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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 *att*B sites (21 to 25 bp) at the ends of a target DNA fragment (or a PCR product) recombine with two *att*P sites of the Gateway donor vector (pDONR), resulting in generation of two *att*L 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 *att*L sites flanking the target DNA in the entry vector recombine with two *att*R sites of a destination vector (pDEST), resulting in creation of two *att*B 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 *att*B and *att*P sites, and also interacts with the eleven base sequences 5'-C/AAGTCACTAT-3' in the P and P' arm of *att*P site. The recognition sequences for the site-specific recombination reactions were engineered to create four different variants each of *att*B, *att*P, *att*L and

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*att*R [3]. Thus, *att*B1 recombines specifically with *att*P1, *att*B2 interacts only with *att*P2, *att*B3 with *att*P3, and *att*B4 with *att*P4. Four variants of recombination sites became a basis for MultiSite Gateway technology for the directional cloning, reading frame-specific recombination and modular assembly of multiple DNA fragments in a single LR clonase reaction [4,5]. This technology enables modular assembly of a promoter, coding, and terminator sequences in the destination vector, selecting from a collection of the multiple sequences in the entry vectors [6,7].

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 ColE1 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]. Becasue the Gateway entry vectors carry the kanamycin-resistance gene for bacterial selection, the pPZP-based destination vectors with the streptomycin/spectinomycin-resistance gene [4-7] 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. Materails and Methods

2.1. Chemicals and Enzymes

Antibiotics (ampicillin, carbenicillin, gentamycin, kanamycin, rifampicin, streptomycin, spectinomycin, tetracycline) and other chemicals used in this experimentwere purchased mainly from Sigma-Aldrich (St. Louis, MO). Restriction endonucleases (*AvrII*, *BamHI*, *BsrGI*, *BstBI*, *EcoRI*, *EcoRV*, *HindIII*, *KpnI*, *MfeI*, *NheI*, *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 polymerasewas 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: *recA*1, *endA*1, *supE*44, *relA*1, Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *gyrA*96, *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 (Sp^{100}) and 50 mg/L (St^{50}), respectively. *A. tumefaciens was* grown on *Agrobacterium media* (*A. media*) containing 2.0 g mannitol, 2.0 g $(NH_4)_2SO_4$, 5.0 g yeast extract, and 100 ml of 10× salt solution per liter. For the 10× salt solution, 109.0 g KH_2PO_4, 1.6 g $MgSO_4{\cdot}7H_2O$, 0.05 g $FeSO_4{\cdot}7H_2O$, 0.11 g $CaCl_2{\cdot}2H_2O$, and 0.02 g $MnCl_2{\cdot}4H_2O$ were dissolved in one liter of H_2O, and the pH of the solution was adjusted to 7.0 with 1.0N 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 media 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 $MgSO_4$ 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], pCAMBIA1301purchased from Cambia (http://www.cambia.org.au, Canberra, Australia), pBluescriptII 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 alkalinelysis method [18] and purified by $CsCl_2$-EtBr gradient centrifugation method [19]. The GENECLEAN kit purchased from BIO101 (Carlsbad, CA) was used to extract DNA from agarose gel [20]. QuickChange Multi Site-Directed Mutagenesis Kit was obtained from Stratagene. Other molecular cloning methods were according to Sambrook and Russell [21].

2.4. Construction of Tetracycline-Based Binary Ti Vectors pLSU12

New binary Ti vectors pLSU11 to 16 have the tetracycline resistance gene *TetC* as a bacterial selection marker. The *TetC* gene was amplified from pBR322 and modified to eliminate fiverestriction enzyme sites, two *NheI* sites, one each of *EcoRV*, *SphI*, and *SalI* sites. The primers used for the mutagenesis were described in details in the Ph.D. thesis of S. Lee [22]. The mutagenesis reactions yielded 5429 bp of pBRVS2ΔNENSS.

