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New Binary Ti Vectors with Co-directional Replicons for Agrobacterium Tumefaciens-mediated Transformation of Higher Plants

Seokhyun Lee
Louisiana State University and Agricultural and Mechanical College, sl144@hanmail.net

NEW BINARY Ti VECTORS WITH CO-DIRECTIONAL REPLICONS FOR *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION OF HIGHER PLANTS

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LIST OF ABBREVIATIONS

A medium : Agrobacterium medium

Amp^R : Ampicillin resistance

BLAST : Basic Local Alignment Search Tool

EDTA : Ethylenediaminetetraacetic acid

EtBr : Ethidium Bromide

EtOH : Ethyl alchohol

GFP : Green Fluorescent Protein

GUS : β-Glucuronidase

Kan^R : Kanamycin resistance

LB medium : Luria-Bertani medium

ORF : Open Reading Frame

PCR : Polymerase Chain Reaction

PEG : Polyethylene glycol

Replication Origin : Replicon

Rif^R : Rifampicin resistance

Sp^R : Spectinomycin resistance

St^R : Streptomycin resistance

Tet^R : Tetracycline resistance

ABSTRACT

Small, high-yielding binary Ti vectors of *Agrobacterium tumefaciens* were constructed. All four basic components of the vector, ColE1 replicon (715 bp) for *Escherichia coli*, VS1 replicon (2,659 bp) for *A. tumefaciens*, a bacterial kanamycin resistance gene (999 bp), and the T-DNA region (170 bp), were modified to reduce the vector size to 4,566 bp and to introduce a number of mutations to increase the copy number and other functionality. The transcriptional direction of VS1 replicon can be the same as that of ColE1 replicon (co-directional transcription), or opposite (head-on transcription) as in the case of widely used vectors (pPZP or pCambia). The new binary vectors with co-directional transcription yielded up to four-fold higher *E. coli* transformation frequency, and 1.2 to 1.7-fold higher in *A. tumefaciens* than the head-on transcription. Compared to the pCambia vector, the new vectors have over ten-fold higher transformation frequencies in *A. tumefaciens* and five-fold greater DNA yields in *E. coli*.

The proper functions of the new T-DNA borders and new plant selection marker genes were confirmed after tobacco leaf discs transformation. Genetic analysis of kanamycin resistance trait among T_1 progeny indicates that the kanamycin resistance genes were integrated stably into a locus or closely linked loci of the nuclear chromosomal DNA of the primary transgenic plants and inherited to the second generation.

A tetracycline-based binary Ti vectors was also constructed to facilitate efficient cloning afforded by the Gateway Technology for *Agrobacterium*-mediated transformation of higher plants. Because the Gateway Technology entry/donor vectors are kanamycin-based, tetracycline-based destination and expression vectors are easily selected for the antibiotic resistance in the *E. coli*. The Gateway expression vector with a *gus* gene was constructed.

The appendices include a manuscript by Su G, Park S, Lee S, and Murai N entitled “Low co-cultivatin temperature at 20℃ improved *Agrobacterium tumefacciens*-mediated

transformation of tobacco leaf discs". Provisional Patent (application number 61328250) entitled "Binary Ti vectors with Co-directional replicons for transformation of eukaryotes and prokaryotes" was applied.

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

The traditional roles of agricultural crops are human food, animal feed, fiber, industrial and renewable materials. Today improved molecular genetic techniques allow introducing selected genes into crop plants to increase their nutritional quality and to produce desired macromolecules such as essential amino acids, vitamins and minerals, and to produce vaccines, antibodies and other biopharmaceuticals from crop plants (Jauhar, 2001).

Biopharmaceuticals have been produced from transgenic bacteria, fungi, and animal cells but higher plants are thought to be inexpensive and safe alternatives. Though proteins produced from transgenic animal cells have better chances to be correctly synthesized and processed including glycosylation and become more suitable for human metabolism, they have low yields and are highly expensive because of complicated culture conditions sensitive to variations in temperature, pH, oxygen, and the metabolites. Moreover, the requirement of protein purification from the serum sets the price very high. As a result, plant-derived products have several advantages when compared to other production systems. First, biopharmaceuticals from transgenic plants could be comparatively high yields because large-scale production is possible. Second, they could be stably stored in seed endosperm or other storage tissues for a long time and can be conveniently extracted. Also, from the aspect of health risks, plant-derived proteins have no or reduced chance to be contaminated by human or mammalian pathogens. This is because plants are not hosts for human disease-causing agents like bacterial endotoxins, fungi, viruses, prions, oncogenic DNA, and other unknown agents (Ma et al., 2003; Cramer, 1996). In addition, proteins produced from crop plants can be directly orally administrated with storage tissues of crops while avoiding expensive purification steps or even be easily taken through standard meals like cereals.

Several transformation methods are used to produce biopharmaceuticals in plants. The commonly used methods are *A. tumefaciens*-mediated transformation (Tzfira and Citovsky, 2006; Barton et al., 1983; Caplan et al., 1983; Herrera-Estrella et al., 1983; Murai et al., 1983), particle bombardment transformation (Kohli et al., 2003; Daniell et al., 2002; Vain et al., 2002; Christou, 1997), polyethylene glycol (PEG)-mediated transformation of protoplast (Datta et al., 1990; Hayashimoto et al., 1990), and recombinant virus infection system.

1.1.1. Particle Bombardment

Particle bombardment transformation is a very widely used plant nuclear transformation method. Almost all major crops can be transformed by this method. Also, because this method does not use Agrobacterium, false-positive expression of reporter gene due to contaminated Agrobacteria can be avoided (Veluthambi et al., 2003). However, the physical damage to cells that occurs during this process lowers the frequency of stable transformants and high copy number of the target gene can exist in multiple chromosomal locations. The multiple copies (up to 40 copies in a single locus) of the transgenic DNA integrated at complex transgenic loci are an inevitable event for direct DNA transfer method (Vain et al., 2002). In addition to the low consistency and requirement of expensive equipment, it has serious problem that the transgenic DNA can be truncated and rearranged, and the multiple copies can be arrayed as concatamers or interspersed in the plant genome (Kohli et al., 2003).

Particle bombardment transformation is also used for plant chloroplast transformation which has advantages such as high levels of transgene expression and polycistronic multigene expression in a single transformation event (De Cosa et al., 2001). Also, because the inserted target gene is only inherited into the chloroplast genome which is not transmitted through the pollen, this maternal inheritance feature can prevent the spread of genetically engineered strain into the field (Zhang et al., 2003; Daniell et al., 2002; Svab et al., 1993).

1.1.2. Virus-mediated Transfection

Plant virus vectors such as *Tobacco Mosaic Virus* (*TMV*), *Cowpea Mosaic Virus* (*CPMV*) *Potato Virus X* (*PVX*), and *Plum Pox Virus* (*PPV*) are used for the recombinant virus-plant transfection system (Canizares et al., 2005). The single strand RNA viruses replicate in the cytoplasm of the host plant, not in the nucleus, and therefore this system is not a transformation but a transfection. Because the recombinant viral RNA does not integrated into the plant genome, the transgenic progeny of the transfected plant does not have the recombinant virus genome. The recombinant plant viruses infect tobacco plants and transiently express a target protein which accumulates in the plant interstitial space. The target protein can be extracted from the interstitial fluid by vacuum centrifugation. Due to the rapid viral infection, the target protein can be collected within several weeks. This fast procedure can be advantageous when compared to other plant transformation methods particularly for the expression of secretory antibodies. Normally, the regeneration after transformation and the growth and production of transgenic plants are very time-consuming. However, the infected tobacco plant collapses quickly, and the quick degradation of the infected tobacco tissue means that the storage time of the targeted tissue in plant is very short (Canizares et al., 2005; Grill et al., 2005; Brennan et al., 1999; Mushegian et al., 1995; Turpen et al., 1995; Kumagai et al., 1993). The recombinant virus-plant transfection system has been very useful for vaccine production in other plant species. The produced vaccines can be stored much easier than tobacco plant tissue and be taken by oral administration (Daniell et al., 2009; Canizares et al., 2005; Grill et al., 2005).

1.1.3. ColE1 Replication Origin

Plasmids, extrachromosomal, circular DNA, can replicate independently from the host chromosomal replication because the replication origin (replicon) differs from the host chromosomal replicon. One of the best characterized plasmid replicon is ColE1 which was

originally from an *E. coli*-originated wild type plasmid ColE1. In the type I mechanism of plasmid replication, which seems to be the most normal and reliable, the plasmid replication begins with transcribing two RNA transcripts 555 bp upstream of the ColE1 replicon. One of the transcripts, RNA II, is transcribed through the replication origin and works as a primer RNA which forms a hybrid with the template DNA at the upstream of the replication origin around position -20 and -265 bp. This RNA II-DNA hybrid is cut off by a host-encoded ribonuclease H at the start point of the replication origin generating a free 3'-hydroxyl group and the initiation of the DNA synthesis begins from this point (Kues and Stahl, 1989; Tomizawa and Masukata, 1987; Selzer and Tomizawa, 1982; Itoh and Tomizawa, 1980). In other alternative replication mechanisms type II and III, the presence of RNase H is not necessary for the hybrid to be used as a primer. However, without the cleavage by RNase H, the DNA synthesis is not as efficient as type I mechanism (Dasgupta et al., 1987). The other RNA transcript is a negative regulatory RNA I which is complementary to the 5'-terminal 108 bases of RNA II and its secondary structure irreversibly binds to the 5'-end of RNA II which has a similar secondary structure. This RNA II-RNA I duplex negatively regulates the initiation of plasmid replication by inhibiting the secondary structure formation of active primer RNA II followed by inhibiting the formation of RNA II-DNA hybrid (Masukata and Tomizawa, 1986; Tomizawa, 1986). To prevent the over-inhibition by RNA I, host-encoded RNase E cleaves the 5'-end of RNAI transcript, which is the critical part for the RNA II-RNA I hybridization and causes the conformational change of the secondary structure of RNA I leading to inactive RNA I inhibitor (Tomcsanyi et al., 1985). There is another negative regulation factor, ROP protein (repressor of primer), which binds to the bridge between RNA II and RNA I and enhances the RNA duplex (Tomizawa et al., 1984).

Once the active RNAII-DNA hybrid is cleaved by RNase H, DNA polymerase I begins to synthesize the leading strand unwinding the template dsDNA and exposes the dnaA boxes and

primosome assembly sites (*pas*). DnaA protein is essential for the initiation of *E. coli* chromosomal replication and for plasmid replication. Although DNA can replicate without it, the absence of DnaA protein and dnaA boxes greatly reduces the DNA synthesis (Parada et al., 1991; Ma el al., 1988; Seufert et al., 1987). The *pas* for leading and lagging strands are also needed for the plasmid replication. The deletion of the lagging strand *pas* reduces the plasmid replication. However, they are not essential for replication and the presence of dnaA boxes significantly improves the low replication efficiency (Masai et al., 1989; Masai et al., 1988; Seufert et al., 1987; Van der Ende et al., 1983).

