Construction of disarmed Ti plasmids transferable between *Escherichia coli* and *Agrobacterium* species

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ABSTRACT

Agrobacterium-mediated plant transformation has been used widely, but there are plants that recalcitrant to this type of transformation. This transformation method uses bacterial strains harboring a modified tumor-inducing (Ti) plasmid that lacks the transfer DNA (T-DNA) region (disarmed Ti plasmid). It is desirable to develop strains that can broaden the host range. A large number of *Agrobacterium* strains have not been tested yet to determine wheter they can be used in transformation. In order to improve disarming method and to obtain strains disarmed and ready for the plant transformation test, we developed a simple scheme to make certain Ti plasmids disarmed and simultaneously maintainable in *Escherichia coli*, and mobilizable between *E. coli* and *Agrobacterium*. To establish the scheme in nopaline-type Ti plasmids, a neighboring seament to the left of the left border sequence, a neighboring segment to the right of the right border sequence of pTi-SAKURA, a cassette harboring the pSC101 replication gene between these two segments, the broad-host-range incP-type *oriT*, and the gentamicin resistance gene were inserted into a suicide-type *sacB*-containing vector. Replacement of T-DNA with the cassette in pTiC58 and pTi-SAKURA occurred at high frequency and with high accuracy when the tool plasmid was used. We confirmed that there was stable maintenance of the modified Ti plasmids in the *E. coli* strain S17-1λpir and conjugal transfer from *E. coli* to Ti-less *Agrobacterium* strains and that the reconstituted *Agrobacterium* strains were competent to transfer DNA into plant cells. As the

modified plasmid delivery system was simple and efficient, conversion of strains to disarmed type was easy and should be applicable in studies to screen for useful strains.

INTRODUCTION

The *Agrobacterium*-mediated transformation has been considered as the most efficient and reliable method for plant biology and biotechnology. This methodology has been established for many plants but not for others. One of the major factors affecting the applicability is the limited number of donor *Agrobacterium* strains, because the method depends exclusively on the host range of the strains.

Wild type *Agrobacterium* strains harboring a tumor-inducing (Ti) plasmid are the causative agent of crown gall tumor disease on dicotyledonous plants (35). The transfer DNA (T-DNA) and virulence gene (*vir*) regions in the Ti plasmid are essential for tumorigenesis. The *vir* gene products nick the T-DNA region at its left border (LB) and right border (RB), and then transfer T-DNA into plant cells. T-DNA contains phytohormone synthesis genes, whose expression causes infected plants to suffer from unregulated growth (5, 26). Hairy root-inducing (Ri) plasmid also has a similar T-DNA system.

The binary vector system (11) is widely used for the *Agrobacterium*-mediated transformation. Binary vectors are small plasmids with a cloning site and a selectable marker gene

between LB and RB (2). To ensure transformation ability without tumorigenicity, *Agrobacterium* strains for the transformation system contain a modified Ti plasmid, which lacks T-DNA (disarmed) but retains the entire *vir* region. Unfortunately, only a small number of Ti plasmids have been disarmed.

Most pathogenic *Agrobacterium* strains are classified into three species: *A. tumefaciens* (biovar 1, *Rhizobium radiobactor*), *A. rhizogenes* (biovar 2, *R. rhizogenes*); and *A. vitis* (biovar 3, *R. vitis*) (33). The genomic organization of the *Agrobacterium* species are diverse (25, 27, 29). Pathogenic strains in each species are variable (1), and some of them might be potentially more effective for transformation than the strain used previously. For instance, *Agrobacterium* strain KAT23 causes tumors in legume plants, including common bean and soybean, very effectively (34). Disarmed Ti or Ri plasmids are either chosen from among mutants or created by homologous recombination with a plasmid designed for this purpose (12, 16, 17). Both methods require either extensive screening efforts or knowledge of the structural and functional information for the plasmids. However, the large size of Ti and Ri plasmids, approximately 200 kbp, makes structural analysis and modification difficult. Complete nucleotide sequence of several Ti and Ri plasmids (for example, pTi-SAKURA, pTiC58 and pRi1724) has been reported (9, 14, 24, 26, 31). Accumulation of such nucleotide sequence information makes targeted replacement easier than it was previously. However, the large size of T-DNA obstructs the double crossover in the removal

process during engineering. In addition to Ti plasmids, chromosomal virulence genes are necessary for plant transformation. It has been pointed out that combining of a Ti plasmid with certain chromosomal backgrounds can markedly influence virulence (8). Thus, the transfer of large plasmids to various *Agrobacterium* strains is another important engineering step, which is still not easy for researchers who are not familiar with *Agrobacterium* biology.

In this study, we describe a simple method and tool plasmids for construcing versatile disarmed nopaline-type Ti plasmids mobilizable from *Escherichia coli* to *Agrobacterium* strains, conversion of nopaline-type *Agrobacterium* strains to disarmed strains using the tool plasmids and simple selection media, and conversion of Ti-less *Agrobacterium* strains to disarmed strainss using the modified Ti plasmids.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in **Table 1**. *E. coli* strains were grown at 37 °C in LB medium (1% Bacto-tryptone, 0.5% NaCl, and 0.5% yeast extract). *A. tumefaciens* strains were cultured at 28 °C in LB medium or IFO medium (1% polypepton, 0.2% yeast extract, and 0.1% $MgSO_4$). *A. rhizogenes* strains were cultured at 28 °C in IFO medium. Antibiotics were added at the following final concentrations: 50 μg/ml gentamicin, 50 μg/ml kanamycin, 30 μg/ml nalidixic acid, 50 μg/ml rifampicin, 50 μg/ml

ampicillin, 50 μg/ml neomycin, and 100 μg/ml spectinomycin.

Plant materials for transformation. *Nicotiana tabacum* SR-1 and *Kalanchoe* sp. were used as host plants for infection and DNA transfer experiments. *N. tabacum* SR-1 was cultured azenically on MS medium solidified with 0.8% agar at 28 °C with continuous light illumination. *Kalanchoe* sp. was cultured in a green house. Leaves were surface-sterilized by 1% sodium hypochlorite for 15 min and rinsed for 2 min with sterile distilled water 4 times before azenic experiments.

Plasmid construction. For the construction of tool plasmids pLRS-GmsacB and pLRS-Gms2, see SUPPLEMENTAL MATERIALS AND METHODS. The 1.4-kbp left fragment (LL) just outside the left border, and the 1.0-kbp right fragment (RR) just outside the right border of T-DNA were derived from pTi-SAKURA (24). The gentamicin-resistance gene (Gm^r) was taken from pUCGm2, *sacB* gene and Km^r gene from. pK18mobsacB (21), IncP type (RK2) *oriT* from pJP5603 (18), and the low copy type pSC101 oriV from pMW119 (Nippon Gene, Tokyo).

A binary plasmid pBIN-GI was prepared as follows. A 2.6-kbp *Hin*dIII-*Eco*RI fragment containing the GUS gene with an intron was taken from pIG221 (15) and inserted into pBIN19 (2).

DNA preparation and analysis. Plasmid DNA was extracted from bacterial cells by the alkaline-SDS method (3). Manipulation of plasmid DNA was performed following standard methods.