The modified *TetC* gene was amplified by PCR from pBRVS2ΔNENSS using two primersTet-F2 and Tet-R1 [22]. Both primers have misplaced bases to introduce *NheI* sites at both ends of the amplified *TetC* gene (1468 bp). The amplified *TetC* gene replaced the *NPTI* gene in binary vectors pLSU2 and 4. To remove the *NPTI* gene from the binary vectors, two primers were designed to perform a reverse PCR. Both primers have misplaced bases to introduce *AvrII* sites atoutside of *NPTI* gene to be removed, and amplified PCR products, pLSU2ΔNPTIand pLSU4ΔNPTI. The amplified 1468 bp of *TetC* gene was ligated into the binary vectors without the *NPTI* gene resulting in new binary vectors pLUS12 and pLSU14 with tetracycline resistance for bacterial selection (6412 and 6648 bp, respectively) (**Figures 1** and **2**).

The *β*-Glucuronidase *GUS* gene was amplified from pCAMBIA1305.2 with primer 1305-1F2 and 1305-1R2 [22]. Both primers introduced new *HindIII* sites at the ends of *GUS* fragment including CaMV35S promoter, *GUS* gene with His6, glycine-rich protein signal peptide, catalase intron and nopaline terminator. After *HindIII* digestion, the amplified 3007 bp fragment were introduced into the expression vectors pLSU12 and pLSU14 at the *HindIII* site 3'-adjacent to the *Hph* or *NPTII* gene producing 9419 bp of pLSU13 and 9655 bp of pLSU15, respectively (**Figure 2**).

2.5. DNA Sequencing of pLSU12

DNA sequences of two strands of new binary vectors pLSU12 were determined with an Applied Biosystems™ 3730xl DNA Analyzerat Eurofins MWG Operon (Huntsville, AL). Twenty eight sequencing primers were designed [22], and synthesized by Sigma. The complete DNA sequence of tetracycline-based binary vector skeleton (pLSU11) is submitted to GenBank (Submission #1398415). In the tetracycline resistance gene at 1142 bp, the nucleotide C was confirmed as T, and in the termination region of Tet^R gene at 1425 bp GCGG were missing from the pBR322 sequence listed in GenBank. C was inserted at 1467 bp, the ligation junction between Tet^R gene and ColE1 replicon. The unexpected G in the RepA region at 3376 bp and the insertion of 16 bp-long fragment CGCGCGGACAAGCTAG in the termination region of Tet^R gene at the ligation junction between VS1 replicon and T-DNA region were determined so as in the sequence of pLSU4.

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 resistanceand ROP genes were excluded by two separate amplification reactions of the pBR322 template, and unique *HindIII* and *BamHI* sites of pBR322 were eliminated

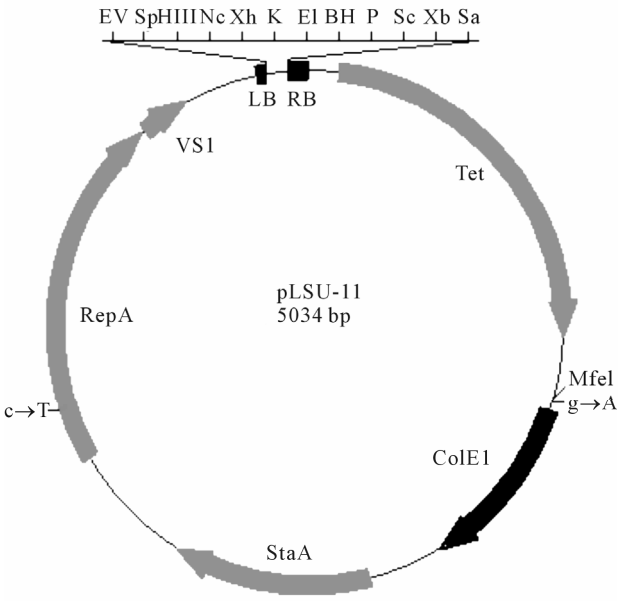

Figure 1. Schematic presentation of backbone structure of tetracycline-based binary Ti vector pLSU11 (5034 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *EcoRV* (EV), *SphI* (Sp), *HindIII* (HIII), *NcoI* (Nc), *XhoI* (Xh), *KpnI* (K), *EcoRI* (EI), *BamHI* (BH), *PstI* (P), *ScaI* (Sc), *XbaI* (Xb), and *SacI* (Sa). The backbone plasmid includes the tetracycline resistance gene (Tet^R), ColE1 origin of replication from pUC19 (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1).

