Tetracycline-Based Binary Ti Vectors pLSU with Efficient Cloning by the Gateway Technology for *Agrobacterium tumefaciens*-Mediated Transformation of Higher Plants

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ABSTRACT

We constructed small high-yielding binary Ti vectors with a bacterial tetracycline resistance gene to facilitate efficient cloning afforded by the Gateway Technology (Invitrogen) for *Agrobacterium tumefaciens*-mediated transformation of higher plants. The Gateway Technology vectors are kanamycin-based, thus tetracycline-based destination and expression vectors are easily selected for the antibiotic resistance in the *Escherichia coli* media. We reduced the size of the tetracycline resistance gene TetC from pBR322 to 1468 bp containing 1191 bp of the coding region, 93 bp of 5’-upstream, and 184 bp 3’-downstream region. The final size of binary Ti vector skeleton pLSU11 is 5034 bp. pLSU12 and 13 have the kanamycin resistance NPTII gene as a plant-selectable marker. pLSU14 and 15 contain the hygromycin resistance HPH gene as a selection marker. pLSU13 and 15 also have the β-glucuronidase (GUS) reporter gene in addition to the plant selection marker. We also constructed a mobilizable version of tetracycline-based binary Ti vector pLSU16 in which the mob function of ColE1 replicon was maintained for mobilization of the binary vector from *E. coli* to *A. tumefaciens* by tri-parental mating. The final size of binary Ti vector skeleton pLSU16 is 5580 bp. New tetracycline-based binary Ti vectors pLSU12 were found as effective as kanamycin-based vector pLSU2 in promoting a 10-fold increase in fresh weight yield of kanamycin-resistant calli after *A. tumefaciens*-mediated transformation of tobacco leaf discs. Using the Gateway Technology we introduced the plant-expressible GUS gene to the T-DNA of binary Ti vector pLSU12. Expression of the β-glucuronidase enzyme activity was demonstrated by histochemical staining of the GUS activity in transformed tobacco leaf discs.

Keywords: *Agrobacterium tumefaciens*; Binary Ti Vectors; Gateway Technology; pLSU; Tobacco Leaf Disk Transformation; Tetracycline Resistance

1. Introduction

Bacteriophage λ relies on the site-specific recombination reaction to integrate the phage DNA by the BP clonase into the bacterial chromosome and excise it out by the LR clonase [1]. The BP clonase reaction for DNA integration is catalyzed by the phage integrase and integration host factor. Two attB sites (21 to 25 bp) at the ends of a target DNA fragment (or a PCR product) recombine with two attP sites of the Gateway donor vector (pDONR), resulting in generation of two attL sites (96 bp) in an entry vector (pENTR) concomitant with transfer and integration of the target DNA [2]. The LR clonase reaction for DNA excision is catalyzed by the phage excisionase, integrase, and integration host factor. Two attL sites flanking the target DNA in the entry vector recombine with two attR sites of a destination vector (pDEST), resulting in creation of two attB sites in an expression vector (pEXPR) and excision/transfer of the target DNA fragment. Succession of four Gateway vectors, donor, entry, destination, and expression vectors are bacterial kanamycin-based plasmids.

The λ clonase recognizes the nine core base sequence 5’-CAACTTNNT-3’ at the recombination points of the attB and attP sites, and also interacts with the eleven base sequences 5’-CTTACGATTGT-3’ in the P and P’ arm of attP site. The recognition sequences for the site-specific recombination reactions were engineered to create four different variants each of attB, attP, attL and...
2.1. Chemicals and Enzymes