Bacterial transformation. Modified shuttle Ti plasmids were extracted from *A, tumefaciens* strains by the modified alkaline-SDS method and purified by EtBr-CsCl gradient ultracentrifugation. Purified shuttle Ti plasmids were introduced into *E. coli* strains by electroporation as described previously (20, 32).

Plasmids were delivered from *E. coli* to *Agrobacterium* strains by conjugal transfer as described elsewhere (28), with some modifications. The *E. coli* and *Agrobacterium* cell mixture was spotted onto LB agar for conjugation of *A. tumefaciens* and IFO agar for conjugation of *A. rhizogenes*. After overnight incubation at 28 °C, cells were resuspended and spread onto appropriate selective agar media.

Plant transformation. Transformation of tobacco leaf disks was carried out according to (6) with some modifications. *Agrobacterium* strains transformed with the binary vector, pBIN-GI, were grown overnight in liquid media supplemented with the appropriate antibiotics. Tobacco leaf disks (1 cm ϕ) were immersed into the *Agrobacterium* suspension (0.8 OD_{660}) for 5 min and co-cultivated for 2 days at 22 °C under continuous fluorescent light illumination. After co-cultivation, the leaf disks were cultivated on MS selective agar with 200 μg/ml claforan, and 100 μg/ml kanamycin at 28 °C with fluorescent light illumination. *Kalanchoe* leaf disks were subjected to the same transformation method with different phytohormone and antibiotics concentrations: 0.5 mg/l benzyladenine, 2.0 mg/l naphthyl acetic acid, and 50 μg/ml

kanamycin.

Quantitative and histochemical analyses of β-glucuronidase activity were carried out according to (13).

RESULTS

Construction of disarmed shuttle Ti plasmids. We designed a simple engineering scheme that can make pathogenic Ti plasmids disarmed, stably maintainable in *E. coli*, and mobilizable between *E. coli* and *Agrobacterium* species. As an example, we used the scheme with nopaline-type plasmids. We first constructed pLRS-GmsacB and pLRS-Gms2 (see **Fig. S1 in the supplemental material**) as tool plasmids to modify nopaline-type Ti plasmids. these tool plasmids are pK18mobsacB containing two fragments, LL and RR, which neighbor to the left of LB and to the right of RB of T-DNA in pTi-SAKURA, respectively, and a cassette containing a gentamicin-resistance gene, the low-copy-number type replication origin (*oriV*) derived from pSC101, and the incP-type transfer origin (*oriT*) sandwiched between LL and RR. The pSC101 replication *ori* should allow the chimeric plasmids to replicate at a very low copy number in *E. coli*.

Two nopaline-type Ti plasmids pTiC58 and pTi-SAKURA were modified using pLRS-GmsacB as shown in **Fig. 1**. First, pLRS-GmsacB in *E. coli* was introduced by conjugation into two pathogenic nopaline-type strains belonging to *A. tumefaciens* (biovar 1). C58rif is the pathogenic strain harboring pTiC58. C58C1 is a Ti-less strain. C58C1 harboring pTi-SAKURA is another pathogenic strain. Because pLRS-GmsacB cannot replicate in *Agrobacterium* cells, the tool plasmid should integrate into the Ti plasmids by homologous recombination at either LL or RR in the transformants (**Fig. 1 panel I**). The *Agrobacterium* transconjugants were resistant to gentamicin

and kanamycin, and sensitive to sucrose due to the Gm^r, Km^r, and *sacB* genes on the fusion plasmids.

Next, the transconjugants harboring the resulting fusion plasmid were cultured on LB agar supplemented with gentamicin and sucrose. Cultivation in a sucrose-containing medium is selects for cells that do not have the *sacB* gene. Loss of the fusion plasmid can occur at a high frequency. Loss of this plasmid converts cells to Gm^s, Km^s, and sucrose-resistant cells. Deletion of the *sacB* gene from the plasmid can take place at high frequency through homologous recombination in two ways: recombination between two RR segments resulting in removal of the pLRS-G*msacB* portion, or, alternatively, between two LL segments resulting in the loss of T-DNA region (**Fig. 1 panel II**). The former recombination converts cells to Gm^s, whereas the latter maintains Gm^r. Thus, colonies on the selective agar plate were expected to have a disarmed type pTi. To confirm the lack of T-DNA in the derivatives of pTiC58 and pTi-SAKURA, for each Ti plasmid four colonies were randomly chosen from the selective agar culture and analyzed by PCR. T-DNA products were not detected in any of the colonies examined, whereas the *virB* gene was detected in every colonies examined in another PCR experiment (data not shown). These results suggest that there was accurate and frequent removal of the long T-DNA region by replacement using pLRS-GmsacB and the simple selection media. The resultant Ti plasmids were named pTiC58-S and pTi-SAKURA-S.

Introduction of the modified Ti into *Agrobacterium* species via *E. coli.* Modified Ti plasmids pTiC58-S and pTi-SAKURA-S were extracted from the *Agrobacterium* strains. The plasmid DNAs were introduced into two *E. coli* strains, S17-1λpir and SURE. In order to check the structural integrity of the modified Ti plasmids during the maintenance in *E. coli*, the plasmid DNAs were extracted from the *E. coli* transformants. The *Eco*RI fragment ladder profiles suggest that pTi-SAKURA-S was maintained stably in S17-1λpir (**Fig. 2 panel I**) and that pTiC58-S was also in the same *E. coli* strain (data not shown). Structural alteration was not detectable even after three serial repetitions of the *E. coli* culture (**Fig. 2 panel II**). In contrast to the plasmids in S17-1λpir, pTi-SAKURA-S suffered from significant deletions in another *E. coli* strain, strain SURE (**Fig. 2 panel I**).

Because S17-1λpir possesses the incP type *tra* genes in its chromosome, it was expected that the S17-1λpir transformants could mobilize the modified Ti plasmids to various bacteria by conjugation. The Ti plasmid-less *Agrobacterium* strain C58C1 was cocultivated with the S17-1λpir transformants harboring the modified Ti plasmids. Resulting Rif^r Gm^r transconjugant frequency was $5x10^{-5}$ for pTiC58-S and $4x10^{-5}$ for pTi-SAKURA-S. Similarly, the modified Ti plasmids were also introduced successfully by conjugation into another Ti plasmid-less *A. tumefaciens* strain, strain MNS-1, and into an Ri plasmid-less *A. rhizogenes* strain, strain A4RL.

Evaluation of reconstructed *Agrobacterium* strains. We performed plant

transformation experiments to confirm the ability of the *Agrobacterium* transconjugants constructed as described above. For this experiment, the *Agrobacterium* transconjugants were transformed with an intron-containing *GUS* reporter plasmid pBIN-GI. The activity of the reconstructed *Agrobacterium* strains for transformation of tobacco leaf disks was as high as that of the original *Agrobacterium* strains in which the Ti plasmids were modified (see **Fig. S2 in the supplemental material**). This result indicates that the modified Ti plasmids maintained T-DNA transfer ability even after the transmission from *E. coli* to *Agrobacterium*.

