severely inhibited on the sucrose medium. After incubation at 28 °C for 2 or 3 days, colonies appeared on the selective agar plates. The colonies were picked and streaked on LB or IFO agar supplemented with kanamycin or neomycin to determine whether the colonies retain the Ti-eviction plasmid.

As a result, in all tested strains, most colonies grown on the sucrose media lost the Ti-eviction plasmids (pK18msr24A or pK18msr) regardless of the presence or absence of the antitoxin genes, whereas the plasmid containing the two intact TA systems (pK18SCat) exhibited recalcitrance to the *sacB*-counter selection (Table 2).

In conclusion, the new Ti-eviction plasmid pK18msr24A, which contains the two antitoxin genes (*tiorf24* and *ietA*), is able to evict Ti plasmids efficiently and is easily removable. In 75% of strains tested in this study, we have obtained Ti-less and Ti-eviction-plasmid-free cells easily. It is expected that the ratio of the Ti-curable strains will increase by addition of the other type antitoxin gene(s) to the Ti-eviction plasmid.

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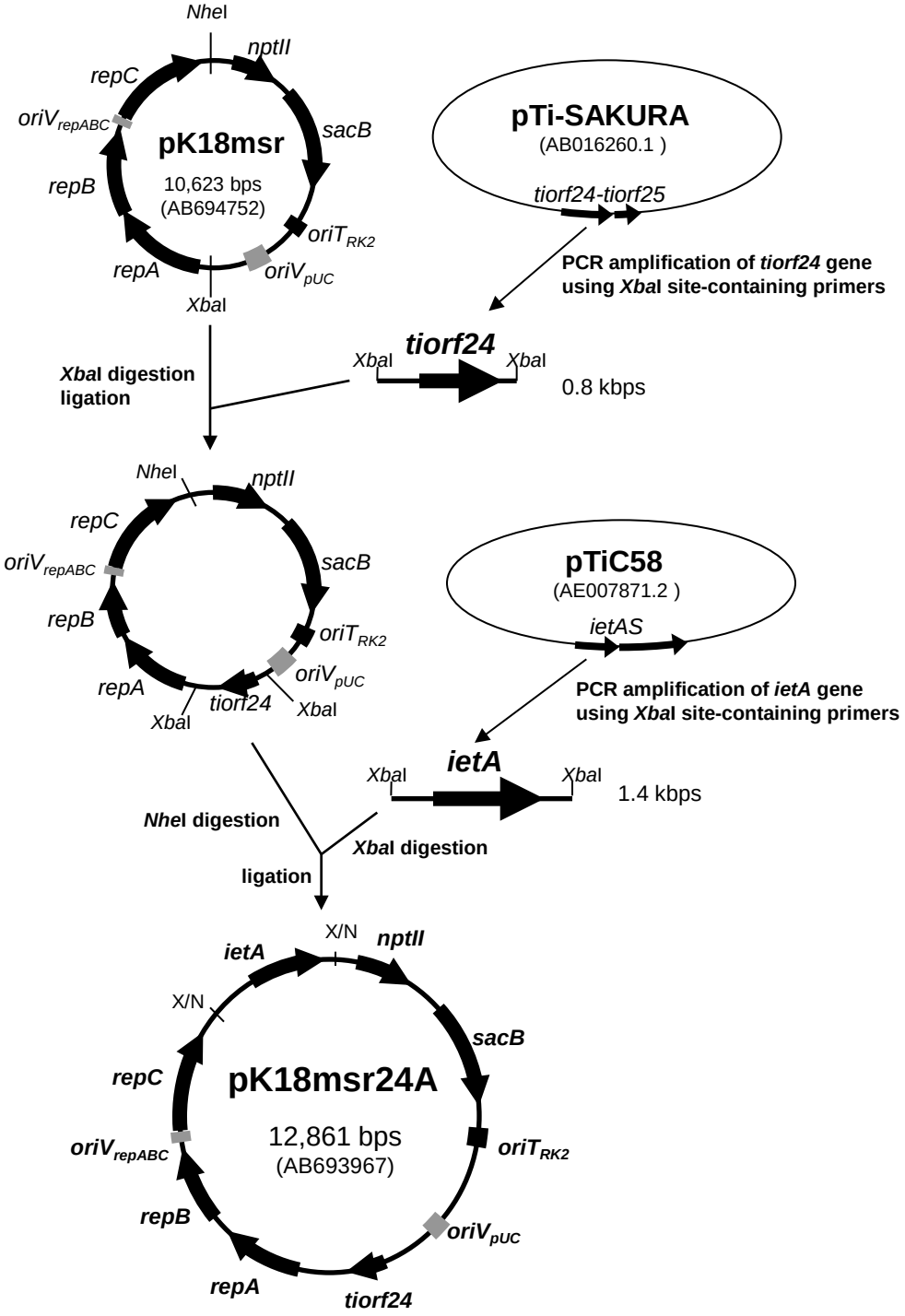
NheI
nptII
repC
oriV repABC
pK18msr
sacB
repB
10,623 bps
(AB694752)
oriT RK2
repA
oriV pUC
XbaI
pTi-SAKURA
(AB016260.1)
tiorf24-tiorf25
PCR amplification of tiorf24 gene using Xbal site-containing primers
Xbal digestion ligation
tiorf24
XbaI
XbaI
0.8 kbps
NheI
nptII
repC
oriV repABC
sacB
repB
oriT RK2
repA
oriV pUC
tiorf24
XbaI
XbaI
pTiC58
(AE007871.2)
ietAS
PCR amplification of ietA gene using Xbal site-containing primers
ietA
XbaI
XbaI
1.4 kbps
Nhel digestion
Xbal digestion
ligation
X/N
nptII
ietA
X/N
sacB
repC
pK18msr24A
12,861 bps
(AB693967)
oriT RK2
oriV repABC
repB
oriV pUC
repA
tiorf24