The MultiSite Gateway methodology was introduced to facilitate the predefined assembly of gene sequences in T-DNA of a binary Ti vector for *A. tumefaciens*-mediated plant transformation [7]. Binary Ti vectors pPZP and pCambia with CoE1 and VS1 replicons were selected for a backbone of the vectors with a pair of any combination of the recombination *att*R1, *att*R2, *att*R3, *att*R4 and *att*R5 in the destination vector [5]. Because the Gateway entry vectors carry the kanamycin-resistance gene for bacterial selection, the pPZP-based destination vectors with the streptomycin/spectinomycin-resistance gene [4,5] were preferred over the pCambia-based vectors with the kanamycin-resistance gene [8,9]. However, the pPZP-based destination vectors are not suited for transformation of *A. tumefaciens* since the most commonly used strains LBA4404, EHA101 and 105 of *A. tumefaciens* contain the avirulent Ti plasmid with the streptomycin-resistance marker. In addition, some strains of *A. tumefaciens* are reported to be resistant to low levels of spectinomycin [10]. Thus, the introduction of pPZP vectors to *A. tumefaciens* is not assured using simple antibiotic selection for streptomycin or spectinomycin, and needs additional steps to circumvent the difficulty. This provides a practical advantage for using the tetracycline-based pLSU binary vectors as destination vectors for efficient cloning of multiple fragments to create expression vectors. The C58 strain of *A. tumefaciens* has a tetracycline-resistance determinant and is resistant to low levels of tetracycline [10].

The objective of this research is to develop new tetracycline-based binary Ti vectors to facilitate efficient cloning by the Gateway Technology. The binary vectors will be tested for transformation of tobacco leaf discs and for expression of the β-glucuronidase *GUS* reporter gene.

2. Materials and Methods

2.1. Chemicals and Enzymes

Antibiotics (ampicillin, carbenicillin, gentamycin, kanamycin, rifampicin, streptomycin, spectinomycin, tetracycline) and other chemicals used in this experiment were purchased mainly from Sigma-Aldrich (St. Louis, MO).

Restriction endonucleases (*AvrII*, *BamHI*, *BsrgI*, *BstBI*, *EcoRI*, *EcoRV*, *HindIII*, *KpnI*, *MfeI*, *MstI*, *PstI*, *PvuII*, *SalI*, *ScaI*, *SphI*, *XbaI* and *XhoI*), Deep Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolab (NEB; Beverly, MA) and/or Bethesda Research Laboratory (BRL; Grand Island, NY). The lysozyme was purchased from Sigma and Ultra Pfu DNA polymerases from Stratagene (La Jolla, CA). The enzymes were treated as instructed by suppliers.

2.2. Bacterial Strains and Plasmid DNA

The XL1Blue-MR strain was purchased from Stratagene (La Jolla, CA). The MR strain has no antibiotic resistance since the F' episome was eliminated while the XL1Blue and XL2Blue strains are tetracycline-resistant. The genotype of the MR strain is as follows: *recA1, endA1, supE44, relA1, Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173*, *gyrA96*, *thi-1*. The EndA* phenotype of XL1Blue-MR strain allows to yield high quality plasmid DNA. The *A. tumefaciens* strain LBA4404 has TiAch5 chromosome which contains rifampicin resistance gene and disarmed Ti plasmid pAL4404 with spectinomycin- and streptomycin-resistance genes [11]. The final concentrations of spectinomycin and streptomycin for selection are 100 mg/L (Sp100) and 50 mg/L (St50), respectively. *A. tumefaciens* was grown on Agrobacterium medium (A. media) containing 2.0 g mannitol, 2.0 g (NH₄)₂SO₄, 5.0 g yeast extract, and 100 ml of 10× salt solution per liter. For the 10× salt solution, 109.0 g KH₂PO₄, 1.0 N KOH. After making a volume to one liter, the 10× salt solution was heated to boil and the precipitates were filtered through Whatman No. 1 filter paper. After the 10× salt solution was added the pH of the solution was adjusted to 7.0 with 1.0N KOH. After making a volume to one liter, the 1× salt solution was heated to boil and the precipitates were filtered through Whatman No. 1 filter paper. After the 10× salt solution was added the pH of the solution was adjusted to 7.0 prior to autoclaving. Transformation of *A. tumefaciens* was conducted in YEB media (Sucrose 5 g, Bacto-Peptone 5 g, Beef Extract 5 g, Yeast Extract 1 g, 0.002 M MgSO₄ per liter). Magnesium ion was omitted from the media when tetracycline was used for bacterial selection.

Plasmids used for experiments were pBR322 [12-14], pUC19 [15,16], pCAMBIA1301 purchased from Cambia (http://www.cambia.org.au, Canberra, Australia), pbEScriptII KS(+) from Stratagene (La Jolla, CA) and pUC4-KIXX and -KSAC from Pharmacia (Uppsala, Sweden) [17].