It has been reported that the plasmid replication can be improved by removing a negative regulation factor. In 1982, Cesareni reported that a single point mutation (G→A), located on the -35 promoter region of the RNA II transcript, can increase the plasmid copy number up to three times (Castagnoli, 1987; Castagnoli et al., 1985; Cesareni, 1982). There is another single point mutation site increasing the plasmid copy number within the RNA II transcript. The single point mutation (G→A1308) is located directly in front of the initiation site of the RNA I transcript of plasmid pUC19 (Chambers et al., 1988). This may be due to the reduced transcription of RNA I or conformational change of RNA I secondary structure leading to the failure of duplex formation with RNA II or the mutation may affect the RNA II transcript and change the secondary structure. If the RNA I does not bind, or reversibly binds to RNA II, all RNA II transcripts form a secondary structure of active RNA primer and the plasmid copy number increases. The deletion of the rop gene located at the downstream of replication origin causes the lack of ROP protein dimer, which is supposed to bind and tighten the binding between RNA II and RNA I transcripts. It also allows the increase of plasmid copy number up to two folds (Chambers et al., 1988).

1.2. *Agrobacterium tumefaciens*

1.2.1. Host Range

The *A. tumefaciens*-mediated transformation method is very useful due to the fact that the molecular mechanism of transformation has been elucidated and the manipulation is simple, easy, and inexpensive (Lindsey, 1992). Prior difficulty of transforming monocot plants with *A. tumefaciens* has been mitigated by inducing the expression of *trans*-acting *virulence* (*vir*) genes of Ti plasmid with acetosyringone, a *vir* gene inducer (Aldemita and Hodges, 1996; Vijayachandra et al., 1995; Hiei et al., 1994). Other monocots such as maize (Frame et al., 2002), barley (Patel et al., 2000; Tingay et al., 1997) and wheat (Jones, 2005) have been also transformed by *A. tumefaciens* and it was shown that *A. tumefaciens* transfers genes to fungi (yeast; Piers et al., 1996) and cultured human HeLa cells, too (Kunik et al., 2001).

1.2.2. Ti Plasmid and Opine Catabolisms

Agrobacterium tumefaciens is a Gram-negative soil bacterium and a plant pathogen causing crown gall tumors at infection sites of host plants. For its oncogenicity in the host plant, two major genetic elements, T-DNA and *virulence* (*vir*) gene region, are required in the tumor-inducing (Ti) plasmid, which is a large (~200 kb) extrachromosomal covalently closed circular DNA. The crown gall tumor on the *A. tumefaciens*-infected plant is formed after the T-DNA is transferred from the bacteria to the plant. The T-DNA is then integrated into the host plant cell nuclear genomes and produces plant growth hormone for tumor formation and opine for bacteria use. The T-DNA contains genes involved in the synthesis of opines and plant hormones.

Opine is an amino acid compound which is the major carbon and nitrogen source for *A. tumefaciens* and is related to the strains of *A. tumefaciens*. When the tumor is formed on the host plant, opine is synthesized from the tumor tissue and imported into *A. tumefaciens*. The imported opine is catabolized specifically by *A. tumefaciens* enzymes. The Ti plasmid sequences from

different *A. tumefaciens* strains are not identical and can not be used as a standard for dividing the classes of *A. tumefaciens* strains. However, the classification of *A. tumefaciens* strains is determined by the specific opine type that each *A. tumefaciens* strain digests, and therfore the Ti plasmids can be grouped into four classes including octopine Ti plasmid such as pTiAch5 from *A. tumefaciens* strain Ach5, nopaline Ti plasmid such as pTiC58 of C58 strain (Kemp et al., 1979), agropine Ti plasmid such as pRiHR1 of HR1 strain (Guyon et al., 1980), and agrocinopine Ti plasmid such as pTiChry5 of Chry5 strain (Ellis and Murphy, 1981).

1.2.3. T-DNA, Borders, and Opine Synthesis Genes

Besides the T-DNA, Ti plasmid also contains two short border sequences flanking T-DNA genes and 35 kb *vir* gene regions, while *A. tumefaciens* chromosome encodes *chromosomal virulence* (*chv*) genes. These factors, the *cis*-acting T-DNA border sequences, the *trans*-acting *vir* genes of Ti plasmid and *chv* genes of chromosome are required for the T-DNA transfer. T-DNA and the virulence genes are shown to exist in *trans* in two separate plasmids. These are called disarmed non-oncogenic Ti plasmids in which the tumor genes are eliminated. The T-DNA left and right border sequences are about 25 bp conserved direct repeated sequences (left border; 5'-GGCAGGATATATTCAGTTGTAAAT, right border; 5'-TGGCAGGATATATGCGGTTGTAA TTC). Adjacent to the right border of octopine T-DNA, there is a 30 bp overdrive sequence (5'-TTGTCTAAATTTCTGTATTTGTTTGTTTG) which stabilizes the high efficiency transfer. If this right border is deleted, the gene transfer is blocked, while deletion of the left border does not affect the transfer. Thus, the T-strand is formed uni-directionally from the right to left border.

1.2.4. Virulence Genes

The *vir* region of Ti plasmid has seven loci, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, and *vir*H and these *vir* genes produce proteins mediating T-DNA transfer into the host plant nucleus. The

expression of *vir* genes are induced by phenolic compounds, such as acetosyringone, which are secreted only from the wounded plant tissue (Tzfira and Citovsky, 2006; Stachel et al., 1985). These compounds are produced at a basal level or not produced at all in the uninjured plants. When they are wounded, the production of these compounds is increased. The *A. tumefaciens*-mediated transformation of a monocot plant has been difficult because the concentration levels of these phenolic compounds are low even when the monocot plants are wounded. Nowadays, by addition of the phenolic compounds, many monocot plants, like rice, can be transformed with *A. tumefaciens*.

1.2.5. Binary Vectors

Binary vectors are T-DNA based vectors with wide host range replication origins (replicons). Because the Ti plasmid is too big to handle, a binary vector system has been developed. A binary vector carries the T-DNA border sequences flanking a multiple cloning site (MCS) and is introduced into *A. tumefaciens* harboring disarmed Ti plasmid. Therefore, for the *A. tumefaciens*–mediated plant transformation, the *Agrobacteria* contain a disarmed helper Ti plasmid carrying the *vir* functions and a binary vector carrying T-DNA borders flanking the foreign gene to be introduced. A binary vector also has a broad host ranged replicon which can control the replication in variety of host bacteria including *A. tumefaciens*. By the interaction of these separate compatible plasmids, the target gene is stably integrated into the plant genome. Due to the presence of an *E. coli* replicon, the binary vector can be manipulated *in vitro* in *E. coli* host which makes it simple. It is easy to handle the binary vector construction in *E. coli*. Thus, a useful binary vector must have a stable broad host range replicon, a selective marker gene such as antibiotic resistance gene, a multi-cloning site between T-DNA borders, and T-DNA left/right border sequences.

For the early binary vectors including pBIN19 (Bevan, 1984), pBI121, or pTJS75 (Schmidhauser and Helinski, 1985), RK2 origin replicon was widely used as broad host range replicon. However, due to its limited functionality, the binary vectors containing derivatives of this replicon become inherently unstable and they have very low replication copy number in *E. coli*. Thus, for construction of more stable binary vectors, other replicons have been developed and used. This includes VS1 replicon from *Pseudomonas aeruginosa* plasmid pVS1 (Deblaere et al., 1987; Itoh et al., 1984) and Ri replicon from *A. rhizogenes* plasmid pRi (Guiney and Yakobson, 1983). Moreover, to get higher copy number in *E. coli*, some binary vectors like the pPZP family vector have been developed by harboring two separate replicons, one for in *E. coli* (ColE1 replicon) and one for *A. tumefaciens* (VS1 replicon). Another disadvantage of early binary vectors was their large sizes. Because the transformation frequency is inversely proportional to the vector size, newly developed binary vectors have smaller vector size by removing unnecessary region. Examples of these vectors include the 12 kb of pCambia series with ColE1 replicon from pBR322 for *E. coli* and VS1 replicon for *A. tumefaciens*, the 4.5 kb of pGreen series with ColE1 replicon from pUC for *E. coli* and pSa replicon for *A. tumefaciens* (Hellens et al., 2000), the 9.0 kb of pPZP series with ColE1 replicon for *E. coli* and VS1 replicon for *A. tumefaciens* (Hajukiewicz et al., 1994), and the 14.5 kb of pCGN series with ColE1 replicon for *E. coli* and Ri replicon for *A. tumefaciens*.

1.3. Biopharmaceuticals Produced in Transgenic Plants

1.3.1. Nutritional Enhancement

The nutritional quality of several crops has been improved through the transgenic plant techniques. For example, a transgenic rice known as “Golden Rice” producing vitamin A, which is present at low levels in non-transformed rice endosperm, was developed (Ye et al., 2000). This transgenic rice was transformed via *A. tumefaciens*-mediated transformation and is genetically

enriched with vitamin A which is thought to be very helpful to reduce diseases caused by Vitamin A-deficiency.

Another major micronutrient problem is iron deficiency. In 1999, Goto et al. reported the accumulation of an iron-storage protein, ferritin, in rice grain by transferring the entire coding sequence of the soybean *ferritin* gene under control of rice *glutelin* gene promoter via *A. tumefaciens*-mediated transformation. Since then, a number of strategies have been employed to increase the iron content in transgenic milled rice endosperm (Lee et al., 2009; Wirth et al., 2009; Khalecuzzaman et al., 2006; Vasconcelos et al., 2003).

1.3.2. Biopharmaceuticals

The first biopharmaceutical product produced in plants was a human growth hormone (Barta et al., 1986). Many other human proteins like human serum albumin have been produced from transgenic plants (Sijmons et al., 1990). Most initial work was conducted with transgenic tobacco leaf tissue which requires expensive purification steps and the protein production was at low levels. For instrance, less than 0.1% of the total soluble protein was produced because of poor protein folding and unstability. Later, the tobacco chloroplast transformation system began to produce human growth hormone and human serum albumin at much higher level (Fernandez-San Millan et al., 2003; Staub, 2000). The human growth hormone was produced in transgenic tobacco chloroplast as 7% of the total soluble protein as active state. It correctly formed disulphide bonds and the human serum albumine produced in transgenic tobacco chloroplast was over 11% of the total soluble protein. The human serum albumin was hyperexpressed by introducing regulatory elements of Shine-Dalgarno sequence and chloroplast untranslated regions that compensated for the excessive proteolytic degradation of albumine. Besides these proteins, many other biopharmaceuticals have been produced in a variety of transgenic plants such as α-inteferon, β-glucuronidase, or human milk protein β-casein (Kusnadi et al., 1998;

Dalsgaard et al., 1997; Chong et al., 1997). The human milk protein β-casein was produced in transgenic potato tuber for improvement of infant nutrition while preventing gastric and intestinal diseases in infants.

Transgenic plant is also an inexpensive alternative for the production of recombinant vaccines (Daniell et al., 2009; Grill et al., 2005). For example, hepatitis B, a virus responsible for chronic liver disease in human has been targeted for developing the recombinant vaccine to induce a humoral immune response since it was first produced in 1992 (Mason et al., 1992). Because yeast or animal cell transformation for the vaccine had many troubles with the cost, transgenic potato expressing hepatitis B surface antigen has been developed (Smith et al., 2003; Richter et al., 2000). This vaccine can be conveniently taken by oral administration with untreated, edible potato tissue (Thanavala et al., 2005).

