Identification of pTi-SAKURA DNA region conferring enhancement of plasmid incompatibility and stability

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In *Agrobacterium tumefaciens*, the stability of Ti plasmids differs depending on the strain. So far, little is known about genes that cause the difference in stability. The *repABC* operon is responsible for replication and incompatibility of Ti plasmids. We constructed recombinant plasmids carrying the *repABC* operon and different portions of pTi-SAKURA. Cells having the recombinant plasmids that harbored a 2.6-kbp *NheI* fragment of pTi-SAKURA were found to be transformed via conjugation 100-fold less frequently with a small incompatible *repABC* plasmid than cells having the recombinant plasmids lacking the 2.6-kbp *NheI* fragment. Since the phenomenon occurred only when the resident and incoming plasmids belonged to the same incompatibility group, it was suggested that the 2.6-kbp *NheI* fragment bears the potential enhancing incompatibility. The fragment contained an operon consisting of two open reading frames, *tiorf24* and *tiorf25*. *tiorf24* is an orphan gene, whereas *tiorf25* is a homologue of a group of plasmid stability genes. Removal of the 2.6-kbp fragment from the resident pTi-SAKURA increased the resident plasmid ejection ratio by the incoming *repABC* plasmid, whereas addition of the fragment to pTiC58 decreased the ejection ratio, and the loss ratio during growth at 37°C. These data suggest that *tiorf24* and *tiorf25* are responsible for the stability of pTi-SAKURA, and reduce, in the host bacterium, the frequency of ejection of the resident plasmid, presumably through an incompatibility mechanism.

Key words: *Agrobacterium*, incompatibility, stability, Ti plasmid

INTRODUCTION

Agrobacterium tumefaciens causes crown gall disease in dicot plants. The tumor-inducing plasmid (Ti) is indispensable for the phytopathogenicity of this bacterium, because it harbors oncogenic genes in its T-DNA region and many virulence genes for infection and T-DNA transport functions. The plasmids are stably maintained at a low copy number in the bacterial cells (at an equimolar ratio with the bacterial linear and circular chromosomes) (Suzuki et al., 2001). The stability of Ti plasmids still differs depending on the strain (Holsters et al., 1982). The stability difference is likely to exert a serious effect on Ti plasmid evolution. The *repABC* locus is sufficient for autonomous replication of the plasmids (Tabata et al., 1989; Li and Farrand, 2000). To date, the nucleotide

sequence of the *repABC* region of Ti has been determined in an octopine-type plasmid, pTiB6S3 (Tabata et al., 1989), and two nopaline-type plasmids, pTi-SAKURA and pTiC58 (Suzuki et al., 1998; Li and Farrand, 2000).

Rhizobiaceae bacteria commonly possess *repABC* plasmids (Rigottier-Gois et al., 1998). The *repABC* region consists of three clustered genes (*repA*, *repB* and *repC*) and a replication origin (*oriV*) required for replication and partitioning. The RepA and RepB proteins are involved in plasmid partitioning and copy number control, which are important for stable inheritance. RepC is an indispensable protein for replication initiation. These genes are organized as an operon (Ramírez-Romero et al., 2000). Thus, the *repABC* region contains factors for autonomous replication and stable inheritance. Some strains of *Rhizobiaceae* bacteria possess more than one *repABC* replicon. For instance, *A. tumefaciens* strain C58 has three *repABC* replicons: pTiC58, pAtC58 and the linear chromosome, each belonging to three different incompatibility

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groups of the *repABC* replicon in this strain (Goodner et al., 2001). It is well known that most Ti plasmids belong to the same incompatibility group, whereas most Ri plasmids belong to another incompatibility group (White and Nester, 1980).

Incompatibility has been defined as a situation where two plasmids harboring a related replication and/or partitioning system are unable to exist in a cell simultaneously without external selection. The incompatibility phenomenon is considered to be caused by competition for a limited number of components for replication, a partitioning mechanism or by disturbance in plasmid copy number correction (Novick, 1987; Austin and Nordström, 1990). Mini-Ti and -Ri plasmids carrying *repABC* genes confer their respective incompatibility characteristics (Nishiguchi et al., 1987; Tabata et al., 1989) and two sites in the *repABC* locus were defined as incompatibility-determining regions in *Rhizobium etli* p42d (Ramírez-Romero et al., 2000).