2.3. Oligodeoxyribonucleotides and Plasmid DNA Manipulation

Oligonucleotides used for PCR, mutagenesis or DNA
sequencing were custom-ordered and synthesized by Sigma-Aldrich (St. Louis, MO). Plasmid DNA was isolated by alkaline lysis method [18] and purified by CsCl-EtBr gradient centrifugation method [19]. The GENE-CLEAN kit purchased from BIO101 (Carlsbad, CA) was used to extract DNA from agarose gel [20]. Quick-CLEAN kit purchased from BIO101 (Carlsbad, CA) was used to extract DNA from agarose gel [20].

2.4. Construction of Tetracycline-Based Binary Ti Vectors pLSU12

New binary Ti vectors pLSU11 to 16 have the tetracycline resistance gene TetrC as a bacterial selection marker. The TetrC gene was amplified from pBR322 and modified to elimi

2.5. DNA Sequencing of pLSU12

DNA sequences of two strands of new binary vectors pLSU12 were determined with an Applied Biosystems™ 3730xl DNA Analyzer at Eurofins MWG Operon (Huntsville, AL). Twenty eight sequencing primers were de-

2.6. Mobilizable Tetracycline-Based Binary Vector pLSU16

The ColE1 replicon and tetracycline resistance gene of pBR322 [13] were amplified as a template for the mobilizable binary Ti vector pLSU16. The ampicillin resistance and ROP genes were excluded by two separate amplification reactions of the pBR322 template, and unique HindIII and BamHI sites of pBR322 were eliminated.

Figure 1. Schematic presentation of backbone structure of tetracycline-based binary Ti vector pLSU11 (5034 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, EcoRV (EV), SphI (Sp), HindIII (HIII), NeoI (Nc), XhoI (Xh), KpnI (K), EcoRI (EI), BamHI (BH), PstI (P), SacI (Sc), XbaI (Xb), and SacI (Sa). The backbone plasmid includes the tetracycline resistance gene (TetrC), ColE1 origin of replication from pUC19 (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1).
yielding 2243 bp pORItet [22]. First, the unique HindIII site at 29 to 34 bp of pBR322 was eliminated by HindIII digestion and filled-in by Klenow fragment producing pBR322-dHindIII. The region from the tetracycline resistance gene to CoIE1 replicon was amplified with primers BR322-1 and BR322-2 producing a smaller 3206 bp pBR-d1. Next, the unique BamHI site in tetracycline resistance gene was inactivated by making a single point mutation using primers BR322-5 and BR322-6. Finally, new PvuII and BstBI sites were introduced by making two single point mutations on 1408 to 1413 bp and 2396 to 2401 bp, and ROP gene was removed with primers BR322-3 and BR322-4 yielding pORItet.

The broad host range replication origin and stability region was amplified from a binary vector pGV941 using primers with new restriction endonuclease sites EcoRI and MfeI. The amplified fragment digested with both restriction enzymes was ligated into 2241 bp to 3 bp EcoRI site of pORItet producing 5429 bp pBRVS1 [22]. The new MfeI sites of the PCR fragment were inactivated by ligation to the EcoRI site of pORItet due to the compatible cohesive end of MfeI to EcoRI.

The T-DNA left and right border sequences originated from the octopine-type Ti plasmid pTi15955 were cloned from pKSLR [22]. Due to the short length of the T-DNA border sequences in pKSLR, it was necessary to make sure that only a single copy of the left/right border sequences are properly inserted into pBRVS1. For this purpose, the kanamycin resistance phenotype was introduced to the binary vector, from the neomycin phosphotransferase II (NPTII) gene of transposon Tn5. Plasmid pUC4-KIXX (Pharmacia) was digested with HindIII and the 1568 bp fragment containing NPTII gene was inserted into HindIII site of pKSLR. This plasmid was named as pLRKIXX and the colonies were double-selected for kanamycin and ampicillin resistance. After kanamycin resistance selection, the MfeI-LB-Kan R-MCS-RB-MfeI fragment was cut with MfeI and inserted into EcoRI site of pBRVS1. This kanamycin resistance gene was removed by HindIII digestion after the insertion of single copy of T-DNA border into pBRVS1 was confirmed. The final product was named aspLSU16 (Figure 3).