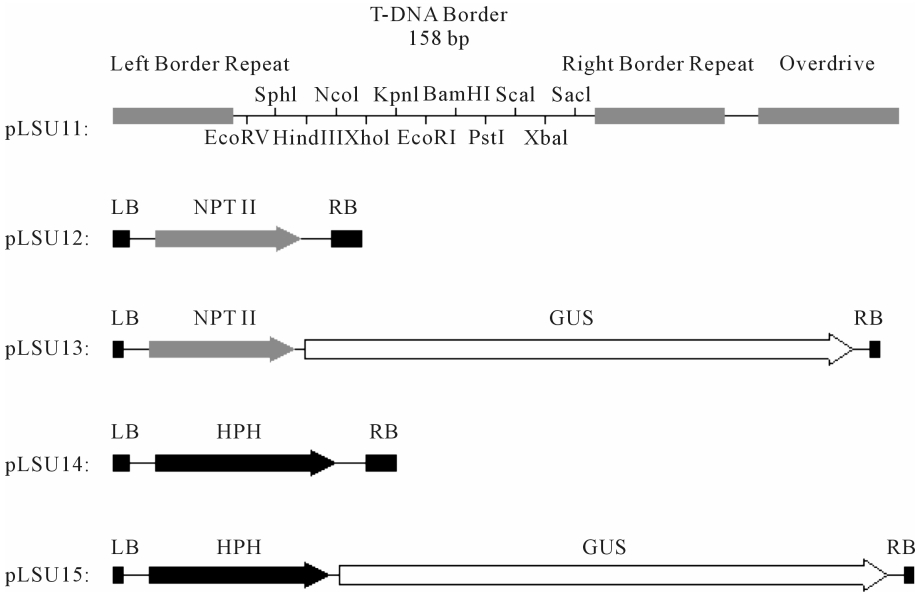

Figure 2. Schematic presentation of the T-DNA region of tetracycline-based binary Ti vectors pLSU11 to 15. pLSU-11 is a basic skeleton vector with the twelve common restriction sites in T-DNA. pLSU12 and 13 have the Neomycin PhosphoTransferase II gene (*NPTII*) adjacent to the left border as a plant selection marker for kanamycin resistance. pLSU14 and 15 contain the Hygromycin B Phosphotransferasae gene (*HPH*) adjacent to the left border as a plant selection marker for hygromycin resistance. pLSU13 and 15 also include the *β*-glucuronidase reporter gene (*GUS*) in addition to the plant selection marker in the T-DNA.

yielding 2243 bp pORItet [22]. First, theunique *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 ColE1 replicon was amplified with primers BR322-1 and BR322-2producinga 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 BR322-4yielding 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 bppBRVS1 [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 kanamycine 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 pBRVSI was confirmed. The final product was named aspLSU16 (**Figure 3**).

2.7. Gateway Technology

The three reading frame cassettes (1711 bp of RfA, 1713 bpof RfB, and 1714 bp of RfC1; Invitrogen (Carlsbad, CA) have the suicidal *ccd*B gene for inhibiting the DNA gyrase activity and chloramphenicol resistance gene (*Cm^R*) flanked by *att*R1 and *att*R2 sites. A*ScaI* 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 *ScaI*site in pLSU12 was digested and dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Then the dephosphorylated vector was ligated with Gateway® cassettes