Ti plasmids have been practically categorized according to their production and catabolism of opines. pTiC58 and pTi-SAKURA are nopaline-type Ti plasmids. The frequency of ejection of pTi-SAKURA in MAFF301001 by transformation via conjugation of the strain with an incompatible small *repABC* plasmid is much lower than that of pTiC58 in the C58 strain (Uraji et al., 2002). The two Ti plasmids are almost identical along 60% of their length (200 kbp), but the remaining regions exhibit no or very low levels of homology. We named the largest nonhomologous region VAR (large variable region) (Suzuki et al., 2000). Because VAR contains many genes whose functions are unidentified (Hattori et al., 2000), we hypothesized that the factor causing the difference of the ejection frequency between the two Ti plasmids is contained within VAR. In the present study, we localized and characterized the factor within VAR of pTi-SAKURA which can enhance incompatibility in small *repABC* plasmids and increase the stability of pTiC58.

MATERIALS AND METHODS

Bacterial strains and culture conditions Bacterial strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* cells were cultured at 28°C in LB medium. *Escherichia coli* cells were cultured at 37°C in LB. When necessary, antibiotics were added to the medium at the following final concentrations: gentamicin (45 µg/ml), kanamycin (50 µg/ml), rifampicin (40 µg/ml), tetracycline (5 µg/ml) and chloramphenicol (35 µg/ml). *A. tumefaciens* cells were also grown on AB minimal salts medium (Chilton et al., 1974) supplemented with nopaline as the sole source of carbon instead of glucose.

Plasmid construction and DNA preparation *E. coli* S17-1 λ pir was used as a host strain for plasmid construc-

tion and conjugal transfer of mobilizable plasmids (Simon et al., 1983; Penfold and Pemberton, 1992). Fosmid clones containing portions of pTi-SAKURA were selected from an *A. tumefaciens* MAFF301001 genomic DNA library (De Costa et al., 2001) by colony hybridization using purified pTi-SAKURA DNA as a probe. Among the 14 recombinant fosmids selected, four clones were chosen for the present study. In order to allow the four plasmids to move into, replicate in and be selected in *Agrobacterium* cells, *repABC* of pTi-SAKURA, *oriT* and *Km^r* genes were inserted into the four fosmids by homologous recombination *in vivo* between plasmids in the *recA⁺* *E. coli* strain LE392. Two fosmid clones, 2K1 and 1F23, contained *repABC* (Fig. 1). They were fused with pJP5603, which contains *Km^r* and *oriT*. The resultant plasmids were named pFK and pFF. The other two fosmids, 1M3 and 2I19, had no *repABC*. They were fused with pJP5603*rep*, which contains *repABC* in addition to *Km^r* and *oriT*. The resultant plasmids were named pFM and pFL. pFK Δ AscI was constructed by AscI digestion of pFK and subsequent self-ligation. pK18msr is a pK18*mobsacB* plasmid carrying a 4.9-kbp HindIII fragment of pMGTrep1 (Uraji et al., 2002); the HindIII fragment contains *repABC*. pN2, pN5, pN7 and pN12 were constructed by inserting each *NheI* fragment of pFK into pK18msr (see Fig. 3). A derivative of pN2, pN2 Δ NspV, was constructed by *NspV* digestion of pN2 and subsequent self-ligation. pHRPN2 and pBINN2 were constructed by inserting a 2.6-kbp *NheI* fragment of pN2 into pHRP311 and pBIN19, respectively. pBINTc was constructed by cloning into pBIN19 a 2.1-kbp *Bam*HI fragment containing a *Tc^r* cassette excised from pHP45 Ω -*Tc*. pN2T-r was constructed by digestion of pN2 with *HindIII* followed by self-ligation and insertion of a 1.7-kbp *Bgl*II fragment of pTiC58 T-DNA. pTiC58px is a fusion plasmid formed *in vivo* in C58 cells between pN2T-r and pTiC58. pN2G-r was constructed by digestion of pN2 Δ NspV with *HindIII* followed by self-ligation and insertion of a 2.8-kbp *AccI*-*NspV* fragment containing a *Gm^r* cassette from pUC19Gm into a unique *NspV* site. pTi-SAKURAmx is pTi-SAKURA with a *tiorf25::Gm^r* mutation, which was formed *in vivo* in MAFF301001rif cells by replacement of *tiorf25⁺* with *tiorf25::Gm^r* of pN2G-r via double crossover between pTi-SAKURA and pN2G-r.

DNA manipulations for plasmid construction were performed using standard procedures (Sambrook et al., 1989).