2.7. Gateway Technology

The three reading frame cassettes (1711 bp of RfA, 1713 bp of RfB, and 1714 bp of RfC1; Invitrogen (Carlsbad, CA) have the suicidal ccdB gene for inhibiting the DNA gyrase activity and chloramphenicol resistance gene (CmR) flanked by attR1 and attR2 sites. A Scal site (Sc) adjacent to the T-DNA right border of the binary vector pLSU12 was used for cloning site of Gateway® reading frame cassette (Figures 1 and 2). First, the Scal site in pLSU12 was digested and dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Then the dephosphorylated vector was ligated with Gateway® cassettes.
Tetracycline-Based Binary Ti Vectors pLSU with Efficient Cloning by the Gateway Technology for Agrobacterium tumefaciens-Mediated Transformation of Higher Plants

Figure 3. Schematic presentation of mobilizable binary Ti vector pLSU16 (5580 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, EcoRV (EV), SphI (Sp), HindIII (HIPI), NcoI (Nc), XhoI (Xh), KpnI (K), EcoRI (EI), BamHI (BH), PstI (P), ScaI (Sc), XbaI (Xb), and SacI (Sa). The backbone plasmid includes the tetracycline resistance gene (\textit{TetR}), ColE1 origin of replication from pBR322 (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1).

RfA, RfB, and RfC1, respectively, producing pLSU17A, 17B, and 17C1 (Figure 4). The ligation products were transformed into \textit{E. coli} strain of the One shot® ccdB Survivaltm2 T1R competent cells with gyrA mutation. Colonies were selected on LB agar plates for 30 mg/L chloramphenicol and 10 mg/L tetracycline under dark.

The insertion of Gateway® cassette includes the tetracycline resistance gene (\textit{TetR}), ColE1 origin of replication from pBR322 (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1).

2.8. Freeze-Thaw Transformation of \textit{A. tumefaciens}

Cells of \textit{A. tumefaciens} LBA4404 strain were grown at 28°C in YEB media. Cells were prepared as described by Hofgen and Willmitzer [23] and as modified as follow. Ten ml of overnight culture were mixed with 50 ml of fresh YEB media and incubated at 250 rpm for six to seven hours until A_{600} reached at 0.5. After cooling on ice for 30 min, cells were harvested by centrifugation at 3000 g for 20 min at 4°C. Cell pellet was washed once in 30 ml of TE buffer and re-suspended in 1 ml of YEB media. One μg of DNA was mixed with 100 μl of cells, and frozen in an ethanol bath at −80°C for two hours to overnight. The frozen cell DNA mixture was thawed at 37°C, mixed with 1 ml of YEB media and incubated at 28°C with gentle rotation at 150 rpm for five hours for stabilization. Aliquots of 100 μl were plated on YEB-agar media containing appropriate antibiotics and incubated at 28°C for two to three days.

2.9. Tobacco Leaf Disc Transformation Mediated by \textit{A. tumefaciens}

\textit{A. tumefaciens}-mediated transformation of tobacco leaf disc was performed as described by Su et al. [24].

2.10. Histochemical Detection of \textit{β}-Glucuronidase Activity

The \textit{β}-glucuronidase activity was detected after histochemical staining of \textit{A. tumefaciens}-infected leaf disc by 5-bromo-4-chloro-3-indolyl \textit{β}-D-glucuronide (X-GlcA).

3. Results

3.1. Tetracycline-Based Binary Ti Vectors pLSU12 and 14

We constructed new tetracycline-based binary Ti vectors by replacing the bacterial kanamyciresistance gene of binary vectors pLSU2 and 4 [25] with the tetracycline resistance gene.
resistance gene, forming pLSU12 and 14, respectively) (Figures 1 and 2). The minimal requirement for the component of tetracycline resistance gene was tested by the tetracycline-resistance comparison and the plasmid stability experiment in E. coli and A. tumefaciens.

3.2. Bacterial Tetracycline Resistance Gene

We used the tetracycline resistance gene TetC from pBR322 for tetracycline selection of bacteria [26]. Tetracycline is a very effective antibiotics since the optimal concentrations for E. coli and A. tumefaciens are 10 and 2 mg/L, respectively. However, there are some limitations in use of the antibiotics because tetracycline is light-sensitive, and is inhibited by magnesium ion included in commonly used bacterial media. The XL1Blue-MR strain of E. coli was used since it has no antibiotic resistance without the F’ episome while the XL1Blue and XL2Blue strains are tetracycline-resistant.