As shown above, pTiC58-S and pTi-SAKURA-S in S17-1λpir were mobilizable into *Agrobacterium* strains, and this enabled us to easily convert *Agrobacterium* strains to a disarmed type. We also tried to evaluate the disarmed Ti plasmids as well as the Ti- and Ri-free strains. As mentioned above, we introduced each of the two disarmed Ti plasmids into two *A. tumefaciens* strains, C58C1 and MNS-1, and one *A. rhizogenes* strain, A4RL. The disarmed-plasmid-containing strains were transformed with a GUS reporter binary plasmid pBIN-GI. Then, transformation of tobacco and *Kalanchoe* leaf disks was carried out with these reconstructed *Agrobacterium* strains. Two weeks after cocultivation with the donor *Agrobacterium* strains, kanamycin-resistant (Km^r) calluses were observed on the tobacco leaf disks. pTi-SAKURA-S was as effective as pTiC58-S in all strains tested (data not shown). Km^r calluses were induced in tobacco frequently by C58C1 strains containing this plasmid, and less frequently by A4RL strains containing the same disarmed

plasmid. However, Km^r calluses were rarely induced by MNS-1 strains having the plasmid. GUS activity in the tobacco leaf disks (**Fig. 3 pane l**) was comparable to the data for formation of Km^r calluses. Regenerated recombinant tobacco plants were obtained from the Km^r calluses and showed GUS activity in their leaves and roots (see **Fig. S3 in the supplemental material**). When we treated *Kalanchoe* leaf disks, however, A4RL strains containing the disarmed plasmid induced higher GUS activity than C58C1 strains containing the same plasmid as shown in **Fig. 3 panel II**. The preference for A4RL of the *Kalanchoe* sp. was in contrast to the preference for C58C1 rather than A4RL of tobacco.

DISCUSSION

In this study, we described a new disarming scheme and construction of versatile disarmed nopaline-type Ti plasmids mobilizable from *E. coli* to *Agrobacterium* strains and then conversion of Ti-less *Agrobacterium* strains to disarmed strains taking advantage of the modified Ti plasmids. Stable maintenance of Ti plasmids both in *E. coli* and during the transfer step is a prerequisite for delivering the disarmed plasmids to many strains of *Agrobacterium* and related genera and subsequent examination of their plant transformation abilities. Several research groups have tried to maintain Ti plasmids in *E. coli*. Native Ti plasmids cannot replicable in *E. coli*, and therefore require additional replication genes functional in *E. coli.* Sprinzl and Geider (23) added

the phage fd *ori* to a nopaline-type Ti plasmid. However, the modified Ti plasmid was inserted into chromosomal DNA of *E. coli*. Velikov and Buryanov (30) added colE1 *ori* to a nopaline-type Ti plasmid, but the modified Ti plasmid was either inserted into chromosomal DNA or maintained as a much smaller plasmid resulting from large deletions.

In this study, we replaced T-DNA with the cassette containing *oriT* derived from RK2 and *oriV* derived from pSC101. This replacement was efficient using the tool plasmid constructed in this study. Two modified Ti plasmids were stably maintained in the *E. coli* strain S17-1λpir. Substitution of the low copy-number *oriV* for high copy-number *oriV* is likely to be effective for stable maintenance in *E. coli*. On the other hand, the modified Ti plasmids were damaged in another *E. coli* strain, strain SURE, due to large deletions, even though SURE was developed using a scheme to increase plasmid structural stability by mutating genes related to DNA recombination and repair pathways (10). In any case, it is clear that *E. coli* strain used is very important for Ti plasmid maintenance..

It was easy to transfer the modified Ti plasmids from S17-1λpir to *Agrobacterium* strains. Moreover, reconstructed *A. tumefaciens* and *A. rhizogenes* strains harboring the modified Ti plasmids successfully transformed plant cells. Therefore, using the *E. coli* strain S17-1λpir harboring the shuttle Ti plasmids, various Ti- and Ri-less *Agrobacterium* strains could be easily converted to disarmed strains useful for plant transformation tests. Plasmid delivery by the

incP-type system conjugation does not require addition of any special inducer molecules and enables transfer to wide range of bacteria, while the conjugation with the *tra* regulon on Ti plasmids requires special inducer, such as agrocinopine (7, 19), which are not available commercially.

Broothaerts *et al*. (4) mobilized pTiEHA101 derivatives that contain incP-type *oriT* using a helper transferable plasmid RP4-4 into *Sinorhizobium meliloti*, *Mesorihzobium loti* and a *Rhizobium* species. They detected T-DNA transfer ability in the transconjugant bacteria. It was necessary to remove the helper plasmid from the transconjugants, because the transconjugants received not only Ti but also the helper plasmid and the latter suppressed the T-DNA transfer ability. The donor *E. coli* strain S17-1λpir employed in this study was easy to select against and moreover is convenient in that it does not deliver the helper incP plasmid to recipient cells.

The C58C1 strains having modified Ti transformed tobacco leaf disks more efficiently than the A4RL strains harboring the same modified Ti did. On the other hand, the latter strains were more effective at transforming *Kalanchoe* leaf disks. These results suggest that various genomic backgrounds of the *Agrobacterium* strains differentially influence the fitness for each plant. There might be strains *Agrobacterium* strains among pathogenic *Agrobacterium* strains that are more efficacious than the commonly used *Agrobacterium* strains. The disarmed Ti plasmids constructed in this study would help the screening for such strains.

Complete nucleotide sequences are available in several different type Ti and Ri plasmids (26). Their difference in the auxiliary *vir* region affects the host range in part. It is worth replacing the LL and RR segments in the tool plasmids with the corresponding segments of various types plasmids in order to develop disarmed strains of a type other than the nopaline one.

In addition to pLRS-GmsacB, we constructed pLRS-Gms2 (see Fig. S2 **in the supplemental material**). The latter tool plasmid is also applicable to disarm nopaline type plasmids and is superior to pLRS-GmsacB since it lacks Ap^r gene in the cassette, and therefore does not increase the resistance to β-lactam antibiotics in the disarmed strains. Using a simple and efficient Ti-curing method which we reported previously (32) and the shuttle Ti plasmids constructed in this study, it would be easy to convert many pathogenic *Agrobacterium* strains disarmed strains, even for researchers who are not familiar with *Agrobacterium* biology.

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REFERENCES

1. **Bautista-Zapanta, J., H. Arafat, K. Tanaka, H. Sawada and K. Suzuki.** 2007. Variation of 16S-23S internally transcribed spacer sequence and intervening sequence in rDNA among the three major *Agrobacterium* species. Microbiol. Res. DOI: 10.1016/j.micres.2007.08.003.

2. **Bevan, M.** 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic. Acids Res. **12**: 8711-8721.

3. **Birnboim, H.C. and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**: 1513-1523.

4. **Broothaerts, W. , H.J. Mitchell, B. Weir, S. Kaines, L.M. Smith, W. Yang, J.E. Mayer, C. Roa-Rodríguez and R.A. Jefferson.** 2005. Gene transfer to plants by diverse species of bacteria. Nature **433**: 629-633.

5. **Citovsky, V. , V. Kozlovsky, B. Lacroix, A. Zaltsman, M. Dafny-Yelin, S. Vyas, A. Tovkach and T. Tzfira.** 2007. Biological systems of the host cell involved in *Agrobacterium* infection. Cell. Microbiol. **9**: 9-20.

