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 are 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 whether 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 segment 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 binaryvector 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 constructing 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% MgSO₄). *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 *HindIII-EcoRI* 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 OD₆₆₀) 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\textsuperscript{r}, Km\textsuperscript{r}, and \textit{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 \textit{sacB} gene. Loss of the fusion plasmid can occur at a high frequency. Loss of this plasmid converts cells to Gm\textsuperscript{s}, Km\textsuperscript{s}, and sucrose-resistant cells. Deletion of the \textit{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-\textit{GmsacB} 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\textsuperscript{s}, whereas the latter maintains Gm\textsuperscript{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 \textit{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-\textit{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 EcoRI 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' Gm' transconjugant frequency was 5×10⁻⁵ for pTiC58-S and 4×10⁻⁵ 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 S17-1pir 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') 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' 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\textsuperscript{r} calluses were rarely induced by MNS-1 strains having the plasmid. GUS activity in the tobacco leaf disks (Fig. 3 pane I) was comparable to the data for formation of Km\textsuperscript{r} calluses. Regenerated recombinant tobacco plants were obtained from the Km\textsuperscript{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 a 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, Mesorhizobium 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 S17-1pir 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 \textit{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\textsuperscript{r} gene in the cassette, and therefore does not increase the resistance to \(\beta\)-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 \textit{Agrobacterium} strains disarmed strains, even for researchers who are not familiar with \textit{Agrobacterium} biology.

\textbf{ACKNOWLEDGMENTS}

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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, S17-1pir and SURE. Plasmid
DNA was extracted from each E. coli transformant culture, then digested with EcoRI before
electrophoretic separation in a 0.8% agarose gel. (I) pTi-SAKURA-S transformant colonies of
S17-1pir and those of SURE were cultivated in liquid medium. (II) Cultivation of one S17-1pir
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.
**Fig. 1**

Transformation
Fusion by homologous recombination

Cultivation in sucrose + gentamicin medium
selective for the deletion product

Disarmed Ti plasmids

(Km\(^r\), Gm\(^r\), sucrose\(^s\))

(Fused plasmid)

(Km\(^s\), Gm\(^r\), sucrose\(^r\))
Fig. 2

(I) pTi-SAKURA-S extracted from E. coli S17-1 λpir SURE

(II) pTiC58-S

Repetition 1st 2nd 3rd 1st 1st 2nd 3rd 1st

+ Gm - Gm + Gm - Gm
Fig. 3

(i) Tobacco leaf disks

(ii) Kalanchoe leaf disks
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 EcoRI and KpnI, and the latter one was treated with PstI and HindIII, 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 PstI fragment excised from pUCGm2 harboring a gentamicin resistance gene. A 1.3-kbp HindIII-XbaI fragment of pJP5603 (1) containing an oriT was inserted into pMW119 (Nippon Gene, Tokyo, Japan). The resulting plasmid, pMW119mob, was digested with XbaI, and pLRS-G was digested with SpeI. The XbaI-cleaved pMW119mob and the SpeI-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 *HindIII-EcoRI* fragment from pLRS-G and inserted into pK18mobsacB (2). The resulting plasmid was digested with *SpeI*, and pMW119mob was digested with *XbaI*. The *SpeI*-cleaved pLRS-GsacB and the *XbaI*-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 *SmaI* fragment from pBSGm, and then ligated with the blunt-ended PCR product. The resulting plasmid was named pMWG. A 1.3-kbp *HindIII* fragment containing oriT was taken from pJP5603, and then inserted into pMWG. The resulting plasmid was named pMWGmob. pLRS-GmsacB was digested with *PstI*, and 8.1-kbp fragment containing LL and RR was self ligated. The resulting plasmid pLRS-sacB was digested with *XbaI*, and pMWGmob was digested with *NheI*. The *XbaI*-cleaved pLRS-sacB and *NheI*-cleaved pMWGmob were ligated to form pLRS-Gms2.


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.
Fig. S1
Fig. S2
<table>
<thead>
<tr>
<th>Genome backbone</th>
<th>C58C1</th>
<th>A48L</th>
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<tr>
<td>Ti plasmid</td>
<td>pTiC58-3</td>
<td>pTi-SAKURA-3</td>
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**Leaf segment**

**Root segment**

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**Fig. S3**