Introduction of plasmid DNA Plasmids were delivered from *E. coli* to *A. tumefaciens* by conjugal transfer as described elsewhere with some modifications (Uraji et al., 2002). Conjugation efficiency was calculated by dividing the number of transconjugant colonies by the number of donor colonies. Conjugal transfer of plasmids from *E. coli* to *E. coli* was carried out as follows. Donor *E. coli*

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>A. tumefaciens</i>		
MAFF301001rif	Wild-type pathogenic strain carrying pTi-SAKURA, Rif ^r	Uraji et al., 2002
MNS-1	pTi-SAKURA-less derivative of the wild-type strain MAFF301001rif	Uraji et al., 2002
C58	Wild-type pathogenic strain carrying pTiC58	Goodner et al., 2001
C58 rif	Wild-type pathogenic strain carrying pTiC58, Rif ^r	This study
C58C1	pTiC58-less and Rif ^r derivative of the wild-type strain C58	Our collection
<i>E. coli</i>		
Sy327 λ pir	Rif ^r Nal ^r λ pir	Miller and Mekalanos, 1988
S17-1 λ pir	Host strain for recombinant plasmid construction and conjugal transfer; Sm ^r λ pir	Simon et al., 1983
LE392	Host strain for homologous recombination; <i>rec</i> ⁺	Sambrook et al., 1989
Plasmids		
pTi-SAKURA	Ti plasmid (206,479 bp) of <i>A. tumefaciens</i> MAFF301001	Suzuki et al., 1998
pTi-SAKURAmx	pTi-SAKURA with <i>tiorf25::Gm^r</i> mutation; Gm ^r	This study
pTiC58	Ti plasmid (214,233 bp) of <i>A. tumefaciens</i> C58	Goodner et al., 2001
pTiC58px	pTiC58 with an insertion of 2.6-kbp <i>NheI</i> fragment of pTi-SAKURA; Km ^r	This study
2K1	Fosmid clone containing 43.5-kbp <i>HindIII</i> fragment of pTi-SAKURA (9104-52625)*; Cm ^r	Our collection
1F23	Fosmid clone containing 43.9-kbp <i>HindIII</i> fragment of pTi-SAKURA (4045-47954); Cm ^r	Our collection
1M3	Fosmid clone containing 42.3-kbp <i>HindIII</i> fragment of pTi-SAKURA (79831-122160); Cm ^r	Our collection
2I19	Fosmid clone containing 37.8-kbp <i>HindIII</i> fragment of pTi-SAKURA (113054-150896); Cm ^r	Our collection
pFK	Co-integrate between 2K1 and pJP5603; Km ^r Cm ^r	This study
pFF	Co-integrate between 1F23 and pJP5603; Km ^r Cm ^r	This study
pFM	Co-integrate between 1M3 and pJP5603rep; Km ^r Cm ^r	This study
pFI	Co-integrate between 2I19 and pJP5603rep; Km ^r Cm ^r	This study
pFK Δ AscI	pFK with a deletion of 19.3-kbp <i>AscI</i> fragment; Km ^r Cm ^r	This study
pK18mobsacB	Mobilizable cloning vector; <i>ColE1-oriV mob sacB</i> (sucrose ^s) Km ^r	Schäfer et al., 1994
pK18msr	pK18mobsacB with <i>repABC</i> of pTi-SAKURA; Km ^r	This study
pMGTrep1	pK18mobsacB with Gm ^r gene cassette and <i>repABC</i> of pTi-SAKURA; Km ^r Gm ^r	Uraji et al., 2002
pK18mssr	pK18msr with 600-bp <i>NlaIII</i> fragment (<i>tiorf25</i>) of pTi-SAKURA; Km ^r	This study
pJP5603	Mobilizable cloning vector; R6K- <i>ori mob</i> Km ^r	Penfold and Pemberton, 1992
pJP5603rep	pJP5603 with <i>repABC</i> of pTi-SAKURA; Km ^r	This study
pJP5608	Mobilizable cloning vector; R6K- <i>ori mob</i> Tc ^r	Penfold and Pemberton, 1992
pJP5608rep	pJP5608 with <i>repABC</i> of pTi-SAKURA; Tc ^r	This study
pHRP311	Broad host range cloning vector; <i>oriT rep-incQ</i> Gm ^r Sp ^r	Parales and Harwood, 1993
pHRPN2	pHRP311 with 2.6-kbp <i>NheI</i> fragment of pFK; Gm ^r	This study
pN2	pK18msr with 2.6-kbp <i>NheI</i> fragment of pFK; Km ^r	This study
pN5	pK18msr with 5.2-kbp <i>NheI</i> fragment of pFK; Km ^r	This study
pN7	pK18msr with 7.5-kbp <i>NheI</i> fragment of pFK; Km ^r	This study
pN12	pK18msr with 11.9-kbp <i>NheI</i> fragment of pFK; Km ^r	This study
pN2 Δ NspV	pN2 with deletion of 456-bp <i>NspV</i> fragment; Km ^r	This study
pHP45 Ω -Tc	Potable Tc resistance gene (<i>tetC</i>) vector; Ap ^r Tc ^r	Fellay et al., 1987
pBIN19	Binary vector; <i>rep-incP oriT</i> Km ^r	Bevan, 1984
pBINN2	pBIN19 with 2.6-kbp <i>NheI</i> fragment (<i>tiorf24</i> and <i>tiorf25</i>) of pTi-SAKURA	This study
pBINTc	pBIN19 with Tc ^r of pHP45 Ω -Tc; Tc ^r Km ^r	This study
pN2T-r	pK18mobsacB with 2.6-kbp <i>NheI</i> fragment of pTi-SAKURA and 1.7-kbp T-DNA fragment of pTiC58; Km ^r	This study
pN2G-r	pK18mobsacB with 4.7-kbp <i>XbaI</i> fragment containing disrupted <i>tiorf25</i> by Gm ^r insertion mutagenesis; Km ^r	This study
pUC19Gm	pUC19 with 2.9-kbp <i>PstI</i> fragment containing Gm ^r gene cassette; Gm ^r Ap ^r	Our collection