6. **Clemente, T**. 2006. *Nicotiana* (*Nicotiana tobaccum, Nicotiana benthamiana*). Methods Mol. Biol. **343**: 143-154.

7. **Ellis, J.G., A. Kerr, A. Petit and J. Tempe.** 1982. Conjugal transfer of nopaline and agropine Ti-plasmids – the role of agrocinopines. Mol. Gen. Genet. **186**: 269-274.

8. **Gelvin, S.B. and C. Liu.** 1994. Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant plant species. Plant Mol. Biol. Manual. **B4**: 1-13.

9. **Goodner, B. , G. Hinkle, S. Gattung, N. Miller, M. Blanchard, et al.** 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science **294**: 2323-2328.

10. **Greener, A.L.** 1996. Cloning host organisms. US Patent 5552314.

11. **Hoekema, A., P.R. Hirsch, P.J.J. Hooykaas and R.A. Schiperoort.** 1983. A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303**: 179-180.

12. **Hood, E.E., G.L. Helmer, R.T. Fraley and M.D. Chilton.** 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol. **168**: 1291-1301.

13. **Jefferson, R.A., T.A. Kavanagh and M.W. Bevan.** 1987. GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**: 3901-3907.

14. **Moriguchi, K., Y. Maeda, M. Satou, N.S.N. Hardayani, M. Kataoka, N. Tanaka and K. Yoshida.**2001. The complete nucleotide sequence of a plant root-inducing (Ri) plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in *Rhizobiaceae*. J. Mol. Biol. **307**: 771-784.

15. **Ohta, S., S. Mita, T. Hattori and K. Nakamura.** 1990. Construction and expression in tobacco of a β-Glucuronidase (GUS) reporter gene containing an intron within the coding sequence plant. Plant Cell Physiol. **31**: 805-813.

16. **Ooms, G., P.J. Hooykaas, R.J. Van Veen, P. Van Beelen, T.J. Regensburg-Tuïnk and R.A. Schilperoort.** 1982. Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid **7**: 15-29.

17. **Palanichelvam, K., P. Oger, S.J. Clough, C. Cha, A.F. Bent and S.K. Farrand.** 2000. A second T-region of the soybean-supervirulent chrysopine-type Ti plasmid pTiChry5, and construction of a fully disarmed vir helper plasmid. Mol. Plant Microbe Interact. **13**: 1081-1091.

18. **Penfold, R.J. and J.M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118**: 145–146.

19. **Piper, K.M. and S.K. Farrand.** 2000. Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. J. Bacteriol. **182**: 1080-1088.

20. **Sawahel, W., G. Sastry, C. Knight and D. Cove.** 1993. Development of an electro-transformation system for *Escherichia coli* DH10B. Biotech. Tech. **7**: 261-266.

21. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small

mobilizable multi-purpose cloning vector derived from the *Escherichia coli* plasmid pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**: 69-73.

22. **Simon, R., U. Priefer and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technol. **1**: 784-794.

23. **Sprinzl, M. and K. Geider.** 1988. Transfer of the Ti Plasmid from *Agrobacterium tumefaciens* into *Escherichia coli* cells. J. Gen. Micobiol. **134**: 413-424.

24. **Suzuki, K., Y. Hattori, M. Uraji, N. Ohta, K. Iwata, K. Murata, A. Kato and K. Yoshida.** 2000. Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. Gene **242**: 331-336.

25. **Suzuki, K., K. Iwata and K. Yoshida.** 2001. Genome analysis of *Agrobacterium tumefaciens*: construction of physical maps for linear and circular chromosomal DNAs, determination of copy number ratio and mapping of chromosomal virulence genes. DNA Res. **8**: 141-152.

26. **Suzuki, K., K. Tanaka, S. Yamamoto, K. Kiyokawa, K. Moriguchi and K. Yoshida.** in press. Ti and Ri plasmids. *In* E. Schwartz (ed.), Microbial Megaplasmids. in Microbiology Monograph series, Springer Verlag, Heidelberg. doi:10.1007/7171_2008_22.

27. **Tanaka, K., H. Urbanczyk, H. Matsui, H. Sawada and K. Suzuki.** 2006. Construction of physical map and mapping of chromosomal virulence genes of the biovar 3 *Agrobacterium*

(*Rhizobium vitis*) strain K-Ag-1. Genes Genet. Syst. **81**: 373-380.

28. **Uraji, M., K. Suzuki, and K. Yoshida.** 2002. A novel plasmid curing method using incompatibility of plant pathogenic Ti plasmids in *Agrobacterium tumefaciens*. Genes Genet. Syst. **77**: 1–9.

29. **Urbanczyk, H., K. Suzuki, K. Yoshida and K. Kondo.** 2003. Physical and gene maps of *Agrobacterium* biovar 2 strains and their relationship to biovar 1 chromosomes. Microbiology **149**: 3035-3042.

30. **Velikov, V.A. and Y.I. Buryanov** 1998. Formation of deletional derivatives of the Ti-plasmid pGV3850 in a conjugative transfer from *Agrobacterium tumefaciens* to *Escherichia coli*. Genetica **34**: 1056-1062.

31. **Wood, D.W., J.C. Setubal, R. Kaul, D.E. Monks, J.P. Kitajima, et al.** 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science **294**: 2317-2323.

32. **Yamamoto, S., M. Uraji, K. Tanaka, K. Moriguchi and K. Suzuki.** 2007. Identification of pTi-SAKURA DNA region conferring enhancement of plasmid incompatibility and stability. Genes Genet. Syst. **82**: 197-206.

33. **Young, J.M., L.D. Kuykendall, E. Martínez-Romero, A. Kerr and H. Sawada** 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajjudie *et al*. 1998

as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. Int. J. syst. Evo.l Microbiol. **51**: 89-103.

34. **Yukawa, K., H. Kaku, H. Tanaka, Y. Koga-Ban and M. Fukuda.** 2007. Characterization and host range determination of soybean super virulent *Agrobacterium tumefaciens* KAT23. Biosci. Biotechno. Biochem. **71**: 1676-1682.

35. **Zambryski, P., J. Tempe and J. Schell.** 1989. Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell **56**: 193-201.