The expression of genes encoding antibodies in plants has many advantages such as the low cost and large-scale production for therapeutic and clinical use. Since Hiatt first demonstrated that functional antibodies could be produced in transgenic plants (Hiatt, 1990; Hiatt et al., 1989), many studies have been done to produce antibodies in plants (Stoger et al., 2004; Stoger et al., 2000; Conrad et al., 1998). Recombinant antibodies include fully assembled whole immunoglobulins, antigen binding fragments of immunoglobulins (IgA, IgG, IgM, etc.), and synthetic single chain variable fragment gene fusions (scFv) (Stoger et al., 2004; Sanchez et al., 1999; Ma et al., 1995; Owen et al., 1992; Hiatt et al., 1989). To maximize antibody expression in transgenic plants, the secretion and accumulation sites of the antibodies have been investigated and determined in *Arabidopsis thaliana* and *Nicotiana tabacum* (Sharp and Doran, 2001; Conrad et al., 1998B; De wilde et al., 1998). Among the cellular compartments investigated, the highest accumulation was observed in the apoplastic space and intercellular

space of leaf mesophyll cells, suggesting the antibodies accumulate in the apoplast through water flow in the vascular bundle.

The major 90% costs of extracting desired macromolecule from plant are the purification steps including milling, aqueous extraction, vacuum filtration, ultrafiltration, ion exchange chromatography, hydrophobic interaction chromatography, and dialysis (Evangelista et al., 1998). Recombinant antibodies can be targeted to accumulate in seeds and storage organs and be stably stored for a long period. Besides, the antibodies can be taken by oral administration in such plant tissues and that would be very efficient in costs and safety (Daniell et al., 2009).

For commercially beneficial and large-scale production of pharmaceutical proteins in transgenic plants, cereal crops such as rice, maize and wheat will be very useful. The cereal grains can be used as storage organs stable for a long period (Stoger et al., 2000). The pharmaceutical proteins in cereal crops can be extracted and purified by traditional milling procedure and thus it will cost much less than other plants such as tobacco because tobacco and other plants may need the removal of toxic compounds like alkaloids and nicotine (Stoger et al., 2000). Thus, cereal crops are a potential alternative for commercial biopharmaceutical production from transgenic plants. Especially rice (*Oryza sativa*) is the major source of food for more than 40% of the world's population and a lot of efforts have been directed to improve the quantity and quality of rice (Shimamoto, 1995).

Though the potential of transgenic plants is very high, there are still many obstacles to be overcome including the relatively poor accumulation of foreign proteins, instability of transgenes, and public fear about the genetically modified foods or drugs (Jauhar, 2001). Thus, biopharmaceuticals derived from transgenic plants should be suitable in both the economic aspect and safety standard and there must be a lot of efforts to get rid of the possibility of health risks. However, those which can be produced inexpensively in transgenic crops will be studied

more and more due to their economic and qualitative advantages. Also, for some cases that should be in non-edible crop plants such as tobacco, less expensive purification method for the foreign macromolecule will be kept on development, as well.

CHAPTER 2. BINARY PLANT TRANSFORMATION VECTOR pLSU-KAN

2.1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium and plant pathogen causing crown gall disease in angiosperms and gymnosperms (Smith and Townsend, 1907). *Agrobacterium*-plant interaction was one of the first systems in which the molecular mechanism for plant pathogenicity has been elucidated in detail (Zaenen et al., 1974; Chilton, 1977). About 20 kb segment of the DNA (T-DNA) in a tumor-inducing plasmid (214 kb Ti plasmid) is transferred from the bacterium to the host plant genome by a Type IV secretion system closely resembling to bacterial conjugal transfers (Zupan and Zambryski, 1985; Sheng and Citovsky, 1996; Gelvin, 2003). The disease phenotype is a manifestation of the expression of bacterial T-DNA in plant cells that result in over-production of the growth hormones cytokinin and auxin.

This natural DNA transfer system has been exploited to introduce genes of agronomic interest into crop plants resulting in the production of genetically modified crops by commercial plant biotechnology industry. Initial approaches of gene transfer were to introduce a target gene into the T-DNA region of Ti plasmid after either a single- (co-integration) or double-homologous recombination between an intermediate vector (pRK290) and Ti plasmid (Zambriski et al., 1983; Murai et al., 1983). A binary plant vector strategy was designed to separate the T-DNA region in a small plasmid from the virulence genes in avirulent T-DNA-less Ti plasmid (Hoekema, 1983). The small plant vectors with the T-DNA region have been simply now called binary Ti vectors (Hellens et al., 2000; Komori et al., 2007).

Binary Ti vectors replicate in both *E. coli* and *A. tumefaciens*. They have two separate replicons, one for replication in *E. coli* and another for *A. tumefaciens*. RK2 origin of replication was widely used as broad host range replicon for the early binary vectors including pBIN19 (Bevan, 1984), pBI121, or pTJS75 (Schmidhauser and Helinski, 1985). However, the RK2

replicon is not well-defined and relatively large, and the binary vectors with RK2 replicon were found to be inherently unstable in *A. tumefaciens* and have very low plasmid yield in *E. coli*. The next generation binary vectors used ColE1 replicon for propagation in *E. coli* and other broad host-range replicons for propagation in *A. tumefaciens*, including Ri replicon from *A. rhizogenes* plasmid pRi (Guiney and Yakobson, 1983), Sa reiplicon from *E. coli* plasmid p15A (Hellens et al., 2000), and VS1 replicon from *Pseudomonas aeruginosa* plasmid pVS1 (Deblaere et al., 1987; Itoh et al., 1984). Most binary vectors are large (> 10 kb) and the size of the plasmid DNA is inversely proportional to the *in vitro* gene manipulation efficiency and transformation frequency.

A broad host-range VS1 replicon of pVS1 originally isolated from *P. aeruginosa* was selected to ensure the stable replication of new binary Ti vectors in *A. tumefaciens* (Itoh et al., 1984). The VS1 replicon has distinctive advantages with its smaller size and more stability over other broad range replicons RK2, pSa and pRi used in the previous binary constructs. Here, I have reduced the size of the REP/STA region of the broad host-range replicon VS1 to a 2,660 bp fragment and demonstrated the stability of the binary vector with this replicon in *A. tumefaciens*. I have also reduced the size of ColE1 replicon to a 715 bp fragment and demonstrated the stability of the binary vector with this replicon in *E. coli*. The final size of the skeleton of new binary vectors was reduced to 4,543 bp, there by increasing the plasmid yield in *E. coli* and transformation efficiency in *E. coli* and *A. tumefaciens*. The binary vectors were shown to be effective in transformation of tobacco leaf disks.

2.2. Materials and Methods

2.2.1. Chemicals, Enzymes, Bacterial Strains, and Plasmid DNA

2.2.1.1. Chemicals and Enzymes

Chemicals used in these experiments were purchased from Amresco (Solon, OH), J. T. Baker Chemical Co (Phillipsberg, NJ), Becton, Dickinson and Company (Sparks, MD), Bethesda

Research Laboratory (BRL; Grand Island, NY), Bio 101 Inc. (Vista, CA), Bio-Rad Laboratories (Hercules, CA), Difco Laboratory (Franklin Lakes, NJ), EM Science (Gibbstown, NJ), EMD Chemicals Inc (Gibbstown, NJ), Fisher Scientific (Fair Lawn, NJ), Mallinckrodt Chemicals (Sweden), Sigma-Aldrich (St. Louis, MO). Antibiotics (ampicillin, carbenicillin, gentamycin, hygromycin, kanamycin, rifampicin, streptomycin, spectinomycin, tetracycline) were purchased from Sigma-Aldrich.

Unless otherwise specified, doubly-deionized sterile water was used for all of the experiments. For dialysis and filter-sterilization, dialysis tubes (Spectrum Medical Industries Inc., Huston, TX), filter paper (Whatman, Maidstone, UK), and 0.2μm Uniflo filter (Schleicher and Schuell, Keen, NH) were used as instructed by the manufacturers.

Restriction endonucleases (*Avr* II, *Bam* HI, *Bcl* , *Bgl* II, *Bst* EI, *Cla* I, *Eco* RI, *Eco* RV, *Hind* III, *Kpn* I, *Mfe* I, *Nde* I, *Nco* I, *Nhe* I, *Pst* I, *Pvu* II, *Sac* II, *Spe* I, *Sph* I, *Xba* I, and *Xho* I), Deep Vent DNA polymerase, Deoxyribonuclease II, and T4 DNA ligase were purchased from New England Biolab (NEB; Beverly, MA) and/or Bethesda Research Laboratory (BRL; Grand Island, NY). Lysozyme was purchased from Sigma and Ultra *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). The enzymes were treated as instructed in the manufacturer's manual.

2.2.1.2. Bacterial Strains and Plasmid DNA

The tetracycline-resistant XL1Blue and XL2Blue strain were purchased from Stratagene. The genotype of the MR strain is as follows: *recA1 endA1 gyrA96(nal^R) hsdR17(r_K^- m_K^+) thi-1 SupE44 relA1 lac glnV44* F'[::Tn*10 proAB*$^+$ *lacI*^{q} *Δ(lacZ)M15*]. The genotype of the XL2-Blue is as follows: *endA1 SupE44 thi-1 hsdR17(r_K^- m_K^+) recA1 gyrA96(nal^R) relA1 lac glnV44* F'[::Tn*10 proAB*$^+$ *lacI*^{q} *Δ(lacZ)M15* Amy Cm^R]. The LBA4404 strain of *A. tumefaciens* was purchased from Invitrogen (Carlsbad, CA). The LBA4404 strain has TiAch5 chromosome with

the rifampicin resistance, and disarmed Ti plasmid pAL4404 with spectinomycin- and streptomycin-resistance (Hokekma et al., 1983). The LBA4404 strain was grown on *Agrobacterium* media (A media) containing, on a basis per liter, 2.0 g mannitol, 2.0 g $(NH_4)_2SO_4$, 5.0 g yeast extract, and 100 ml of 10X salt solution (109.0 g/L 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 $MnCl_2 \cdot 4H_2O$). The 10X salts were dissolved in H_2O, pH was adjusted to 7.0 with 1 M KOH, and the volume of solution was adjusted to one liter. The 10X salt solution was heated to boiling and the precipitates were filtered through Whatman #1 filter paper. After addition of the 10x salt solution the pH of the A media was adjusted to 7.0 prior to autoclaving. The LBA4404 strain was maintained on A medium with 30 mg/L of rifampicin (Rif^{30}), 100 mg/L of spectinomycin (Sp^{100}), and 50 mg/L of streptomycin (St^{50}) at 28°C.

Plasmids used for experiments were pBR322 (Bolivar, 1978; Sutcliffe, 1978A; Sutcliffe, 1978B), pUC19 (Chambers et al., 1988; Yanisch-Perron et al., 1985). pCambia-1301, -1305.2, and -2301 were obtained from Cambia (http://www.cambia.org.au, Canberra, Australia). pBluescriptII KS(+) was purchased from Stratagene, and pUC4-KIXX and -KSAC were purchased from Pharmacia (Barany, 1985).