*) Nucleotide coordinate numbers indicate the position of the fragment in pTi-SAKURA.

S17-1 *λpir* cells harboring a plasmid and recipient *E. coli* Sy327 cells were cultured in LB medium until the cell density reached an OD₆₀₀ of 1. Approximately 1.0×10^8 donor cells were washed with 0.9% (w/v) NaCl and then mixed with the same number of recipient cells in 5 ml of LB medium. After incubation at 37°C for 1 hour, 50-μl aliquots were plated onto LB agar supplemented with rifampicin and other antibiotics that were selective for recipient cells that had acquired incoming plasmid.

Electroporation was performed as described by Wise et al. (2006) using a Cell-Porator (BRL, Gaithersburg U.S.A) with Bio-Rad Pulser cubettes (1 mm electrode gap) under the following conditions: voltage, 300 V; capacitance, 330 μF; impedance, low ohms; charge rate, fast; voltage boost resistance, 4 K. After electroporation the cells were immediately re-suspended in 1 ml of SOC medium, and incubated at 28°C with gentle shaking for 1 hour.

Ejection ratio in conjugation In order to determine the ejection ratio of the resident plasmid after introduction of another plasmid, 100 transconjugant colonies selected for incoming plasmid markers were streaked onto LB agar plates with antibiotics to which the resident and incoming plasmids conferred resistance. When the resident plasmid was a Ti plasmid, growth on AB minimal agar with nopaline (2 mg/ml) as the sole source of carbon was used instead of examination of antibiotic resistance, because pTi-SAKURA and pTiC58 code for nopaline utilization genes. After incubation at 28°C for 2 days, growth on the selective agar media was examined to distinguish transconjugants that still retained the resident plasmid from resident-plasmid-less transconjugants.

Loss ratio in successive cultivation *Agrobacterium* cells were cultured overnight in LB at 37°C. Then, 1×10^8 cells were inoculated into fresh LB to repeat the overnight culture. The serial cultivation was repeated 5 times in total. The final culture was diluted and spread on LB agar to establish single colonies. The presence and absence of Ti plasmid in the colonies were checked by colony hybridization using *virB* gene fragment as a probe.

Homology and motif/domain search Searches for DNA and protein homologs in the DNA databases were carried out using the FASTA program (<http://www.ddbj.nig.ac.jp/search/fasta-j.html>). Motif/domain search was performed using the MOTIF program (<http://motif.genome.jp/>) and the Pfam program (<http://www.sanger.ac.uk/Software/Pfam/>).

Extraction of RNA and RT-PCR Logarithmically growing cells were lysed by vortexing with glass beads in the presence of SDS, phenol and chloroform principally as

described elsewhere (Suzuki et al., 1989). RNA was purified from the cell extract by treatment with phenol/chloroform, then treatment with DNase and proteinase followed by ethanol precipitation. For cDNA synthesis, 10 μg of the total RNA were used as a template. cDNA synthesis was carried out using reverse transcriptase ReverTra Ace (Toyobo, Osaka) in accordance with the manufacturer's instructions. An oligonucleotide primer (5'-tegaagctcatcgtgccacatct-3') complementary to the 3' end region of the *tiorf25* open reading frame (ORF) was used as a primer for the reverse transcription (Fig. 3B). Subsequent PCR amplification was performed using KOD Dash DNA polymerase (Toyobo, Osaka) in accordance with the standard method. The gene-specific primer and a forward primer (5'-tatgcgaggcgcatccgaagg-3'), the corresponding sequence of which is located in the middle of *tiorf24*, were used for the PCR amplification (Fig. 3B).