1 **Table.1** Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype or characteristics	Reference or source
Escherichia coli		
S17-1λpir	Tp^r Sm^r λpir *tra*$^+$ *recA*	22
SURE	Km^r Tc^r *recB recJ sbcC umuC relA1*	Stratagene, La Jolla
Agrobacterium tumefaciens		
C58rif	Rif ^{r} mutant of the pathogenic strain C58 carrying pTiC58	Our collection
C58C1	Ti plasmid less and Rif ^{r} derivative of C58; Rif ^{r}	Our collection
C58C1(pTi-SAKURA)	C58C1 harboring pTi-SAKURA	Our collection
MNS-1	Ti plasmid less MAFF301001rif ; Rif ^{r}	28
Agrobacterium rhizogenes		
A4RL	Ri plasmid less A4; Nal^r	Our collection
Plasmid		
pK18mobsacB	Mobilizable plasmid; Km^r *sacB oriT*	21
pUCGm2	pUC19 harboring Gm^{r}gene	Our collection
pMW119	Low copy number pSC101-replicon; Ap^r	Nippon Gene, Tokyo
pJP5603	Mobilizable plasmid; Km^r	18
pLRS-GmsacB	pK18mobsacB containing LL, RR, pSC101-replicon and pJP5603 *oriT*; Km^r Gm^r Ap^r *sacB*	This study
pLRS-Gms2	pLRS-GmsacB lacking Ap^r; Km^r Gm^r *sacB*	This study
pTiC58-S	pTiC58 containing Gm^r Ap^r pSC101-*rep oriT* instead of T-DNA region	This study
pTi-SAKURA-S	pTi-SAKURA containing Gm^r Amp^r pSC101-*rep oriT* instead of T-DNA region	This study
pBIN19	Binary vector with *nptII* driven by P*nos*; Km^r	2
pIG221	pUC19 with intron-containing GUS gene	15
pBIN-GI	pBIN19 with intron-containing GUS gene; Km^r	This study

2

FIGURE LEGENDS

Fig. 1 Conversion of pathogenic Ti plasmids so that they are disarmed and transferable between *E. coli* and *Agrobacterium*. The modification of pTiC58 and pTi-SAKURA consists of two steps. (I) pLRS-GmsacB was inserted in vivo into pTiC58 and pTi-SAKURA by homologous recombination at either RR or LL. (II) Cells harboring the fused plasmid DNA were cultivated on LB agar containing sucrose and gentamicin in order to select for the subsequent cross over products. Only the recombinant that excluded the T-DNA portion was selected by cultivation on the medium.

Fig.2 Stability of the modified Ti plasmids. pTiC58-S and pTi-SAKURA-S were extracted from *Agrobacterium* cells, and then introduced into two *E. coli* strains, S17-1λpir and SURE. Plasmid DNA was extracted from each *E. coli* transformant culture, then digested with *Eco*RI before electrophoretic separation in a 0.8% agarose gel. **(I)** pTi-SAKURA-S transformant colonies of S17-1λpir and those of SURE were cultivated in liquid medium. **(II)** Cultivation of one S17-1λpir transformant was repeated serially 3 times. The presence (+Gm) or absence (-Gm) of gentamicin in the medium is indicated.

Fig. 3 Evaluation of plant transformation efficiency of reconstructed *Agrobacterium* strains with

different genome backbones. (I) Expression of GUS activity in tobacco leaf disks transformed with re-constructed *Agrobacterium* strains harboring pBIN-GI. (II) Expression of GUS activity in *Kalanchoe* leaf disks cocultivated with reconstructed *Agrobacterium* strains harboring pBIN-GI. Cell extracts of the leaf disks were prepared. The filled bars indicate the relative GUS activity of leaf disks transformed with C58C1 harboring pTiC58-S and pBIN-GI. The open bars indicate specific GUS activity. The data averages and with standard deviation of three independent experiments (5 leaf disks each). 4MU, 4-methylumbelliferone.

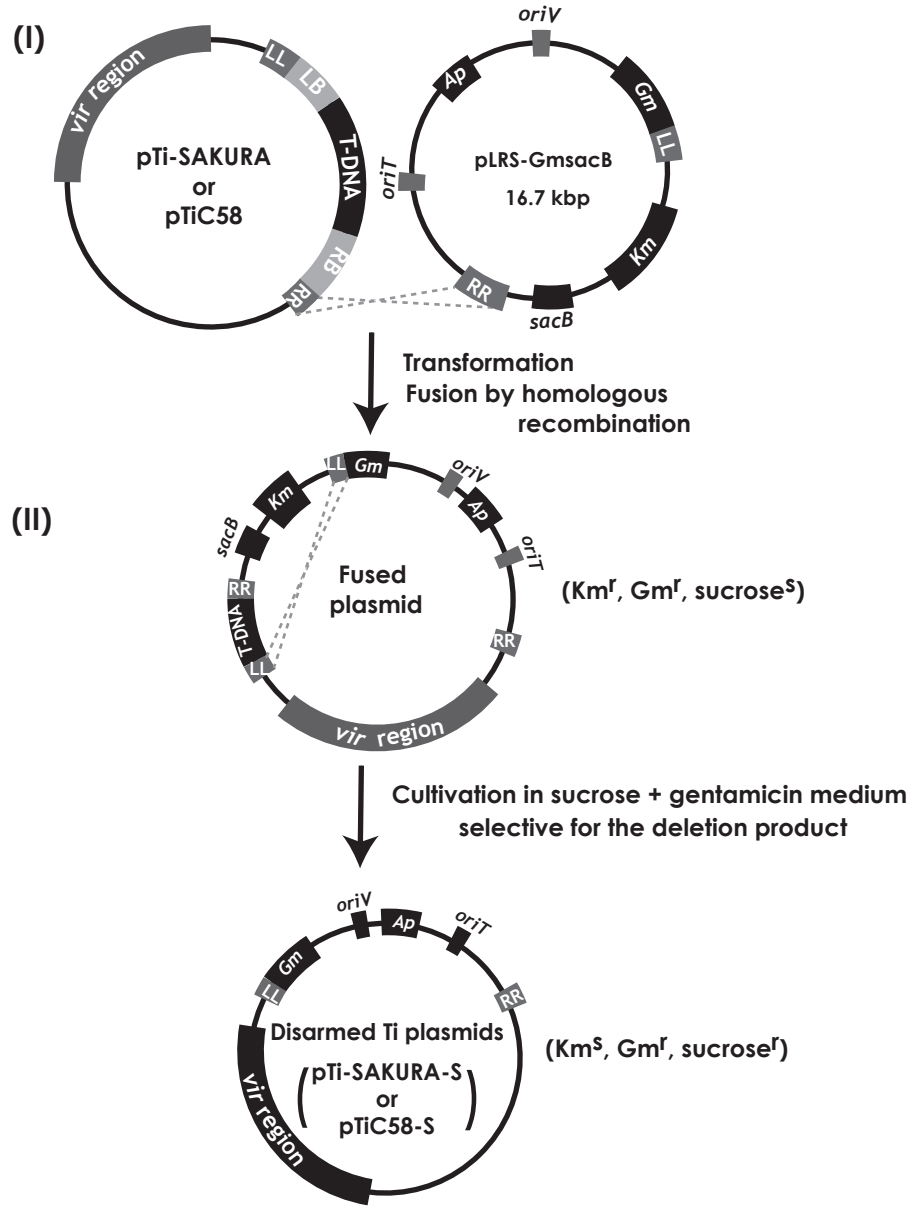
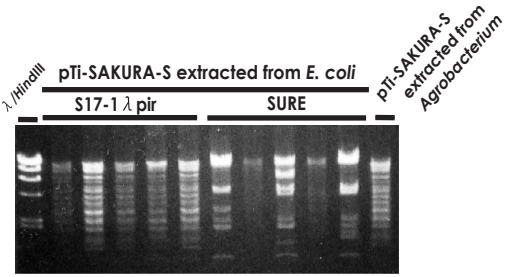