2.2.1.3. Oligodeoxyribonucleotides and Plasmid DNA Manipulation

Oligonucleotides used for PCR, mutagenesis, or DNA sequencing were custom-ordered and synthesized by Sigma-Aldrich and listed in Supplementary Tables B1 and B2. Plasmid DNA was isolated by the alkaline lysis method (Bimboim and Doly, 1979) and purified by the $CsCl_2$-EtBr gradient centrifugation method (Hansen and Olsen, 1977). The GENECLEAN kit purchased from BIO101 (Carlsbad, CA) was used to extract DNA from agarose gel (Vogelstein and Gillespie, 1979). The Quick Change Multi Site-Directed Mutagenesis Kit was purchased

from Stratagene and other molecular cloning methods were conducted according to Sambrook and Russell (2001).

2.2.2. Bacterial Kanamycin Resistance Gene *npt*I

The *neomycin phosphotransferase* I (*npt*I) gene from transposon Tn*903* has an 816 bp ORF (Grindley and Joyce, 1980; Oka et al., 1981). There are 227 bp inverted repeat sequences at both ends of the *npt*I gene fragment of Tn*903* in pUC4-KSAC (Barany, 1985). Primer KSACKF1 at the promoter region was designed to contain the last 19 bp of 5'-inverted repeat and transcription initiation sites including the -35 element TTGTGT (1,061 to 1,066 bp) and TATA box TACATT (1,084 to 1,089 bp). The -35 element and the TATA box were located at the 101 bp and 78 bp 5'-upstream, respectively, from the initiation codon of the *npt*I gene. Two large stem-loop structures were found in the transcriptional termination region (Supplementary Figure B2). Two primers, KSACKR1 and KSACKR2 were designed to hybridize, respectively, at the 3'-downstream end of each stem-loop structures to minimize the size of the amplified fragment. Four common restriction endonuclease sites, *Xba* I, *Sma* I, *Hind* III, and *Cla* I were located in the *npt*I coding region, and eliminated by site-directed mutagenesis producing p2-7ΔXSHC. To facilitate vector construction, a restriction enzyme site for *Bam* HI was added on primer KSACKF1 (aaagcc→GGaTcc; upper cases are the introduced single point mutations) and an *Mfe* I site was added on primers KSACKR1 (cgattt→cAattG) and KSACKR2 (gaacgg→CaaTTg). Primer KSACKF1 is 5'-cgatttattcaacGGaTccacgttgtgtctc-3' (*Bam* HI site is underlined) and corresponds to nucleotide (nt) 1,039 to 1,069 of p2-7ΔXSHC. Primer KSACKR1 is 5'-tcagcaaaagttcAattGattcaacaaagccgcc-3' (*Mfe* I site is underlined), corresponding from 2,068 to 2,035 nt of p2-7ΔXSHC. Primer KSACKR2 is 5'-ctttgccacgCaaTTgtctgcgttgtcg-3' (*Mfe* I site is underlined), corresponding from 2,123 to 2,096 nt. Amplification using KSACF1 and KSACR1 or R2 yielded the 999 bp fragment of KSACK5 and

1,056 bp of KSACK6, respectively (Supplementary Figure B4). The shorter 999 bp fragment of *npt*I gene was selected for final construction.

2.2.3. ColE1 Replicon from pUC19

The ColE1 replicon used for construction was isolated from pUC19 using two primers at the 5'-side and three at the 3'-side of the replicon. Primer ColR3 hybridizes to the 5'-side of the promoter region of RNA II and introduces a single point mutation (g1428**A**) in -35 element TTGAGA to increase the copy number. Primer ColF2 hybridizes to downstream of the plasmid replication origin (Ori) including dnaA and dnaA' boxes and ColF3 hybridizes to further downstream including L-*pas* which is primosom assembly site for the lagging strand (Supplementary Figure B3). To ligate these amplified fragments with KSACK5 and KSACK6 in the same transcriptional direction, restriction enzyme site *Mfe* I and *Bam* HI sites were introduced in the primers. *Mfe* I site was added on primer ColR3 (caaagg→caa**TT**g) and *Bam* HI was added on ColF2 (agctca→**G**g**A**tc**C**) and ColF3 (gcgggg→g**GATCC**). Primer ColR3 is 5'-agaaaagat**caaTTg**atcttcttga**A**atccttttttctgcg-3' (*Mfe* I site is underlined and single point mutation is boxed) and corresponds from nucleotide 1,476 to 1,435 of pUC19. The single point mutation on -35 element also produced Δ*Sau3*A1 (*Mbo* I; gatc→**A**atc) to verify the introduction of mutation. Primer ColF2 is 5'-gagcggtatc**G**g**A**tc**C**ctcaaaggcgg (*Bam* HI site is underlined), corresponding from 737 to 763 nt of pUC19. Primer ColF3 is 5'-ccaacgcg**GATCC**agaggcg gtttgc-3' (*Bam* HI site is underlined), which binds between 648 and 673 nt of pUC19. The amplified products are 715 bp of Col3 using primers ColF2 and ColR3, and 807 bp of Col4 amplified with primers ColF3 and ColR3. The PCR products Col3 and Col4 were ligated with KSACK5 and KSACK6 resulting in 1,714 bp of pKC53, 1,806 bp of pKC54, 1,771 bp of pKC63, and 1,863 bp of pKC64 (Supplementary Figure B4). pKC53 was selected for final construction since no difference was found between pKC53 and 54 in transformation frequency of *E. coli*.

2.2.4. Broad Host-range Replicon pVS1

The broad host-range replicon VS1 was isolated from pGV941 kindly provided by Marc Van Montagu, Universiteit Gent, Gent, Belgium. A single point mutation to increase the copy number of VS1 replicon was introduced to replace alanine with valine at 4,659 bp (c4659t) of *rep*A ORF. A tetracycline resistant binary vector pBRVS1 (Supplementary Figure B1) was re-circularized by inverted PCR using primers BRVSF2 and BRVSR2. Primer BRVSF2 is 5'-cgagatcacagaaggcaagaaccc-3' binding between nucleotide 4,675 and 4,698 of pBRVS1. Primer BRVSR2 is 5'-cggtacatccaatc**A** **A** **C**tagctcgatct-3' (Val codon is capital letters, boxed capital letter A is the introduced point mutation, and inactivated *Nhe* I site is underlined), corresponding from 4,674 to 4,647 nt of pBRVS1. The amplified product was named as pBRVS2 (Supplementary Figure B5).

The *rep*A and *sta*A region was amplified from pBRVS2 using primers SRL-F1 and SRL-R1. Primer SRL-F1 was designed to contain the 607 bp upstream region of the *sta*A open reading frame including the deduced -10 and -35 elements. SRL-F1 is 5'-aggcgtgaagtttggcTGATCAcctaccctcaccccg-3' (*Bcl* I site is underlined), corresponding from 2,303 to 2,339 nt of pBRVS2. The VS1 origin of replication consists of two 8 bp of dnaA boxes surrounding four 21 bp direct repeats and 92 bp of AT-rich region, serving as an entry site for the replication machinery. Primer SRL-R1 was designed to include the VS1 origin of replication and downstream 141 bp region of VS1 replicon. SRL-R1 is 5'-cttgtccgcgcGGATCCagattgcctggccgtagg-3' (*Bam* HI site is underlined), corresponding from 5,414 to 5,380 nt of pBRVS2. The amplified products of *sta*A and *rep*A regions were digested with *Bcl* I and *Bam* HI, producing 3,079 bp fragment. Then the amplified Sta/Rep region was ligated into the *Bam* HI site in pKC53 and pKC54, resulting in 4,793 bp of pKCVS53 and 4,885 bp of pKCVS54, respectively (Supplementary Figure B5). Further cloning process including the

insertion of T-DNA region and plant selection markers, hygromycin resistance gene and kanamycin resistance gene, eventually produced 6,572 bp binary vector of pCVH-Long and 6,343 bp of pCVN2-Long (Supplementary Figure B9).

To reduce the size of the Sta/Rep region from pCVH-Long, new primers CV-F7 and CV-R6 were designed to eliminate the excessive upstream region of the *sta*A ORF. pCVH-Long and pCVN2-Long were re-circularized by inverted PCR (Supplementary Figure B9). Primer CV-F7 is 5'-ccggccagct**AgATcT**ctgaagaaaccgag-3' (*Bgl* II site is underlined), binding between 2,125 and 2,154 nt of pCVHS-Long, or corresponding position in pCVN2S-Long to contain the 126 bp region upstream of the *sta*A ORF including -10 and -35 elements. Primer CV-R6 is 5'-tgagggtagg**TGATCA**ctcaaaggcggtaatacggttatcc-3' (*Bcl* I site is underlined), binding between 1,730 and 1,690 nt which is located 30 bp behind the dnaA box of ColE1 replicon. The amplified products were digested with *Bgl* II and *Bcl* I eliminating a 420 bp excessive 5' end of *sta*A ORF and then self-ligated, producing 6,152 bp of pCVHS and 5,923 bp of pCVN2S (Supplementary Figure B10). After complete DNA sequence analysis of double strands, the complete size of the binary Ti vector pCVHS was determined to be 6,173 bp and the binary Ti vector pCVHS was renamed as pLSU-4 (Figure 2).

A *Cla* I site at the middle of *rep*A gene was inactivated by a single base mutation (atcgat→at**T**gat) while maintaining the amino acid codon Isoleucine. The product was amplified by inverted PCR using primer BRVSF4 and BRVSR4 and was re-circularized by blunt end self-ligation producing a 4,258 bp of pKCVS53ΔClaI. Primer BRVSF4 is 5'-cgattacttttg**ATTgat**cccggcatcggc-3' (Ile ATT codon is capital letters and the boxed capital letter **T** is the mutation to inactivate underlined *Cla* I site), binding from 3,696 to 3,726 nt of pKCVS53. Primer BRVSR4 is 5'-gggtgaaccgtcagcacgtccgggttcttgc-3', binding between 3,695 and 3,665 nt of pKCVS53.