RESULTS

Mapping of the locus that enhances incompatibility in pTi-SAKURA The large variable region (VAR) was thought to contain a region that makes pTi-SAKURA more resistant to interference by incoming incompatible *repABC* replicons than pTiC58. In order to localize the responsible region, we constructed five *repABC* plasmids. As shown in Fig. 1, pFk and pFf contained a portion of the pTi-SAKURA VAR region and pFm harbored another portion of VAR, whereas pFl carried the *vir* region without

Fig. 1. Structure of pTi-SAKURA and the four derivative fosmid clones used in this study. The gray region indicates the *repABC* operon. The hatched region indicates the large variable region (VAR). Arches outside of the Ti plasmid circle indicate the regions that are contained in each fosmid clone. Shown in parentheses are the fosmids with *oriT* and *Km^r* genes. *repABC* was also added in pFl and pFm.

VAR, and a small vector plasmid, pK18msr, possessed only *repABC* (negative control). We used pTi-SAKURA as a positive control in the next experiment.

The mini Ti plasmids and the vector were introduced into a Ti-less strain, MNS-1. Then, the incoming *repABC* plasmid, pJP5608*rep*, was introduced by conjugation, in order to evaluate the five plasmids. As shown in Fig. 2A, MNS-1 harboring pFk or pFf exhibited low transconjugant efficiency, like that harboring pTi-SAKURA. In contrast, strains possessing pFm or pFi exhibited high efficiency, which was as high as that of MNS-1 containing the vector plasmid. The difference in efficiency between the two plasmid groups was greater than about 20-fold.

Using the cells with mini Ti as a recipient, all of the transconjugant colonies examined (100 colonies) were sensitive to kanamycin, suggesting that the resident mini Ti plasmid, which determines kanamycin resistance, was excluded in every transconjugant (data not shown).

pFk and pFf share a 39-kbp segment of pTi-SAKURA (Fig. 1). Thus, the common overlapping region shared by the two plasmids should contain the genes responsible for the effect described above. We localized the effective

region to a 2.6-kbp *NheI* fragment using a series of deletion plasmids and subclone plasmids, as shown in Fig. 3A.

To discriminate whether the suppression takes place during the conjugational entry process or vegetative replication process after the conjugation, we introduced the incoming plasmid by electroporation in place of conjugation. As shown in Fig. 2B, the 2.6-kbp *NheI* fragment decreased the efficiency of transformation via electroporation 7.4-fold. This result indicates that the effect by the 2.6-kbp *NheI* fragment is not due to inefficient cell-cell interaction during conjugation.

In order to know whether the 2.6-kbp *NheI* fragment affects conjugation efficiency when the fragment is present in the incoming plasmid in the conjugation assay, pN2 was introduced into the MAFF301001rif strain by conjugation. The transconjugant efficiency of pN2 was about ten times higher than that of vector plasmid (Fig. 2C). Thus, the fragment is effective even when it is on the incoming plasmid.

On examination of the nucleotide sequence of pTi-SAKURA (Hattori et al., 2000; Suzuki et al., 2000), the 2.6-kbp *NheI* fragment was found to cover nucleotide

Fig. 2. Frequency of transconjugant formation induced by incompatible plasmids. (A) *Agrobacterium tumefaciens* MNS-1 harboring various resident plasmids was used as recipient for conjugation with donor *E. coli* S17-1 λ *pir* (pJP5608*rep*). Each value is the average of at least three independent experiments. Thin vertical bars represent standard deviations. (B) Introduction of pMGTrp1 into the C58C1 harboring plasmid with or without the 2.6-kbp *NheI* fragment by electroporation. (C) Results of reciprocal experiments. *A. tumefaciens* MAFF301001rif strain was used as conjugal recipient, *E. coli* S17-1 λ *pir* harboring plasmid with or without 2.6-kbp *NheI* fragment was used as conjugal donor. + and - in parenthesis indicate presence and absence of the 2.6-kbp *NheI* fragment, respectively.

positions 23587 to 26207 in pTi-SAKURA. The fragment codes for two complete ORFs (*tiorf24* and *tiorf25*) (Fig. 3B). In order to confirm that the two ORFs are responsible for suppressing *repABC* plasmid incursion, deletion mutants were constructed (Fig. 3B). Cells with a plasmid lacking either one of the two ORFs (pN2Δ*NspV*, pK18mssr) allowed entry of the incoming plasmid with similar efficiency to that observed in cells with the vector plasmid (Fig. 2A and Fig. 3B).