(I)
vir region
LL
LB
T-DNA
RB
RR
pTi-SAKURA
or
pTiC58
oriV
Ap
Gm
LL
oriT
pLRS-GmsacB
16.7 kbp
Km
RR
sacB
Transformation
Fusion by homologous
recombination
(II)
LL
Gm
oriV
Ap
oriT
RR
vir region
LL
T-DNA
RR
sacB
Km
Fused
plasmid
(Km^r, Gm^r, sucrose^s)
Cultivation in sucrose + gentamicin medium
selective for the deletion product
oriV
Ap
oriT
Gm
LL
RR
vir region
Disarmed Ti plasmids
pTi-SAKURA-S
or
pTiC58-S
(Km^s, Gm^r, sucrose^r)

Fig. 1

(I)


λ/HindIII
pTi-SAKURA-S extracted from E. coli
S17-1 λ pir
SURE
pTi-SAKURA-S extracted from Agrobacterium

(II)

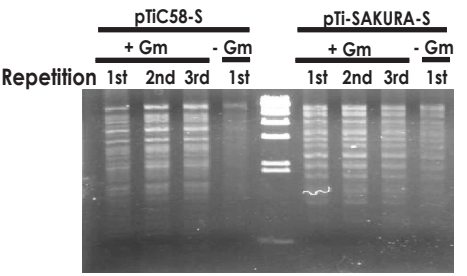
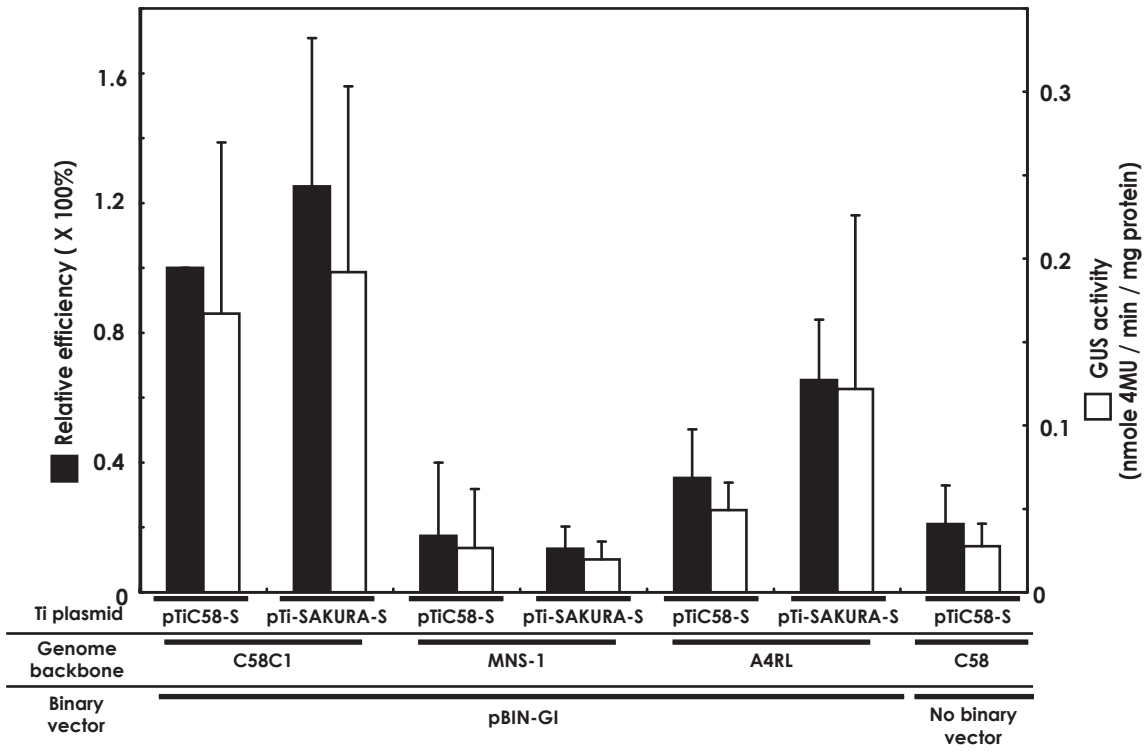

pTiC58-S
pTi-SAKURA-S
+ Gm
- Gm
+ Gm
- Gm
Repetition
1st
2nd
3rd
1st
1st
2nd
3rd
1st