2.2.5. T-DNA Borders with the Overdrive from pTi15955

The left (LB), right border (RB) and the overdrive sequences of the octopine-type Ti-plasmid pTi15955 were used to delimit the T-DNA region of the new binary Ti vector. The left border oligonucleotides (82 bp long), ended with *Mfe* I and *Xba* I, while the right border and overdrive oligonucleotides (85 bp long), ended with *Xba* I and *Sac* II. A multiple cloning site (MCS) for ten common restriction endonuclease sites (Left border-*Hind* III-*Nco* I-*Xho* I-*Kpn* I-*EcoR* I-*Bam* HI-*Pst* I-*Sca* I-*Xba* I-*Sac* I-right border) was introduced inside of the T-DNA borders. The sequences of LB-MCS and RB-MCS are as follows:

LB MCS 1 and 2:

5′ CAATT*GCAGGATATATTCAGTTGTAAAT* AAGCTT CCATGG CTCGAG GGTACC GAATTC GGATCC CTGCAG

3′ GTTAA*CGTCCTATATAAGTCAACATTTA* TTCGAA GGTACC GAGCTC CCATGG CTTAAG CCTAGG GACGTC

*Mfe*I ←LB repeat fr pTi15955→ *Hind*III *Nco*I *Xho*I *Kpn*I *Eco*RI *Bam*HI *Pst*I

AGTACT T 3′ : LB MCS1 (78 nt)

TCATGA AGATC 5′ : LB MCS2 (82 nt)

*Sca*I *Xba*I

RB MCS 1 and 2:

5′ CTAGA GAGCTC*TGGCAGGATATATGCGGTTGTAATT*CATTTTTA*TTGTCTAAATTTCTGTATTTGTTTGTTTG*CAATTG

3′ T CTCGAG*ACCGTCCTATATACGCCAACATTAA*GTAAAAATAACAGATTTAAAGACATAAACAAACAAAC GTTAAC

*Xba*I *Sac*I ←RB repeat fr pTi15955→ ←Overdrive from pTi15955→ *Mfe*I

CCGCGG 3′ : RB MCS1 (85 nt)

GGCGCC 5′ : RB MCS2 (81 nt)

*Sac*II

The first pair LB MCS1 and 2 were annealed *in vitro* and ligated into the *Eco* RV and *Xba* I site of pBluescript KS(-), resulting in pLBMCS (Supplementary Figure B6). The second

pair RB MCS1 and 2 were annealed *in vitro* and ligated into *Xba* I and *Eco* RV sites of pBluescript KS(-), producing pRBMCS. The right border and overdrive region of pRBMCS were digested between the *Xba* I and *Sac* II sites and inserted into pLBMCS producing 3.0 kb of pTDNA (Supplementary Figure B6). The *Mfe* I site just outside of the left border was found inactivated. To re-constitute the *Mfe* I site by PCR, I designed two new primers, TDNA1-revised and TDNA2-revised (Supplementary Figure B6). The primer TDNA1-revised is 5'-gatccgcggcaattgcaaaca-3', corresponding to the sequence from 656 to 677 nt of pTDNA and contains *Mfe* I and *Sac* II site at the ends. The primer TDNA2-revised is 5'-gatatcgatcttgatcaattggcagga-3', corresponding from 837 to 810 nt and contains *Mfe* I site, a half of *Eco* RV and *Hind* III sites, and *Cla* I site at the terminal. This 182 bp PCR product, *Cla* I-LB-MCS-RB-overdrive-*Mfe* I-*Sac* II fragment was digested with *Cla* I and *Sac* II and inserted into the *Sac* II/*Cla* I sites of pBluescriptII KS(+) producing pKSLR (Supplementary Figure B8).

2.2.6. New Plant Selection Markers, Kanamycin and Hygromycin Resistance Genes

Hygromycin B phosphotransferase (*hph*) gene and *neomycin phosphotransferase* II (*npt*II) gene were isolated from pTRA151 and pSMGtml (Zheng et al., 1991) (Supplementary Figure B7). The *Nco* I, *Sca* I, *Nhe* I sites in pTRA151 and *Pst* I, *Sph* I, *Nco* I, *Nhe* I sites in pSMGtml were inactivated by single point mutation without changing the amino acid codons using Multi Site-Directed Mutagenesis Kit (Stratagene). On pTRA151, the primer TRANco is 5'-cggagGC**G**atggatgcga-3' : Alanine codon is capital letters, boxed capital letter G is the introduced single point mutation, and inactivated *Nco* I site is underlined. The primer TRASca is 5'-tgcgtagaaGT**T**ctcgccgatagtgg-3' : Valine codon is capital letters, boxed capital letter T is the single point mutation, and inactivated *Sca* I site is underlined. The primer TRANhe is 5'-agtgttctaagc**GG**gcctggtaataatctatacgagc-3' : boxed capital letters GG are the introduced point mutations, and inactivated *Nhe* I site is underlined. The multi-site mutation produced 4,413 bp of

pTRAΔNSN (Supplementary Figure B7). Four other primers were designed for pSMGtml to produce 4,268 bp of pSMGΔPSNN. The primer SMGPst is 5'-ctgaatgaaCT**C**caggacgaggcagc-3' : Leucine codon is capital letters, boxed capital letter C is the introduced point mutations, and inactivated *Pst* I site is underlined). The primer SMGSph is 5'-caaggcgCG**G**atgcccgacg-3' : Arginine codon is capital letters, boxed capital letter G is the introduced point mutations, and inactivated *Sph* I site is underlined. The primer SMGNco is 5'-cgtcgtgAC**A**catggcgatgcctgc-3' : Threonine codon is capital letters, boxed capital letter A is the introduced point mutations, and inactivated *Nco* I site is underlined. The primer SMGNhe is 5'-agtgttctaagc**GG**gcctggtaataatctatacgagc-3' : boxed capital letters GG are the introduced point mutations, and inactivated *Nhe* I site is underlined.

The modified *hph* coding region was amplified from pTRAΔNSN by PCR using two primers hphBgl-F2 (5'-ctctagaggatccc**AgATCT**caatgagatatgaaaaagcctgaactcacc-3'), binding to pTRAΔNSN between 1,809 and 1,760 nt, and hphBgl-R2 (5'-ggatccggtcg**AG**atctactctattccttttgccctc-3'), binding between 735 and 770 nt of pTRAΔNSN. Both primers have misplaced bases (capital letters) to introduce *Bgl* II sites (underlined) at both ends of the amplified *hph* gene (1,044 bp). To amplify the 815 bp fragment of *npt*II coding region from pSMGΔPSNN, primers NPT2Bgl-F1 and NPT2Bgl-R1 were designed. Primer NPT2Bgl-F1 (5'-tgccc**A**gatc**T**tcgcatgattg-3') binds to pSMGΔPSNN between 529 and 550 nt, and has two misplaced bases to introduce *Bgl* II site and inactivate *Bam* HI site. Primer NPT2Bgl-R1 (5'-ggtcatttcga**GAT**c**TG**gagtcccgctc-3') binds between 1,365 and 1,338 nt, and introduces *Bgl* II site at the end of *npt*II gene (Supplementary Figure B7).

The coding regions of *hph* and *npt*II genes were placed under the transcriptional control of promoter and terminator region of *tumor morphology large* (*tml*) gene of *A. tumefaciens* pTi15955. To introduce these promoter and terminator regions into the new binary vector,

pTRA151 was amplified by two primers TmlBcl-F2 and TmlBcl-R2, and self-ligated on *Bcl* I site producing 3,268 bp of pTml (Supplementary Figure B7). Primer TmlBcl-F2 (5'-tccgcat**G**at**CA**ccgtcgccgacacctaataaagtc-3') binds to pSMGΔPSNN from 1,528 to 1,563 nt and has three misplaced bases (capital letters) to introduce *Bcl* I sites (underlined) at the end of tml promoter region. Primer TmlBcl-R2 (5'-tgttcaatcatgcga**T**gatc**A**gggcagtgttggatgtactac-3') binds between 554 and 513 nt of pSMGΔPSNN, and introduces a *Bcl* I site (underlined) while inactivating the *Bam* HI site at the beginning of *tml* terminator region. The amplified *hph* gene and *npt*II gene has *Bgl* II sites on both ends of the fragments which has compatible cohesive ends with *Bcl* I. The introduction of *hph* and *npt*II coding regions in pTml produces 4,312 bp of pTmlH and 4,083 bp of pTmlN2, respectively (Supplementary Figure B7).

The *hph* gene and *npt*II gene with the *tml* promoter and terminator were amplified using the same two primers and introduced into *Hind* III site of multi-cloning site adjacent to the left border of T-DNA (Supplementary Figure B8). The *hph* gene was amplified from pTmlH with primers tmlP-H3II and tmlT-H3II. These primers have *Hind* III sites in the primer sequences and the amplified 1,613 bp of fragment was ligated into *Hind* III site in pKSLR. Primer tmlP-H3II is 5'-agctcggtaccc**A**ag**CT**ttatgcagcatttttttggtgtatg-3' (capital letters are misplaced bases), binding to pTmlH from 237 to 278 nt, and introducing *Hind* III site (underlined) at the 5'-end of *tml* promoter. Primer tmlT-H3II is 5'-ccgggtacc**A**agct**T**g**C**tcgtggtgatattaaagagag-3' and binds between 1,876 and 1,839 nt of pTmlH. This primer has three misplaced bases (capital letters) to introduce *Hind* III site (underlined) and to inactivate *Sac* I site at the end of *tml* terminator region. The resulting plasmid from this amplification is 4,679 bp of pLRH. The *npt*II gene was amplified using the same primers, the amplified fragment ligated to *Hind* III site of pKSLR, resulting in 4,450 bp pLRN2.

The *Hind* III site at the end of *tml* terminator region was selected as the insertion site of *β-glucuronidase* gene and thus the other *Hind* III site at the beginning of *tml* promoter region was removed (Supplementary Figure B8). Primers LRSphF1 and LRSphR1 were designed to inactivate the promoter-side *Hind* III and to introduce new *Eco* RV and *Sph* I sites in the multi-cloning site of border sequence. The plasmid pLRH and pLRN2 were amplified by inverted PCR with LRSphF1 and LRSphR1 and then recircularized by self-ligation at the newly synthesized *Sph* I site. The 49 nt primer to amplify the antisense sequence of pLRH template is LRSphF1 (5'-aaggggaaaaggggGCATGCGATATCatttacaactgaatatatcctgc-3'), and binds between 2,407 and 2,430 nt. The new *Eco* RV and *Sph* I sites (bold capital letters) were designed in the unbound 5' end portion (underlined) of the primer. The other 45 nt primer LRSphR1 (5'-ggaaaaggggGCATGCtatgcagcatttttttggtgtatgtgggc-3') binds to the pLRH template between 2,401 and 2,373 nt. Like the primer LRSphF1, the new *Sph* I site (bold capital letters) was designed in the unbound 5' end portion (underlined) of the primer. The amplified product was digested with *Sp*h I and then self-ligated resulting in 4,685 bp of pLRHS (Supplementary Figure B8). The same primers were used for the removal of the excessive *Hind* III site at the 5'-end of *tml* promoter and the introduction of new *Eco* RV and *Sph* I sites in the MCS of pLRN2. After self-ligation at the new *Sph* I site, 4,456 bp of pLRN2S was produced.

The T-DNA border sequences harboring the *hph* gene in pLRHS was amplified with primers KSRLF1 and KSRLR1 (Supplementary Figure B9). The primer KSRLF1 is 5'-agctccacAgATCTaattgcaaac-3' (capital letters are misplaced bases), binding outside of overdrive sequence of T-DNA border and introduces a new *Bgl* II site (underlined) while inactivating the *Mfe* I and *Sac* I sites. The other primer KSRLR1 is 5'-cggtatcgatctAgatcTattggcagg-3' (capital letters are misplaced bases), binding right next to the left border repeat sequence. Primer KSRLR1 introduces another new *Bgl* II site (underlined) and

removes the *Mfe* I and *Bcl* I sites. The amplified product was then digested with *Bgl* II and the 1,779 bp of T-DNA border sequences harboring *hph* genes was ligated into the *Bam* HI site of binary vector pKCVS53. Due to the compatible cohesive ends between *Bgl* II and *Bam* HI sites, both enzyme sites became inactivated after ligation and the newly constructed plasmid was 6,152 bp of pCVH. The T-DNA borders harboring the *npt*II gene was amplified with the same primers from pLRN2. After digestion with *Bgl* II, the amplified 1,550 bp fragment was ligated with pKCVS53 producing 5,923 bp of pCVN2.