Effect of the 2.6-kbp *NheI* region on intact Ti plasmids The 2.6-kbp *NheI* fragment affected whole Ti plasmids in a manner different from the effect on the small plasmids. C58rif, which harbors pTiC58, allowed

introduction of a *repABC* plasmid as efficiently as MAFF301001rif, which harbors pTi-SAKURA (Table 2). However, the ejection ratio of pTiC58 in transconjugants (47%) was higher than that of pTi-SAKURA in transconjugants (6%) (Table 2). Here the ejection ratio was defined as the percentage of transconjugant cells which lost the resident plasmid. Addition of the 2.6-kbp *NheI* fragment to pTiC58 (see pTiC58px in Table 2) decreased the ejection ratio of pTiC58 in transconjugants down to 1%, although the addition had only a negligible effect on the transconjugant efficiency. Furthermore, a similar phenomenon was also observed in MAFF301001rif. A *tiorf25* mutant, pTi-SAKURAmx, was ejected more frequently (53%) than wild-type pTi-

Fig. 3. Transconjugant efficiency of cells harboring a VAR portion of pTi-SAKURA. Left panel, physical map; right panel, efficiency of transconjugant colony formation. (A) Recombinant fosmid clones and subclone plasmids. Gray arrows indicate the *repABC* operon. Hatched boxes indicate the region shared by pF and pK. Restriction site: N, *NheI*; A, *Ascl*. (B) Enlarged physical map of the 2.6-kbp *NheI* fragment. Two small arrows indicate the locations and directions of the two oligonucleotide primers used for RT-PCR (Fig. 4).

Table 2. Influence of addition and deletion of the 2.6-kbp *NheI* fragment to Ti plasmids

Resident plasmid		Transconjugant efficiency (conjugant/donor)	Ejection ratio (%) of resident plasmid	Loss ratio (%) of resident plasmid
pTi-SAKURA	(+)*	1.4×10^{-4}	6	NT
pTi-SAKURAmx	(-)	1.2×10^{-4}	53	NT
pTiC58	(-)	2.7×10^{-4}	47	90
pTiC58px	(+)	2.1×10^{-4}	1	0

The ejection ratio is the percentage of colonies which lost the resident plasmid after conjugation. The loss ratio is the percentage of colonies that lost the resident plasmid after successive cultivation (see MATERIALS AND METHODS section).
* presence (+) and absence (-) of the 2.6-kbp *NheI* region.
NT means Not Tested.

SAKURA (6%). As shown in Table 2, the ejection ratio of the *tiorf25::Gm^r* mutant (pTi-SAKURAmx) of pTi-SAKURA was as high as that of pTiC58. The mutation had only a negligible effect on suppression of the entry of *repABC* plasmids. These results indicate that the 2.6-kbp *NheI* fragment is responsible for the lower ejection ratio of pTi-SAKURA than of pTiC58. In contrast to the intact Ti plasmids, pN2, the small plasmid harboring the 2.6-kbp *NheI* fragment showed a significantly higher ejection ratio (4%) than pTi-SAKURA (data not shown).

In order to examine whether the addition of the 2.6-kbp *NheI* fragment affects the segregational stability of pTiC58, we measured the loss ratio of pTiC58 with or without the fragment after serial cultivation. After 5 repetitions of culturing at 37°C, 90% of the colonies lost pTiC58. In contrast, no colony lost pTiC58px under the same experimental conditions (Table 2). This result shows that the 2.6-kbp *NheI* fragment increased the segregational stability.

Gene expression and mode of action of *tiorf24* and *tiorf25* Transcription of *tiorf24* and *tiorf25* *in vivo* was investigated by RT-PCR experiments. RNA isolated from cells harboring various plasmids was subjected to reverse transcription as described in MATERIALS AND METHODS. The oligonucleotide primers used for PCR were located at the 3'-end of *tiorf25* and in the middle of *tiorf24*, respectively (Fig. 3B). As shown in Fig. 4, a PCR product of about 550-bp in size was observed when total

Fig. 4. RT-PCR analysis of transcription from *tiorf24* and *tiorf25* genes. RNAs extracted from *A. tumefaciens* MNS-1 harboring a plasmid were used as templates for the reverse transcriptase reaction. PCR amplification was carried out using the oligonucleotide primers shown in Fig. 3B and MATERIALS AND METHODS.