Fig. 2

(I) Tobacco leaf disks



(II) *Kalanchoe* leaf disks

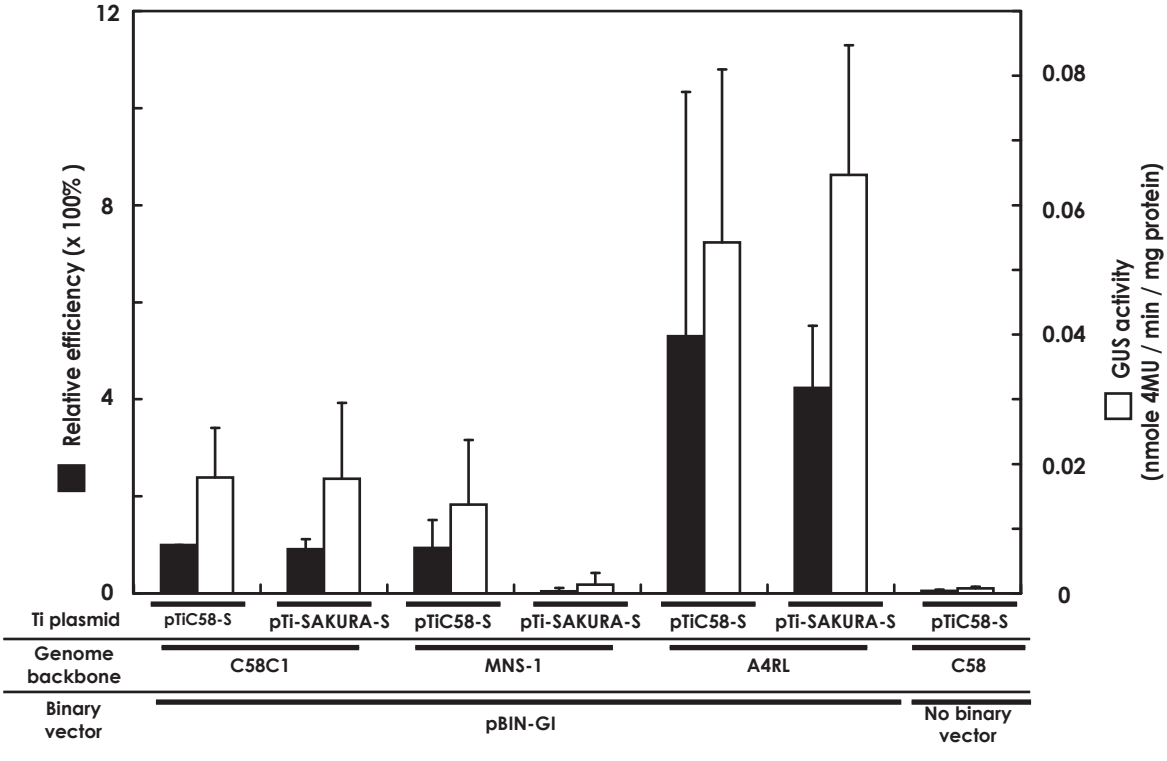


Fig. 3

SUPPLEMENTAL MATERIALS AND METHODS

Construction of tool plasmids for modification of nopaline type plasmids

pLRS-GmsacB was constructed as follows. The left fragment (LL) just outside the left border and the right fragment (RR) just outside the right border of T-DNA in pTi-SAKURA (3) were amplified by PCR using two pairs of primers: LL-Fw (5′-GAATTGAGAAAGCG-3′) and LL-Rv (5′-AAGGGGTACCGTTGTAAGGCGGCAG-3′), and RR-Fw (5′-GGAACTGCAGAAAAGAGCGTTTATTAG-3′) and RR-Rv (5′-TACGGACGCGACTTCTC-3′), respectively. The former PCR product was digested with *Eco*RI and *Kpn*I, and the latter one was treated with *Pst*I and *Hin*dIII, respectively. The resulting two fragments were inserted into pK19mob (2) to form pLRS. pLRS-G was made of pLRS by inserting a 2.8-kbp *Pst*I fragment excised from pUCGm2 harboring a gentamicin resistance gene. A 1.3-kbp *Hin*dIII-*Xba*I fragment of pJP5603 (1) containing an *oriT* was inserted into pMW119 (Nippon Gene, Tokyo, Japan). The resulting plasmid, pMW119mob, was digested with *Xba*I, and pLRS-G was digested with *Spe*I. The *Xba*I-cleaved pMW119mob and the *Spe*I-cleaved pLRS-G were ligated to produce pLRS-Gm. A portion containing LL, RR, and the gentamicin-resistance gene was excised as

a 4.8-kbp *Hin*dIII-*Eco*RI fragment from pLRS-G and inserted into pK18mobsacB (2). The resulting plasmid was digested with *Spe*I, and pMW119mob was digested with *Xba*I. The *Spe*I-cleaved pLRS-GsacB and the *Xba*I-cleaved pMW119mob were ligated. The resulting plasmid was named pLRS-GmsacB.

Another tool plasmid pLRS-Gms2 was constructed as follows. The entire portion of pMW119 except for ampicillin resistance gene was prepared by PCR using a pair of primers: P0043-amprdel-f (5'-GGTTATTGTCTCATGAGCGG) and P0044-amprdel-r (5'-GAGTTTTCGTTCCACTGAGC). The PCR product was treated with Klenow fragment. Gentamicin resistance gene was excised as a 2.8-kbp *Sma*I fragment from pBSGm, and then ligated with the blunt-ended PCR product. The resulting plasmid was named pMWG. A 1.3-kbp *Hin*dIII fragment containing *oriT* was taken from pJP5603, and then inserted into pMWG. The resulting plasmid was named pMWGmob. pLRS-GmsacB was digested with *Pst*I, and 8.1-kbp fragment containing LL and RR was self ligated. The resulting plasmid pLRS-sacB was digested with *Xba*I, and pMWGmob was digested with *Nhe*I. The *Xba*I-cleaved pLRS-sacB and *Nhe*I-cleaved pMWGmob were ligated to form pLRS-Gms2.

SUPPLEMENTAL REFERENCE

1. **Penfold, R.J., and J.M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118**: 145–146.

2. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vector derived from the *Escherichia coli* plasmid pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**: 69-73.

3. **Suzuki, K. , Y. Hattori, M. Uraji, N. Ohta, K. Iwata, K. Murata, A. Kato, and K. Yoshida.** 2000. Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. Gene **242**: 331-336.

SUPPLEMENTAL FIGURE LEGENDS

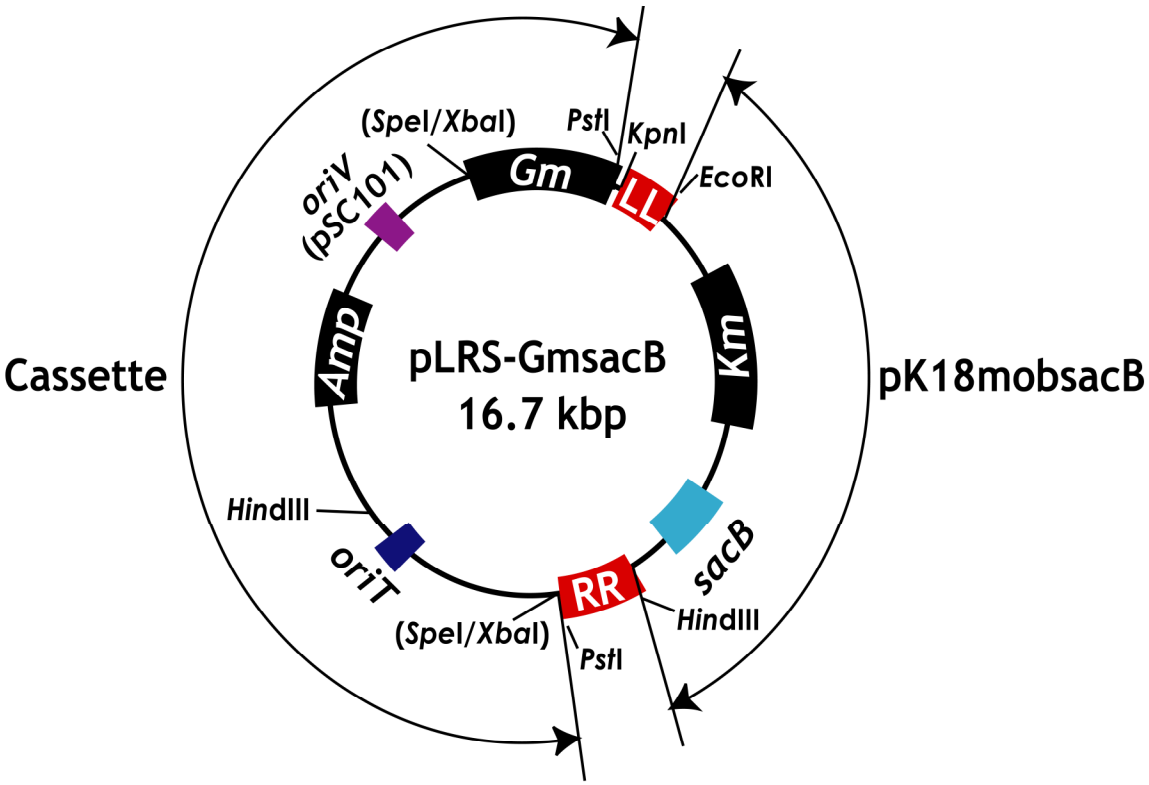
Fig. S1 Structure of the tool plasmids for the modification of nopaline type Ti plasmids. The plasmid pLRS-GmsacB harbors a gentamicin-resistance gene (Gm), the low copy number pSC101 replication gene (*oriV*), the RK2 plasmid transfer origin (*oriT*) and ampicillin-resistance gene (Ap) in the replacement cassette, and 1.4-kbp sequence flanking the left border of T-DNA (LL) and a 1.0-kbp sequence flanking the right border of T-DNA (RR) for homologous recombination with the target region in nopaline type plasmids, and kanamycin-resistance gene (Km), levansucrase gene (*sacB*) in the vector portion.