Five restriction enzyme sites for two NheI sites, one each of EcoRV, SphI, and SalI site were eliminated from the TetC coding and 5’-upstream regions by single point mutations without alternation of the amino acid codons, so that these restriction sites remain unique in the multi-cloning site of T-DNA. Based on the sequence analysis of TetC gene, we deduced the minimal size of gene extending from the 5’-upstream region including −35 and −10 elements to the 3’-downstream region following the small stem-loop structures presumably acting as a transcription termination signal. The new truncated TetC gene of 1468 bp contains 1191 bp of the coding region with 93 bp of 5’-upstream region to the initiation codon, and 184 bp 3’-downstream from the termination codon. This truncated gene confers the resistance up to 100 mg/L of tetracycline as effective as the wild-type gene, but less effective at 200 mg/L in E. coli. The truncated TetC gene was used to replace the bacterial kanamycin resistance NPTI gene from binary vectors pLSU2 and 4, generating pLSU12 and 14 [22].

DNA sequence analysis of pLSU12 indicated that all single point mutations introduced to the tetracycline resistance (TetR) gene were confirmed as expected. How-

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ever, we found one and 16 bp insertions at the junctions of ligation reactions and 4 bp deletion in the tetracycline resistance gene as noted in Materials and Methods. DNA sequence of the binary vector skeleton pLSU11 was deposited to GenBank at submission number 1398415.

3.3. Mobilizable Tetracycline-Based Binary Ti Vector pLSU16

We also constructed amobilizable version of tetracycline-based binary Ti vectors pLSU16 in which the mob function of CoIE1 replicon was maintained for mobilization from *E. coli* to *A. tumefaciens* by tri-parental mating assisted by pRK2013. The final size of vector skeleton pLSU16 is 5580 bp long consisting of the Tc gene, CoIE1 and VS1 replicons, and T-DNA (Figure 3).

3.4. *A. tumefaciens*-Mediated Transformation of Tobacco Leaf Discs

The tetracycline-based binary Ti vector pLSU12 in *A. tumefaciens* was used for transformation of tobacco leaf discs after four-day co-cultivation. Transformated leaf discs were selected for in the presence of 300 mg/L of kanamycin for four weeks. Stable expression of introduced kanamycin-resistance gene was evident by up to 10-fold increase in fresh weight yield in g of treated tobacco leaf discs (Table 1). The tetracycline-based pLSU12 was as effective as the kanamycin-based pLSU2 in the growth promotion assay.

3.5. Gateway Technology Expression Vector with GUS

The tetracycline-based binary vector pLSU12 was used to generate Gateway expression vectors. Three different reading frame cassettes flanked by attR1 and attR2 sites (1711 bp of RfA, 1713 bp of RfB, and 1714 bp of RfC1) were ligated to the *SacI* site (Sc) of T-DNA of pLSU12, producing Gateway destination vectors, pLSU17A, 17B, and 17C1 (Figure 4). Each reading frame cassettes contain the chloramphenicol resistance gene (Cm<sup>8</sup>) and the suicidal *ccdB* gene inhibiting the DNA gyrase activity (topoisomerase II). The ligation products were transformed to the *E. coli* strain DB3.1 containing gyrA mutation, and the Gateway destination vectors were isolated from colonies after simple selection for tetracycline and chloramphenicol resistance. Aplant-expressible β-glucuronidase (*GUS*) gene in a entry vector was used to replace the *Cm<sup>8</sup>* and *ccdB* gene in the destination vector using the LR clonase-catalyzed recombination reaction of the attL1/attL2 sites of the donor vector with the attR1/attR2 sites of the destination vector. A pLSU expression vector with the GUS gene was isolated from *E. coli* colonies by simple selection for tetracycline resistance.