RNA extracted from MAFF301001rif and MNS-1 harboring pN2 or pBINN2 was used, whereas no such fragment was produced using total RNA from MNS-1 without the plasmids. Increased expression was observed in cells harboring pBINN2, which contained the 2.6-kbp *NheI* fragment on a multicopy-type vector. These data indicate that *tiorf24* and *tiorf25* are transcribed as a single mRNA.

In order to determine whether the *tiorf24* and *tiorf25* genes can act on plasmids not only in a *cis* but also in a *trans* manner, an incQ-type replicon, pHRPN2, which has the 2.6-kbp *NheI* fragment, was introduced into MNS-1 harboring a *repABC* vector plasmid, pK18msr. MNS-1 harboring pHRPN2 and pK18msr allowed entry of incoming *repABC* plasmid at the same level as cells with pK18msr only (Fig. 2A). These results show that *tiorf24* and *tiorf25* are able to act effectively only in a *cis* manner. In addition, we investigated whether the 2.6-kbp segment of pTi-SAKURA was effective even if the incoming and resident plasmids were of different replication types. MNS-1 harboring pN2 allowed entry of an incQ-type replicon, pHRP311, and an incP-type replicon, pBINTc, with the same efficiency as MNS-1 and MAFF301001rif (Fig. 5A and B). This observation indicates that the resident and incoming plasmids must have the same type of replicon for the two ORFs to exert their effect. This confirmed that the effect of the 2.6-kbp segment was caused by incompatibility enhancement.

We next examined whether the incompatibility-enhancing effect of *tiorf24* and *tiorf25* takes place even when the two genes are located on replicons other than the *repABC* type or even when the two genes are contained in *E.*

Fig. 5. Efficiency of introduction of incQ and incP replicons into *A. tumefaciens* and *E. coli*. Cells harboring a resident plasmid were subjected to conjugation with donor *E. coli* S17-1 λ pir harboring an incoming plasmid shown in the upper part of each panel. *A. tumefaciens* MNS-1 (panels A, B and C) and *E. coli* Sy327 λ pir (panel D) harboring a resident plasmid as shown underneath each bar were used as recipient.

Table 3. Homologues of the Tiorf25 protein

Accession No.	Gene or ORF	Organism	Amino acid Identity (%)	E value	Feature
CAD84464	<i>NE0553</i>	<i>Nitrosomonas europaea</i> ATCC 19718	50	5e ⁻¹²	PIN domain
EAQ19904	<i>PnapDRAFT_1562</i>	<i>Polaromonas naphthalenivorans</i> CJ2	37	1e ⁻⁹	Similar to <i>Agrobacterium tumefaciens</i> Yle
ABC93127	<i>RHE_PB00085</i>	<i>Rhizobium etli</i> CFN 42	44	7e ⁻⁸	Putative plasmid stabilization Protein
AAK64896	<i>Sma045</i>	<i>Sinorhizobium meliloti</i> 1021	33	1e ⁻⁷	Homologous to possible plasmid stability locus Yle from <i>A. tumefaciens</i> octopine-type Ti plasmid
BAC47699	<i>Bll2434</i>	<i>Bradyrhizobium japonicum</i> USDA 110	41	2e ⁻⁷	Plasmid stability protein
CAJ30068	<i>MgI417</i>	<i>Magnetospirillum gryphiswaldense</i>	43	3e ⁻⁷	Putative plasmid stability-like protein
AAL03856	<i>RC1318</i>	<i>Rickettsia conorii</i> Malish 7	31	4e ⁻⁷	Similarity to plasmid stability protein

coli. The 2.6-kbp *NheI* fragment on the incP vector pBIN19 (see pBINN2) suppressed the entry of another incP-type replicon pBINTc into MNS-1 16-fold. However, such suppression was not detected in *E. coli* (Fig. 5C and D). These results show that the 2.6-kbp *NheI* fragment confers incompatibility-enhancing effect to the resident plasmid when the same type replicon is introduced in *Agrobacterium* cells, but that the fragment does not contain an incompatibility-determining region itself.