Another tool plasmid pLRS-Gms2 is the same with pLRS-GmsacB, but lacks Ap gene.

Fig. S2 Expression of GUS activity in tobacco leaf disks cocultivated with *Agrobacterium* strains. Tobacco leaf disks were transformed with *Agrobacterium* strains harboring pBIN-GI, and then cell extracts of the leaf disks were prepared as described in materials and method. The data represent average values of three independent experiments (5 leaf disks each). The thick and thin vertical bars indicate GUS activity and standard deviation values, respectively. Asterisk (*) indicates the

Agrobacterium strains in which Ti plasmid was modified.

Fig. S3 GUS expression in regenerated tobacco plants. Leaf and root segments were taken from tobacco plants regenerated from the leaf disks transformed with reconstructed *Agrobacterium* strains, then stained histochemically with X-Gluc, and then washed with 99% ethanol. Thick bars in each picture indicate 2 mm in scale.


Cassette
pK18mobsacB
pLRS-GmsacB
16.7 kbp
(SpeI/XbaI)
PstI
KpnI
EcoRI
oriV
(pSC101)
Gm
LL
Km
Amp
HindIII
oriT
RR
sacB
(SpeI/XbaI)
PstI
HindIII

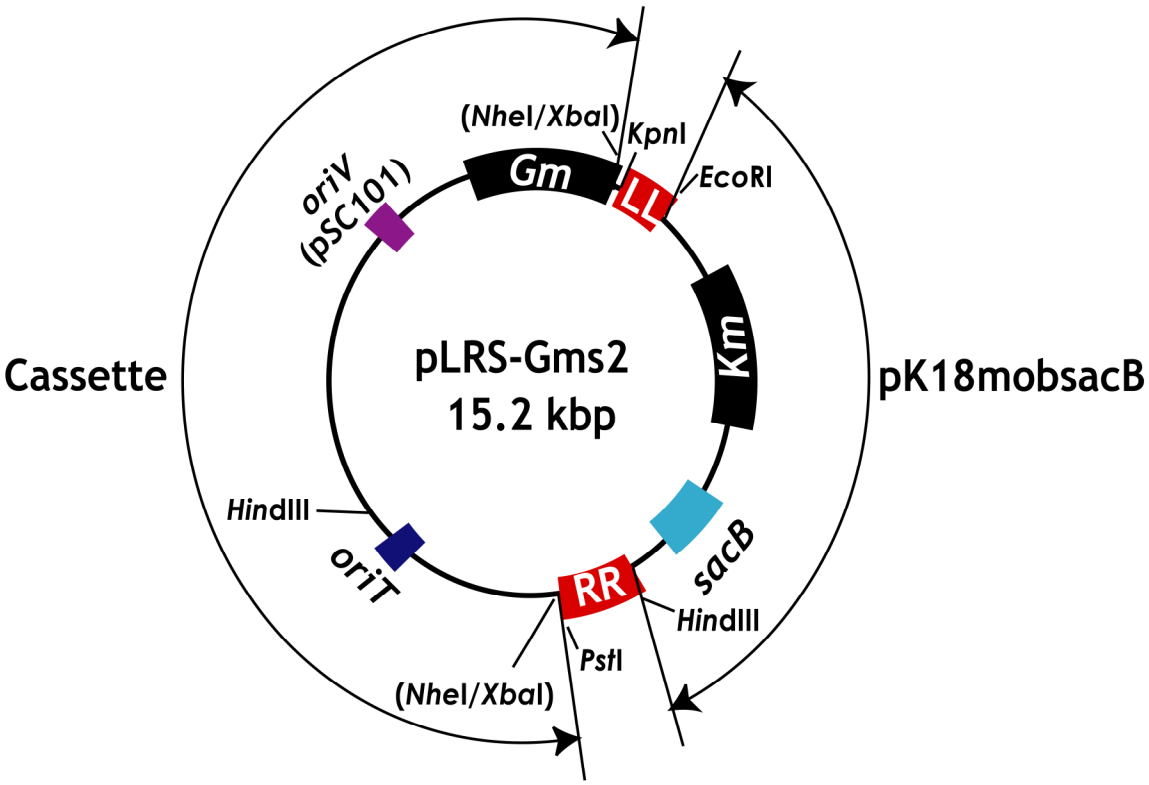

Cassette
pK18mobsacB
pLRS-Gms2
15.2 kbp
(NheI/XbaI)
KpnI
EcoRI
oriV
(pSC101)
Gm
LL
Km
HindIII
oriT
RR
sacB
(NheI/XbaI)
PstI
HindIII

Fig. S1

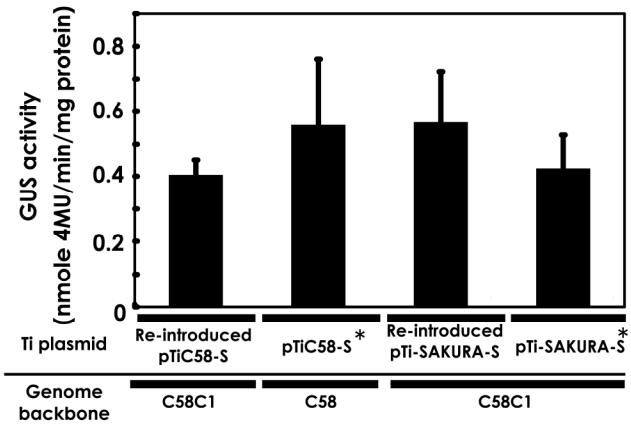

GUS activity
(nmole 4MU/min/mg protein)
0.8
0.6
0.4
0.2
0
Ti plasmid
Re-introduced pTiC58-S
pTiC58-S*
Re-introduced pTi-SAKURA-S
pTi-SAKURA-S*
Genome backbone
C58C1
C58
C58C1

Fig. S2

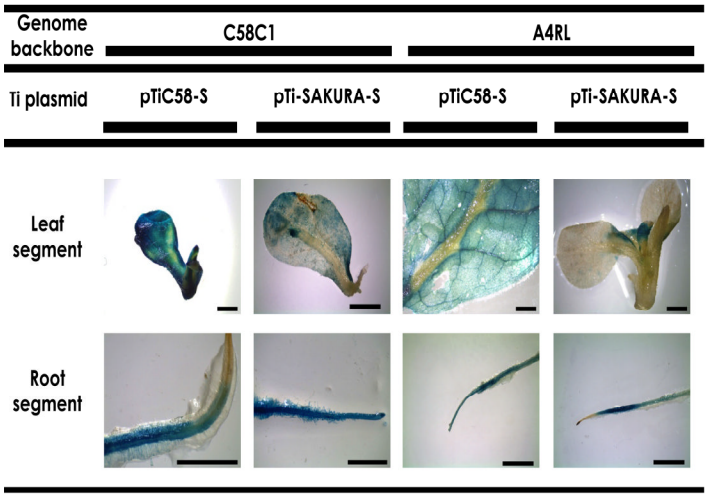

Genome backbone
C58C1
A4RL
Ti plasmid
pTiC58-S
pTi-SAKURA-S
pTiC58-S
pTi-SAKURA-S
Leaf segment
Root segment

Fig. S3