Fig. 1

Fig. 1 Construction of the reinforced Ti-eviction plasmid, pK18msr24A. Black arrows indicate genes and their directions. Black and gray boxes show transfer origin (*oriT*) and vagetative replication origin (*oriV*), respectively. The genes are the following: neomycin-kanamycin phosphotransferase II gene (*nptII*), levansucrase gene (*sacB*), transfer origin from plasmid RK2 ($oriT_{RK2}$), replication origin from plasmid pUC18 ($oriV_{pUC}$, for replication in *E. coli*), antitoxin gene of *tiorf24-tiorf25* from pTi-SAKURA (*tiorf24*), replication genes and origin from pTi-SAKURA (*repA*, *repB*, *repC* and $oriV_{repABC}$, for replication in *Agrobacterium*), antitoxin gene of *ietAS* from pTiC58 (*ietA*). The GenBank/DDBJ accession numbers of the nucleotide sequence data of each plasmid are shown in parentheses.

Table 1. Induction of Ti-free cells by the Ti-eviction plasmids.

Recipient strain	Ti-eviction plasmid		
	pK18msr (none)	pK18SCat (*tiorf24*, *tiorf25*,*ietA*, *ietS*)	pK18msr24A (*tiorf24*, *ietA*)[a]
	Ti plasmid-free colony (%)[b]		
MAFF301001	34	85	80
C58	56	100	100
Ch-Ag-2	22	93	100
Ch-Ag-10	0	79	95
Pch-Ag-2	0	100	100
Pch-Ag-4	1	97	96
Pl-Ag-1	0	50	40
P-Ag-5	16	22	19
Ro-Ag-10	0	0	0
Ro-Ag-13	0	0	2

[a] TA system genes in the plasmids are indicated in parentheses.

[b] The Ti plasmid loss frequency is the percentage of colonies that lost the Ti plasmid after conjugal transfer of a Ti-eviction plasmid from donor *E. coli* cells.

Table 2. Loss of the Ti-eviction plasmids by *sacB* counter-selection.

Strain	Ti-eviction plasmid		
	pK18msr (none)	pK18SCat (*tiorf24*, *tiorf25*,*ietA*, *ietS*)	pK18msr24A (*tiorf24*, *ietA*)[a]
	Ti-eviction-plasmid-free colony (%)[b]		
MAFF301001	96	32	100
C58	100	31	99
Ch-Ag-2	97	3	100
Ch-Ag-10	Nt[c]	1	100
Pch-Ag-2	Nt	0	100
Pch-Ag-4	98	0	100
Pl-Ag-1	Nt	0	99
P-Ag-5	100	0	99
Ro-Ag-10	Nt	Nt	Nt
Ro-Ag-13	Nt	Nt	99

[a]TA system genes in the plasmids are indicated in parentheses.

[b]The percentage of kanamycin or neomycin sensitive colonies among colonies grown on the selective sucrose agar.

[c]Nt means not tested.