2.2.7. GUS Reporter Gene

The *β-glucuronidase* gene was amplified from pCambia1305.2 with primers 1305-1F2 and 1305-1R2. The 28 nt primer 1305-1F2 is 5'-tgagcttggat**A**ag**C**ttgtcgtttcccg-3' (capital letters are misplaced bases) which was designed to binds to pCambia1305.2 from 11,260 to 11,287 nt, just outside of CaMV35S promoter region. At the end of nopaline synthase polyA sequence, the 38 nt primer 1305-1R2 (5'-cgccaatata**AG**ct**T**tcaaacactgatagtttaattcc-3') binds from 2,371 to 2,334 nt of pCambia1305.2 (capital letters are misplaced bases). Both primers introduced new *Hind* III sites (underlined letters) at the ends of GUS cassette fragment including CaMV35S promoter, GUS gene with His6, glycine-rich protein signal peptide, catalase intron and nopaline terminator (Supplementary Figure B10). After *Hind* III digestion, the amplified 3,007 bp fragment was introduced into the expression vectors pCVH and pCVN2 at *Hind* III site next to *hph* or *npt*II gene producing 9,159 bp of pCVHG and 8,930 bp of pCVN2G (Supplementary Figure B10).

2.2.8. DNA Sequence Analysis

DNA sequences of two strands of the binary Ti vectors pLSU-4 were determined with an Applied Biosystems™ 3730xl DNA Analyzer at Eurofins MWG Operon (Huntsville, Alabama**).** Twenty six sequencing primers were designed as listed in Supplementary Table B2, and

synthesized by Sigma-Aldrich. The complete DNA sequence of binary vector skeleton pLSU-1 is submitted to the GenBank (Accession number HQ608521). The determined sequences were compared with the expected sequences using Blast. A sixteen bp-long CGCGCGGACAAGCTAG was inserted at the ligation site between the VS1 replicon and T-DNA region, and GATC were inserted at 1,721 bp, the ligation site between the ColE1 and VS1 replicon. In addition, a G was inserted at 3,008 bp in the RepA region. In hygromycin resistance gene, the nucleotides at 5,069 and 5,072 bp were referred as T (Zheng et al., 1991), but both nucleotides were found to be A.

2.2.9. Freeze-thaw Transformation of *A. tumefaciens*

Cells of *A. tumefaciens* LBA4404 strain were grown at 28°C in YEB broth (on a basis per L, 5 g sucrose, 5 g peptone, 5 g beef extract, 1 g yeast extract and 2 mM $MgSO_4$) because cells were less aggregated in this media. When the *Agrobacteria* aggregates during liquid cell culture, the harvested cells were very difficult to resuspend completely and the *Agrobacteria* transformation frequency was poor and non-consistant. Cells were prepared as described by Hofgen and Willmitzer (1988) and modified as following. Ten mililiter of overnight culture were mixed with 50 ml of fresh YEB media and incubated at 28^{o}C with 250 rpm, for six to seven hours until the A_{600} reached 0.5. After cooling on ice for 30 min, cells were harvested by centrifugation at 3,000 *g* for 20 min at 4°C. The Cell pellet was washed once in 30 ml of TE buffer and re-suspended in 1 ml of YEB media. One microgram of DNA was mixed with 100 µl of cells, and frozen in an ethanol bath at -80^{o}C 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.

2.2.10. Tobacco Leaf Disk Transformation Mediated by *A. tumefaciens*

A. tumefaciens-mediated transformation of tobacco leaf disc was performed according to Burow et al. (1990) as modified by Park (2006) and Su (2010).

2.2.11. T_1 Progeny Analysis

Three independent calli originated from a separate tobacco leaf disc were regenerated to mature plants for each binary vector. Flowers were self-pollinated by hand and intact seed pods were harvested and stored separately in microfuge tubes at -80°C. Seeds were planted at 100 seeds per plate on T-media (on a basis per liter, 0.1 g *myo*-inositol, 0.4 mg thiamine-HCl, 30.0 g sucrose, pH 5.6, 10 g agar, and 4.4 g Sigma MS salts (Murashige and Skoog, 1962)) containing 300 mg/L of kanamycin and grown at 25°C in an incubator under continuous illumination for four weeks.

2.2.12. Statistical Analysis

The results in Tables 1 to 6 were subjected to one-way ANOVA program of Statistical Analysis System (SAS) software, SAS Online ver. 9.2 from SAS Institute (Cary, NC). Means were compared using Fisher's least significant difference (LSD) test (α=0.05). Table 7 was subjected to χ^2 test to determine the difference between the theoretical ratio and experimental ratio (P=0.05).

2.3. Results

I constructed a series of binary vectors pLSU to reduce the plasmid size to 4,543 bp and to increase the cloning efficiency and plasmid yield in *E. coli* and *A. tumefaciens*. I tested the minimal requirements for the components of plasmid replication origin and the antibiotic resistance genes in *E. coli* and *A. tumefaciens*. The overall scheme of the construction of the kanamycin-based binary Ti vector is illustrated in Supplementary Figure B1.

2.3.1. ColE1 Replicon, Size Reduction and Higher Copy Number Mutations

I reduced by 416 bp the size of ColE1 from 1,131 to 715 bp and introduced mutations to increase the copy number presumably by three fold. The 715 bp region of ColE1 replicon from pUC19 was chosen as a starting replicon in *E. coli* for the new binary Ti vectors. The pUC19 ColE1 replicon was preferred rather than the pBR322 ColE1 replicon as in pPZP or pCambia plasmids since the pUC19 ColE1 replicon has a new single point mutation (g1308**A**) at the 5' end of RNA I coding region, resulting in the three folds increase in the plasmid copy number compared to the pBR322 ColE1 replicon (Chambers et al., 1988; Yanisch-Perron et al., 1985). A second new single point mutation (g1428**A**) was introduced in -35 element TTGAGA of RNA II transcript to increase further up to three folds the plasmid copy number (Castagnoli, 1987; Castagnoli et al., 1985; Cesareni, 1982). The pPZP and pCambia plasmids contain the 1,131 bp ColE1 replicon from pBR322 (122 to 2,534 bp) and the *bom* site for mobilization by triparental mating. The *bom* site was eliminated to reduce the plasmid size and because *A. tumefaciens* can be transformed effectively by electroporation or freeze-thaw procedures.

The 5'- and 3'-sides of elements in the ColE1 replicon were essential for stable maintenance through ten consecutive liquid subcultures (Supplementary Figures B3 and B12). At the 5'- side of ColE1 replicon RNA I and RNA II transcripts were absolutely required for the plasmid replication as expected. Truncation of the RNA I transcript resulted in less stable replication only after five consecutive growths cycles. The extracted plasmid with truncated RNA I transcript showed significantly reduced yield on gel compared to the plasmid yield extracted before five consecutive liquid subcultures. At the 3'-side of elements, it was tested whether to include the dnaA and dnaA' boxes, or primosome assembly sites (L-*pas* and H-*pas*). Both primosome assembly sites for the lagging strand and the leading strand were not necessary for stable maintenance (Supplementary Figures B3 and B12). I concluded the minimum length

of ColE1 replicon for stable replication in *E. coli* includes RNA I/RNA II transcripts on 5'-side and dnaA/dnaA' boxes on the 3'-side.

The stability of the truncated ColE1 replicon of 715 bp was tested by plasmid maintenance experiment as shown in the Supplementary Figure B12. All four plasmids maintained stably in the presence of two antibiotics during total 140 hrs of consecutive liquid subcultures and overall, the pKCVS53 constructs had shown better growth than pCambia1301 or pUC19. After 140 hrs of consecutive liquid subcultures, the plasmid DNA was extracted by alkaline lysis miniprep and cut with restriction endonuclease. The correct sizes and DNA yields of the plasmid DNA were confirmed on agarose gel electrophoresis compared to the plasmid DNA extracted from the conolies of the initial subculture. The plasmid DNA yields have not been changed during 140 hrs of consecutive liquid subcultures.

2.3.2. Bacterial Kanamycin Resistance *npt*I Gene

The *neomycin phosphotrasferase* I gene (*npt*I) from Tn903 (pUC4-KSAC) was chosen for the bacterial kanamycin resistance because the *npt*II gene from Tn5 was used for the plant kanamycin selection marker. A third *npt*III gene from *Streptoccocus faecalis* was used for pBin19 and its Ti binary vector derivatives (Bevan 1984). There was no sequence similarity among these three *npt* genes based on BLAST analysis. Four common restriction endonuclease sites for *Xba* I, *Sma* I, *Hind* III, and *Cla* I were eliminated from the *npt*I coding region by site-directed mutagenesis. Based on the sequence analysis of the *npt*I gene, the minimal size of the *npt*I gene was deduced to extend from the 36 bp upstream of the -35 promoter elements, to the 3'-end following the second stem-loop structure presumably acting as a transcription termination signal (Supplementary Figure B2). The new truncated *npt*I gene of 999 bp contains 816 bp of the coding region, 109 bp of 5' upstream region, and 74 bp downstream region. This truncated *npt*I

gene confers the kanamycin resistance up to 1,500 mg/L as effective as the wild-type gene (Supplementary Figure B11).

2.3.3. Broad Host-range Replicon VS1 and Size Reduction

I reduced the size of VS1 replicon from 3.8 kb fragment to the 2,660 bp fragment consisting of 126 bp of 5' upstream sequence, 630 bp of the *sta*A coding region, 50 bp of 5'-sequence, 216 bp of the ORF3 coding region, 158 bp of 5'-sequence, 1,107 bp of *rep*A coding region, 65 bp of 5'-sequence, 195 bp of *ori*V sequemce (5'-dnaA box, direct repeats, A/T-rich sequence, and 3'-dnaA box), and 142 bp of 3' downstream sequence. The binary Ti vectors with the truncated VS1 replicon of 2,660 bp were stably maintained with more than 98 % efficiency in *A. tumefaciens* without antibiotic selection for four consecutive liquid subcultures (Table 1). The stability of the binary vector pLSU was as good as or better than pCambia vector which has a longer 3.8 kb VS1 replicon. An alanine in *rep*A ORF replaced with valine at 4,659 bp (c4659t) was reported to increase the plasmid DNA copy number by two to three folds in *P. fluorescens* as in plasmid pME294 and pME6012 (Heeb et al., 2000; Itoh et al., 1988). I introduced this amino acid replacement presumably to increase the copy number of new binary Ti vector in *A. tumefaciens* (c4659t; Supplementary Figure B5). The VS1 replicon was further reduced by eliminating the 109 bp 3'-downstream region of *ori*V. However, this construct reduced the transformation frequency in *A. tumefaciens* in less than one half compared to the other binary vectors containing the original size 3.8 kb of the STA/REP replicon in pBRVS1, pBRVS2, or pCambia1305.2. The *Cla* I site in the *rep*A gene was also inactivated. However, the *Cla* I-inactivated binary vector did not produce any colony after transformation of *A. tumefaciens*, while the intact *Cla* I site-containing vectors yielded abundant colonies. The *Cla* I inactivation reaction (atcgat→at**T**gat) should maintain the same amino acid codon (underlined), but might have altered the secondary structure of RNA transcript or introduced additional mutation(s).