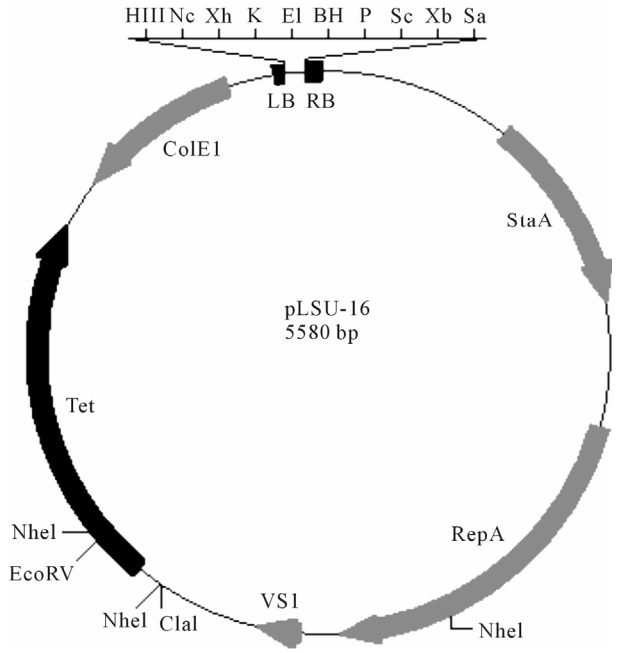

Figure 3. Schematicpresentation of mobilizable binary Ti vector pLSU16 (5580 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *EcoRV* (EV), *SphI* (Sp), *HindIII* (HIII), *NcoI* (Nc), *XhoI* (Xh), *KpnI* (K), *EcoRI* (EI), *BamHI* (BH), *PstI* (P), *ScaI* (Sc), *XbaI* (Xb), and *SacI* (Sa). The backbone plasmid includes the tetracycline resistance gene (*Tet*^{R}), 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 *E. coli* strain of the One shot®*ccd*B Survival™2 T1^R competent cells with *gyr*A 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 was determined by *BsrGI* restriction enzyme whose recognition site is located in the *att*R1 and *att*R2 sites.

The plant-expressible *β*-Glucuronidase (*GUS*) gene was amplified by PCR from pCAMBIA1305.2, using two primers containing *att*B1 and *att*B2 sites at the ends. A donor vector pDONR 221 has the *ccd*B and *Cm*^{R} gene flanked by the *att*P1 and *att*P2 sites. BP Clonase II enzyme catalyzed the BP recombination reaction between the *att*B sites of the GUS gene and the *att*P sites of pDONR 221. After incubation for one hour at 25˚C, the Proteinase K solution was added and incubated for 10 min at 37˚C. The reaction products were transformed in *E. coli* TOP10/P3 One Shots and selected for kanamycinat 50 mg/L. Colonies were picked and transferred to a replica plate under selection of chloramphenicol 30 mg/L and kanamycin 50 mg/L. If the site-specific recombination happened between *att*B1/B2 and *att*P1/P2 sites, the replica colonies are sensitive to chloramphenicol selection because the *Cm*^{R} gene was removed by recombination, yielding a new entry vector pENTR-GUS.

The destination binary vectorpLSU17A was mixed with pENTR-GUS and Gateway® LR Clonase™ II enzyme mix, and the LR recombination reaction was performed at 25˚C overnight. After the Proteinase K treatment the reaction products were transformed in E. coli TOP10/P3. Selection for tetracycline resistance at 10 mg/L and for chloramphenicol sensitivity at 30 mg/L yielded colonies containing the expression vector pLSU17A-GUS.

2.8. Freeze-Thaw Transformation of *A. tumefaciens*

Cells of *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 1ml 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 *A. tumefaciens*

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

2.10. Histochemical Detection of *β*-Glucuronidase Activity

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

3. Results

3.1. Tetracycline-Based Binary Ti Vectors pLSU12 and 14

We constructed new tetracycline-basedbinary Ti vectors by replacing the bacterial kanamycinresistance gene of binary vectors pLSU2 and 4 [25] with the tetracycline