<table>
<thead>
<tr>
<th>pLSU binary Ti vectors</th>
<th>Plate average of FW yield in g</th>
<th>Increase in FW in g per plate over vector-less control</th>
<th>% leaf discs with increased FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector-less control</td>
<td>0.91 (0.20)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pLSU2</td>
<td>12.06 (5.57)</td>
<td>10.96 (5.54)</td>
<td>92</td>
</tr>
<tr>
<td>pLSU12</td>
<td>8.21 (1.48)</td>
<td>7.09 (1.48)</td>
<td>98</td>
</tr>
<tr>
<td>pLSU12-long</td>
<td>11.24 (7.23)</td>
<td>10.12 (7.23)</td>
<td>96</td>
</tr>
</tbody>
</table>

3.6. GUS Reporter Gene Expression in Tobacco Leaf Discs

The plant-expressible *GUS* gene in the T-DNA of binary vector pLSU17A was introduced to tobacco after *A. tumefaciens*-mediated transformation. Expression of the *GUS* gene was demonstrated by histochemical staining of GUS activity in transformed tobacco leaf discs.

4. Discussion

We previously constructed a series of kanamycin-based binary Ti vectors pLSU1 to 5 to improve the transformation frequency and plasmid yield in *E. coli* and *A. tumefaciens* for *A. tumefaciens*-mediated transformation of higher plants [22,25]. Transcriptional direction of STA/REP replicon for *A. tumefaciens* can be the same as that of CoIE1 replicon for *E. coli* (co-directional transcription), or opposite (head-on transcription) as in the case of widely used vectors (pPZP or pCambia). New binary pLSU vectors with co-directional transcription yielded in *E. coli* up to four-fold higher transformation frequency than those with the head-on transcription. Here we converted these kanamycin-based vectors to the tetracycline-based binary vectors pLSU11 to 15 to exploit the user-friendly features of the Gateway<sup>®</sup> Technology for efficient cloning. With further introduction of Multi-site Gateway methodology, different combinations of attR1,
**attrR2**, **attrR3**, **attrR4**, and **attrR5** will be inserted in T-DNA region of pLSU and the high-throughput modular assembly of promoter, terminator, and coding region of target gene will be suitable for global analysis of plant gene functions in a genomic scale.

The Gateway Technology relies on use of four kanamycin-based plasmid vectors in quick succession from the donor, entry, destination to expression vectors. Many destination vectors for transformation of higher plants used as a vector skeleton pCambia, pGreen, or pBin19 which has a bacterial kanamycin-resistance gene [8,9,27]. The use of the same kanamycin-based vectors made impossible the simple antibiotic selection of colonies to distinguish the destination vectors from donor/entry vectors after LR clonase reaction. To overcome this difficulty, the entry vector should be linearized before LR recombination or the proper expression vector should be selected based on the plasmid DNA size or restriction enzyme sites by labor-intensive DNA purification. An alternative approach used the suicidal characteristic of **ccdB** gene in destination binary vectors [28]. After LR recombination reaction the *E. coli* transformants only have either the proper expression vector or unreacted entry vector because transformants harboring unreacted destination vector or entry vector with recombined chloroamphenicol resistance gene and **ccdB** gene cannot survive due to the activation of **ccdB** gene. Thus, the plasmid DNA isolated from the survived clones should be the mixture of entry vector and expression binary vector. After transformation to *A. tumefaciens* with the mixed plasmid, the transformants harboring the entry vector which does not have replication origin for *A. tumefaciens* cannot survive. The survived colony harboring the proper expression vector can be further used for plant transformation. However, the identity of expression binary vector generated by this method might not easily verified since it is difficult to purify plasmid DNA from *A. tumefaciens*.

The major group of destination vectors is based on pPZP200 vector which has streptomycin/spectinomycin resistance gene [4-6,29]. The streptomycin selection is often not suitable for *Agrobacteria*-mediated transformation because the widely used *A. tumefaciens* strain LBA4404 has the streptomycin/spectinomycin-resistance gene in the avirulent Ti plasmid, although these streptomycin selectable vectors can be used for plant transformation using particle bombardment method or *Agrobacteria*-mediated transformation using other *A. tumefaciens* kanamycin-resistant strain, EHA101.

The new tetracycline-based, Gateway-compatible binary vectors pLSU are more user-friendly in this aspect. With further introduction of Multi-site Gateway methodology, the high-throughput modular assembly of promoter, terminator, and coding region of target gene will be suitable for global analysis of plant gene functions in a genomic scale.

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### REFERENCES


Abbreviations

A. media, Agrobacterium media;
FW: fresh weight;
GUS: β-glucuronidase;
REP and STA: the replication and stability region of VS1 replicon, respectively.