Table 1. Binary vectors pLSU was stably maintained in *A. tumefaciens* during four day-long consecutive liquid subcultures without antibiotics selection. After first and fourth liquid subcultures, *Agrobacteria* were grown on solid A media without antibiotics selection for three days, respectively. Then, from each plate, 150 colonies were picked and transferred to solid A media under selection of kanamycin 100 μg/ml. The colony numbers resistant to kanamycin were shown on the table. The percentage of kanamycin-sensitive colonies on the table indicates the replica colonies lost their binary vectors during four consecutive liquid subcultures. Standard deviations are in parentheses.

A. Initial screening

Binary vectors	Percent loss of kanamycin resistance (150 colonies per plate)			
	#1	#2	#3	Average
pLSU-4	150 0 %	150 0 %	149 0.67 %	149.67 (0.58) 0.22 %
pCambia1305.2	149 0.67 %	149 0.67 %	148 1.33 %	148.67 (0.58) 0.83 %

B. After four consecutive growth cycles in liquid subcultures

Binary vectors	Percent loss of selection marker (150 colonies per plate)			
	#1	#2	#3	Average
pLSU-4	145 3.33 %	149 0.67%	148 1.33 %	147.33 (2.08) 1.78 %
pCambia1305.2	144 4 %	147 2 %	146 2.67 %	145.67 (1.53) 2.89 %

2.3.4. Transcriptional Directions of the ColE1 and VS1 Replicons

The broad host-range VS1 replicon (2,660 bp) contains three transcription units of *sta*A, *rep*A and VS1 replicon and the three genes transcribe in the same direction. It was examined whether the transcriptional direction of ColE1 replicon (RNA II) in relation to that of VS1 replicon would affect the transformation efficiency of the combined replicons in *E. coli* and *A. tumefaciens*. The direction of transcription of two replicons can be the same (co-directional transcription), or the opposite (head-on transcription). In the widely-used binary vectors pPZP and pCambia, the transcriptional directions of ColE1 and VS1 replicons are opposite (head-on transcription). The effect of transcriptional direction was tested after introduction of T-DNA border sequences, plant selection markers, and GUS reporter gene into the pLSU vectors. In *E. coli*, the binary vectors with the co-directional transcriptional replicons yielded two to four-fold higher transformation frequency than the construct with the head-on trascriptional replicons as described in Table 2. In *A. tumefaciens*, the effect of co-directional transcription was positive in 1.2 to 1.7-fold higher transformation frequency than head-on direction, although it was not as prominent as in *E. coli* (Table 3). These results indicates that the same transcriptional direction of broad host range replicon enhanced the plasmid replication efficiency of ColE1 replicon more profoundly in *E. coli* than in *A. tumefaciens*. DNA yields of the new vectors were three to five-folds greater than pCambia vector in *E. coli*, even though the effect of transcriptional direction was not observed (Table 4).

The level of kanamycin resistance conferred by the *npt*I gene was not influenced by the difference of transcriptional direction. There was little difference in colony numbers among the transformants plated on LB media containing kanamycin 50, 100, 200, 400, 800, 1600, and 2000 mg/L, whether the transcriptional direction of the VS1 and ColE1 is same or opposite (Supplementary Figure B11).

Table 2. Transcriptional directions between ColE1 and VS1 replicons were compared in transformation frequency of *E. coli* strain XL2Blue. pLSU4-C and 4-H represent Co-directional or Head-on transcriptional orientation of ColE1 and VS1 replicons, respectively. The bacteria were transformed per 100 pg of plasmid DNA (three independent transformations per plasmid, respectively). The direction of transcription of two replicons can be the same (co-directional transcription), or the opposite (head-on transcription). In pCambia the transcriptional directions of ColE1 and VS1 replicons are opposite (head-on transcription). The effect of transcriptional direction was tested after introduction of T-DNA border sequences, plant selection markers, and GUS reporter gene into the plasmid pKCVS53. Standard deviations are in parentheses. Superscript alphabets indicate significantly different values that were determined by the *t* test ($P<0.05$).

Plasmid	T-DNA	Transcriptional direction between ColE1/VS1 replicon	Average colony per 100 pg DNA (N=3)	Ratio (Co-directonal/ Head-on)	Ratio to pCambia control
pLSU4-C	Hyg^R	Co-directional	397 (28) [a]	2.9	4.1
pLSU4-H		Head-on	136 (2) [c]		1.4
pLSU5-C	Hyg^R + GUS	Co-directional	174 (14) [b]	2.9	1.8
pLSU5-H		Head-on	60 (14) [e]		0.6
pCambia1305.2		Head-on	97 (2) [d]	-	-
pLSU2-C	Kan^R	Co-directional	282 (16) [a]	1.7	3.2
pLSU2-H		Head-on	165 (7) [c]		1.9
pLSU3-C	Kan^R + GUS	Co-directional	198 (8) [b]	3.9	2.2
pLSU3-H		Head-on	51 (9) [e]		0.6
pCambia2301		Head-on	89 (20) [d]	-	-

Table 3. Transcriptional directions between ColE1 and VS1 replicons were compared in transformation frequency of *A. tumefaciens* strain LBA4404. pLSU4-C and 4-H represent Co-directional or Head-on transcriptional orientation of ColE1 and VS1 replicons, respectively. The bacteria were transformed per one microgram of plasmid DNA (five independent transformations per plasmid, respectively). The direction of transcription of two replicons can be the same (co-directional transcription), or the opposite (head-on transcription). In pCambia the transcriptional directions of ColE1 and VS1 replicons are opposite (head-on transcription). The effect of transcriptional direction was tested after introduction of T-DNA border sequences, plant selection markers, and GUS reporter gene into the plasmid pKCVS53. Standard deviations are in parentheses. Superscript alphabets indicate significantly different values that were determined by the *t* test ($P<0.05$).

Plasmid	T-DNA	Transcriptional direction between ColE1/VS1 replicon	Average colony per 1 μg DNA (N=5)	Ratio (Co-directonal/ Head-on)	Ratio to pCambia control
pLSU4-C	Hyg^R	Co-directional	2042 (113) [a]	1.3	12.5
pLSU4-H		Head-on	1540 (157) [b]		9.4
pLSU5-C	Hyg^R + GUS	Co-directional	930 (41) [c]	1.4	5.7
pLSU5-H		Head-on	670 (21) [d]		4.1
pCambia1305.2		Head-on	164 (35) [e]	-	-
pLSU2-C	Kan^R	Co-directional	7317 (279) [a]	1.8	24.3
pLSU2-H		Head-on	4189 (195) [b]		13.9
pLSU3-C	Kan^R + GUS	Co-directional	1269 (109) [c]	1.2	4.2
pLSU3-H		Head-on	1049 (129) [c]		3.5
pCambia2301		Head-on	301 (69) [d]	-	-

Table 4. Transcriptional directions between ColE1 and VS1 replicons were compared in plasmid yield of *E. coli* strain XL2Blue. pLSU-4C and -4H represent Co-directional or Head-on transcriptional orientation of ColE1 and VS1 replicons, respectively. The direction of transcription of two replicons can be the same (co-directional transcription), or the opposite (head-on transcription). In pCambia the transcriptional directions of ColE1 and VS1 replicons are opposite (head-on transcription). The bacteria were transformed and three single colonies of each plasmid constructs were grown for 16 hrs in 25 ml LB media with kanamycin 50 μg/ml. Plasmid DNA was extracted by QIA Midiprep kit. DNA yields represent the average of three independent samples. Standard deviations are in parentheses.

Plasmid	Transcriptional direction between ColE1/VS1 replicon	Average DNA yield (μg) (N=3)	Ratio to pCambia control
pLSU2-C (5.9 kb)	Co-directional	68 (22) [b]	2.7
pLSU2-H (5.9 kb)	Head-on	81 (6) [b]	3.2
pLSU3-C (8.9 kb)	Co-directional	77 (33) [b]	3.0
pLSU3-H (8.9 kb)	Head-on	114 (28) [a]	4.5
pCambia2301 (11.6 kb)	Head-on	25 (2) [c]	-

2.3.5. New T-DNA Borders and Plant Selection Markers

A new T-DNA region was delimited by the right (with the overdrive) and left border sequences from the octopine-type pTi15955. The T-DNA region contains a multi-cloning site for 12 common restriction endonuclease sites (Left border-*Eco* RV-*Sph* I-*Hind* III-*Nco* I-*Xho* I-*Kpn* I-*Eco* RI-*Bam* HI-*Pst* I-*Sca* I-*Xba* I-*Sac* I-Right border-overdrive) (Figure 2). A plant selection marker, either hygromycin (*hph*) or kanamycin resistance gene (*npt*II), and/or β-Glucuronidase gene (*gus*) were placed adjacent to the left border sequence to ensure that after selection, transformed cells would contain the full length of T-DNA from the right to left boarder. Both *npt*II and *hph* coding regions were placed in between *Sph* I and *Hind* III restriction sites under transcriptional control of the 285 bp promoter and 284 bp terminator regions of *tm*l (tumor morphology welarge) gene from pTi15955 (Supplementary Figure B7). The GRP-GUS Plus-His6 cassette isolated from pCambia1305.2 was placed in *Hind* III restriction site (Supplementary Figure B10).

I constructed pLSU-1, 2, 3, 4, and 5 with the composition of the T-DNA region as illustrated in Figure 2. pLSU-1 is a basic vector skeleton with the 12 common restriction sites in T-DNA (Figure 1). pLSU-2 and pLSU-3 have the kanamycin resistance *npt*II gene as a plant selection marker. pLSU-4 and pLSU-5 contain the hygromycin resistance *hph* gene as a plant selection marker. pLSU-3 and pLSU-5 also have the *gus* reporter gene in addition to the plant selection marker (Figure 3).

The DNA sequence analysis of pLSU-4 indicated that all single point mutations introduced to ColE1 replicon, VS1 replicon, kanamycin resistance genes *npt*I and *npt*II, and the T-DNA region was confirmed as expected. However, I found 16 and 4 bp new insertions at two ligation sites as noted in Material and Methods. DNA sequence of pLSU-1 was deposited to Genbank at accession number HQ608521.