Homology analysis using the FASTA program indicated that *tiorf24* is an orphan ORF, having no homologous ORF in the databases searched, and that *tiorf25* is a homolog of *stbB*, which is a part of the *stbABCD* gene cluster. *stbABCD* is a plasmid stability locus in *Pseudomonas syringae* pv. *tomato* (Hanekamp et al., 1997). Neither *Pseudomonas stbB* nor any *stbB* homologues (Table 3) have been characterized functionally yet. No matching motif was detected by MOTIF analysis. However, domain searching using the Pfam program detected the PIN (twitching motility protein PilT N-terminus) domain in the Tiorf25 protein (score = 23.0 bits, E value = 8e⁻⁰⁷).

DISCUSSION

This study showed that the region which is responsible for plasmid stability as well as incompatibility-enhancement in pTi-SAKURA is located on a 2.6-kbp *NheI* fragment, which contains *tiorf24* and *tiorf25*. The 2.6-kbp *NheI* fragment has the ability to inhibit transformation by an incoming plasmid only when the resident and incoming plasmids belong to the same incompatibility group. This inhibition occurred regardless of the plasmid introduction procedure (conjugation or electroporation). Thus, inhibition should occur by a mechanism that operates after cell-cell contact. Because the 2.6-kbp *NheI* fragment was effective on *repABC* replicons and an

incP replicon (Fig. 2A and Fig. 5C), its effect is not directly involved in replicon-specific replication, stabilization, or incompatibility mechanisms. Since two different replication type plasmids containing the 2.6-kbp *NheI* fragment (pN2 and pHRPN2) can replicate independently in the same *Agrobacterium* cell (data not shown), it is unlikely that this region is an incompatibility-determining site, for example one that interacts directly with cellular centromere-like components. Addition of the 2.6-kbp *NheI* fragment to pTiC58 also decreased the loss ratio during growth at 37°C. The 2.6-kbp *NheI* fragment determines higher plasmid stability, and plasmid incompatibility enhancement may be reflected by increasing segregational stability.

The 2.6-kbp *NheI* fragment contains two complete ORFs (*tiorf24* and *tiorf25*). Our RT-PCR experiment showed that the two ORFs are transcribed as a single mRNA. *tiorf24* is an orphan gene, whereas *tiorf25* is a homologue of a plasmid stabilization protein (Table 3). The putative plasmid stabilizing genes listed in Table 3 are homologues of *stbB* on the B-plasmid of *Pseudomonas syringae* pv. *tomato* (Hanekamp et al., 1997). It has been reported that the *stbB* gene is encoded in the plasmid stabilization cluster (*stbABCD*), but its function is still unknown. These data also suggest that the incompatibility-enhancing effect of *tiorf24* and *tiorf25* is caused by a plasmid stabilization mechanism. Our domain search using the Pfam program detected the PIN (twitching motility protein PilT N-terminus) domain in Tiorf25. PIN is a compact domain of about 100 amino acid residues, with two conserved aspartate residues. We also detected the PIN domain in all proteins listed in Table 3. *Leptospira interrogans* gene *vapC* has a PIN domain and is homologous to the putative stabilizer genes listed in Table 3. The *vapC* gene encodes a toxic protein of the toxin-antitoxin system (TA system), which also involves the antitoxin gene *vapB*

(Zhang et al., 2004). These genes are closely located, suggesting an operonic structure, as generally found in TA systems. Although the *Leptospira* TA system is located in the chromosome, the region has the ability to stabilize an unstable plasmid when added to the plasmid. Because *tiorf25* also contains a PIN domain and together with adjacent *tiorf24* comprises an operon, the Ti plasmid stabilizing effect of two ORFs might be caused by a TA mechanism.

The difference in the plasmid ejection ratio in cells containing pTi-SAKURA and pTiC58 in response to introduction of incompatible *repABC* replicons is contingent on *tiorf24* and *tiorf25*. Furthermore, differences in exclusion and permissiveness for *repABC* plasmid incursion were significant between intact Ti plasmids and smaller plasmids such as pN2 (data not shown). It is likely that an additional stabilization factor causes the difference in permissiveness between intact Ti and small *repABC* plasmids. An alternative explanation is the contribution of plasmid size differences, since the stability of yeast artificial chromosomes depends on their size (Zakian et al., 1986), and *repABC* replicons are generally huge plasmids or chromosomes, such as the linear chromosome of *A. tumefaciens* C58 (about 2,000 kbp), pSymb of *Sinorhizobium meliloti* (about 1,700 kbp) and Ti and Ri plasmids (about 200 kbp) (Galibert et al., 2001; Goodner et al., 2001; Moriguchi et al., 2001).

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