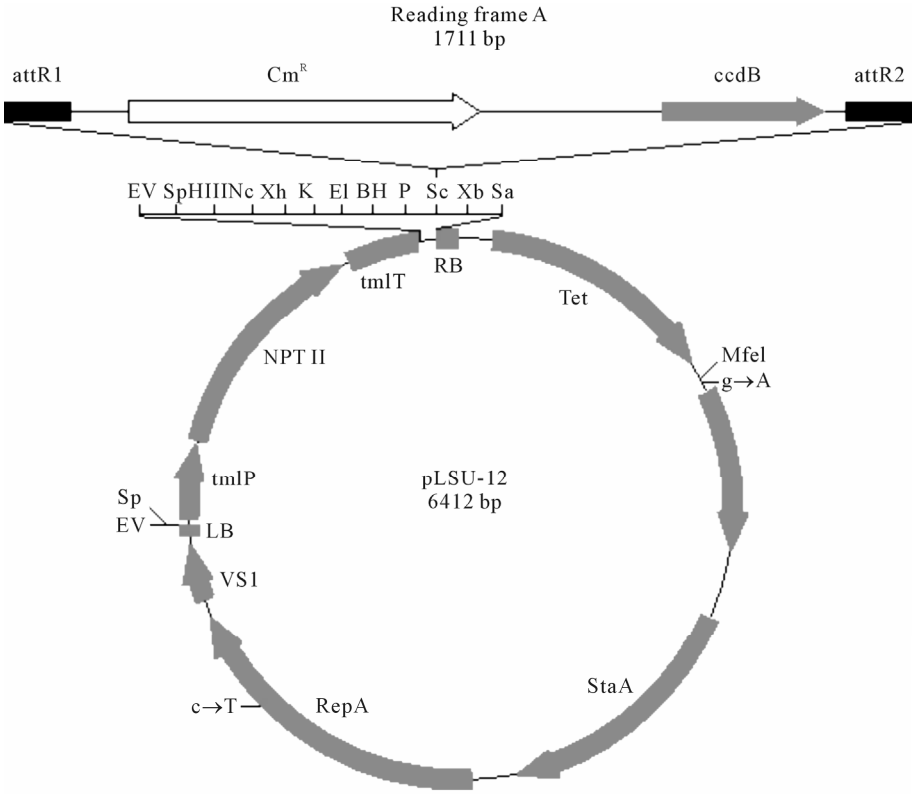

Figure 4. Schematic presentation of Gateway destination vector pLSU17A. At the top is the reading frame A fragment with two clonase recognition sites (attR1 and attR2), chloramphenicol resistance gene (*Cm*^{R}), and *ccd*B gene that inhibits the DNA gyrase (topoisomerase II). T-DNA is limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *EcoRV* (EV), *SphI* (Sp), *HindIII* (HIII), *NcoI* (Nc), *XhoI* (Xh), *KpnI* (K), *EcoRI* (EI), *BamHI* (BH), *PstI* (P), *ScaI* (Sc), *XbaI* (Xb), and *SacI* (Sa). The kanamycin resistance gene *NPTII* of pLSU12 is located between the *SphI* and *HindIII* sites. The backbone plasmid includes the tetracycline resistance gene (*Tet*^{R}), ColE1 origin of replication (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1).

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. tumefaicnes* 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 incommonly 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.

Fiverestriction 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 multicloning 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 (*Tet*^{R}) gene were confirmed as expected. How-

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 tetracyline-based binary Ti vectors pLSU16 in which the mob function of ColE1 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, ColE1 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. tumefaciencs* was used for transformation of tobacco leaf discs after four-day co-cultivation. Transformed 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 *att*R1 and *att*R2 sites (1711 bp of RfA, 1713 bp of RfB, and 1714 bp of RfC1) were ligated to the S*caI* 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^R) and the suicidal *ccd*B gene inhibiting the DNA gyrase activity (topoisomerase II). The ligation products were transformed to the *E. coli* strain DB3.1 containing *gyr*A 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^R and *ccd*B gene in the destination vector using the LR clonase-catalyzed recombination reaction of the *att*L1/*att*L2 sites of the donor vector with the *att*R1/*att*R2 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 and chloramphenicol sensitivity.

Table 1. Tetracycline-based binary Ti vector pLSU12 was compared with kanamycin-based binary vectors pLSU2 in its effect on the increase in final fresh weight yield of kanamycin-resistant calli of tobacco leaf disks. Leaf disks were co-cultivated for four days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU. Leaf disks were selected at 25˚C on shoot medium containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for two weeks. Fresh medium was prepared for additional two weeks of selection. Co-cultivation was performed from 1/11 to 1/15, the first selection from 1/15 to 1/29 and the second selection from 1/29 to 2/12/2010. Each treatment had five plates with 10 leaf disks per plate. Numbers in parentheses indicate standard deviations.

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

3.6. GUS Reporter Gene Expression in Tobacco Leaf Discs

The plant-expressible *GUS* gene in the T-DNA of binary vector pLSU17Awas 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 ColE1 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® Technology for efficient cloning. With further introduction of Multi-site Gateway methodology, different combinations of *attR*1,

*attR*2, *attR*3, *attR*4, *attR*5 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 coloniesto 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 bylabor-intensive DNA purification. An alternative approach used the suicidal characteristic of *ccd*B 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 *ccd*B gene cannot survive due to the activation of *ccd*B 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. tumefacins*.

The other 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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Abbreviations

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