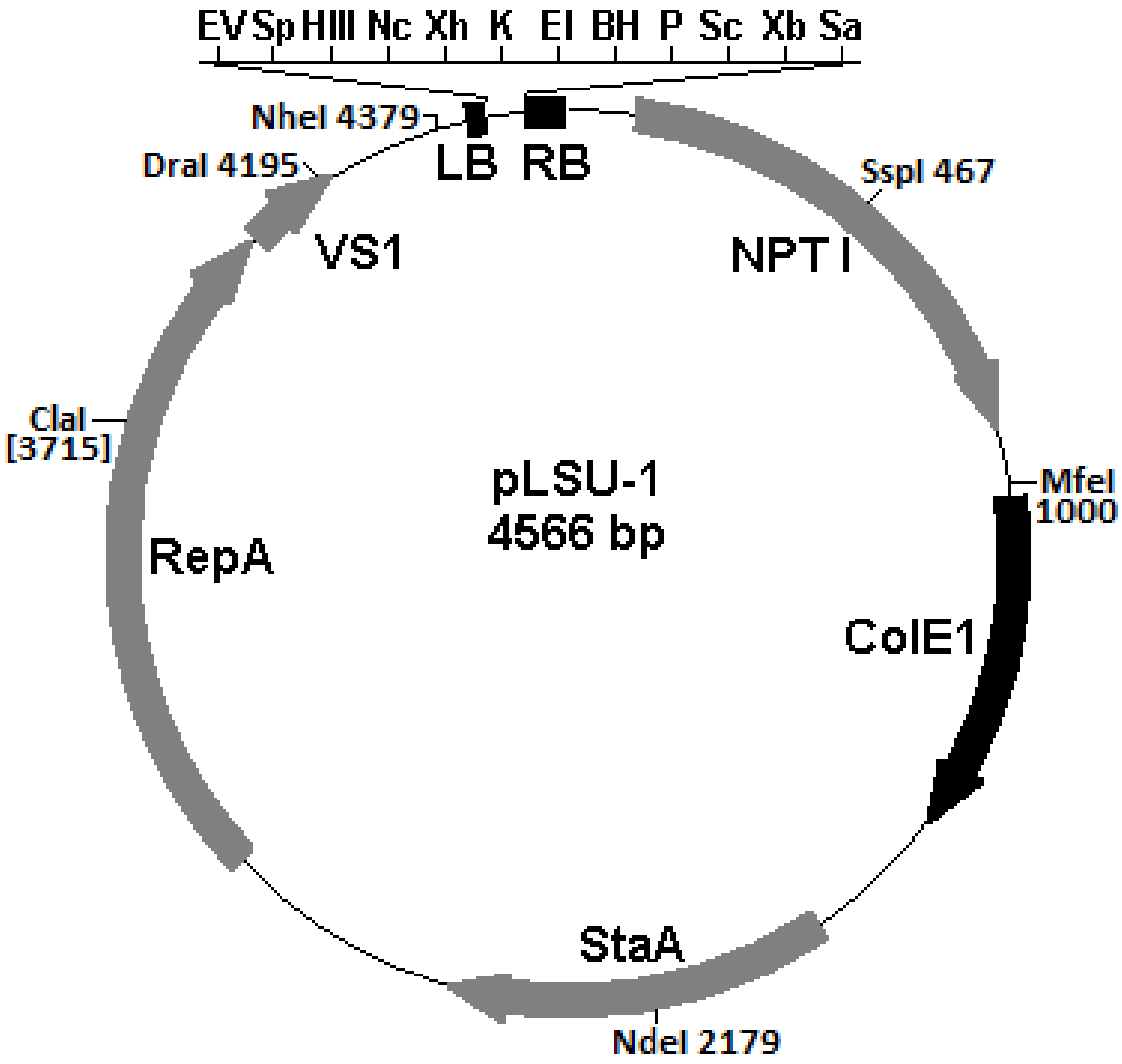

Figure 1. Schematic representation of backbone structure of binary Ti vector pLSU-1 (4,566 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *Eco* RV (EV), *Sph* I (Sp), *Hind* III (HIII), *Nco* I (Nc), *Xho* I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sac* I (Sa). The backbone plasmid include the *neomycin phosphotransferase* I (NPTI), ColE1 origin of replication (ColE1), Stability region A (StaA), Replication region A (RepA), and VS1 origin of replication (VS1). DNA sequence of pLSU-1 was deposited to Genbank at accession number HQ608521.

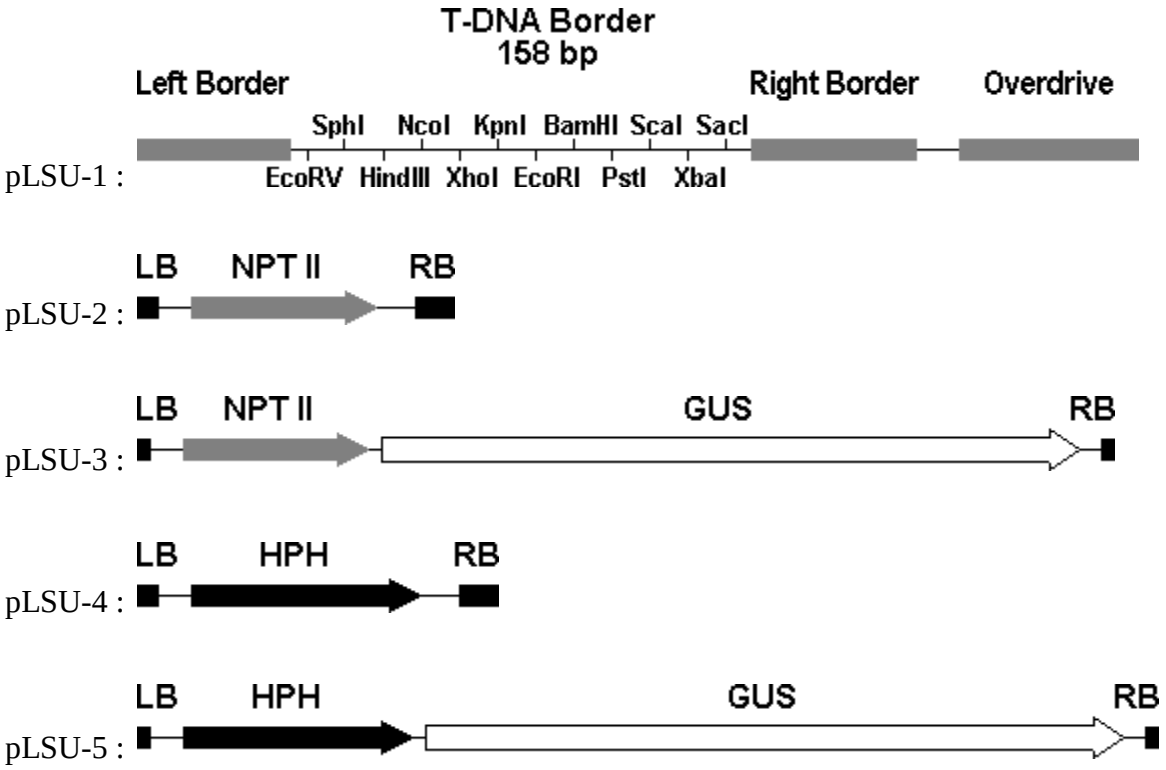

Figure 2. Schematic representation of the T-DNA region of binary Ti vectors pLSU-1 to -5. pLSU-1 is a basic skeleton vector with the twelve common restriction sites in T-DNA. pLSU-2 and pLSU-3 have the *neomycin phosphotransferase* II gene (NPTII) adjacent to the left border as a plant selection marker for kanamycin resistance. pLSU-4 and pLSU-5 contain the *hygromycin B phosphotransferasae* gene (HPH) adjacent to the left border as a plant selection marker for hygromycin resistance. pLSU-3 and pLSU-5 also include the *β-glucuronidase* reporter gene (GUS) in addition to the plant selection marker in the T-DNA. The restriction enzyme sites: *Mfe* I (1,000 bp), *Cla* I (3,715 bp)

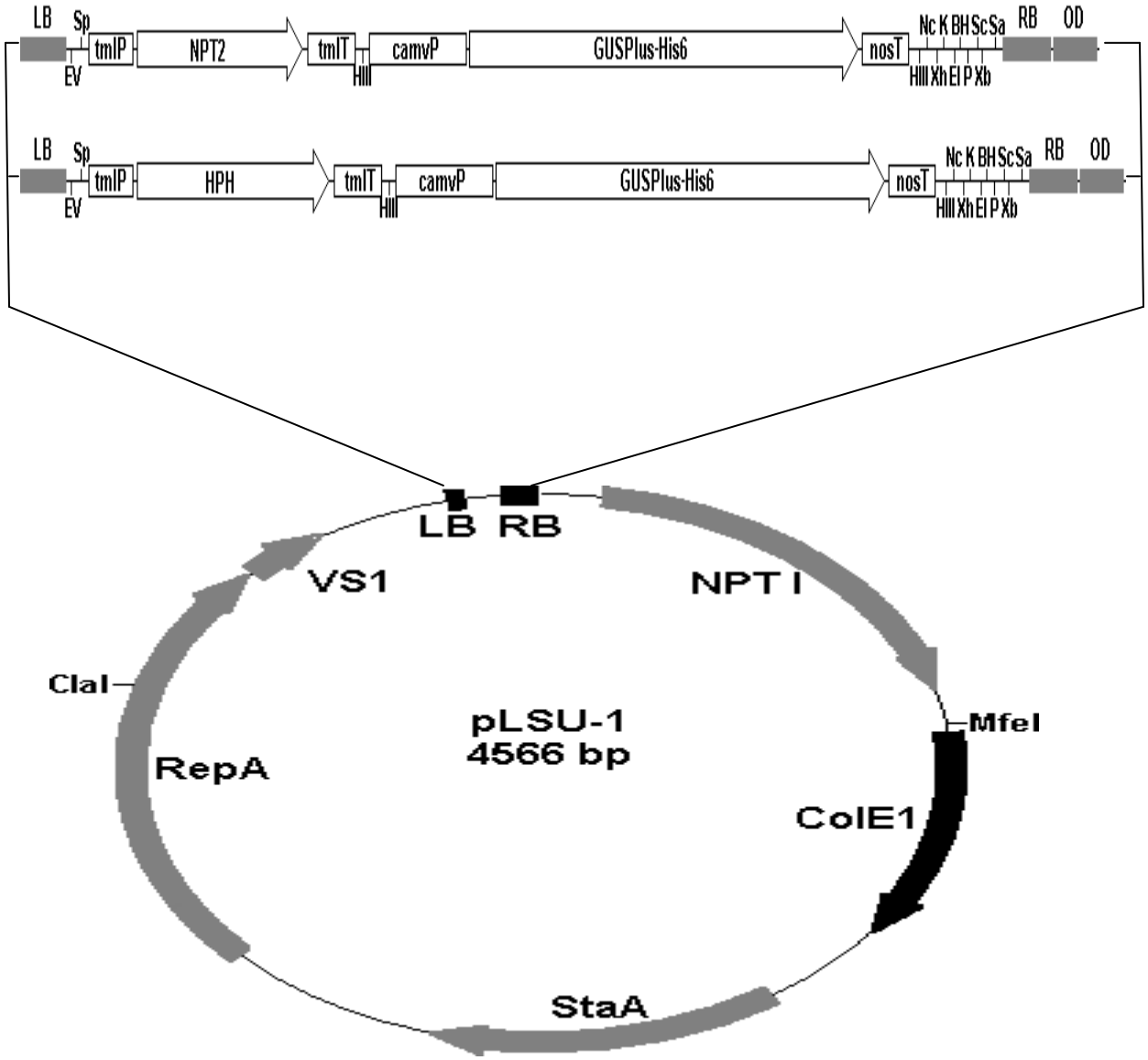

Figure 3. Detailed diagrams of the T-DNA regions of pLSU-3 and pLSU-5. Plant expression of the *npt*II (NPT2) and *hph* gene (HPH) are under transcriptional control of the promoter (tmlP) and terminator region (tmlT) of *tumor morphlogy large* (*tml*) gene from pTi15955. The GUS reporter fragment including CaMV35S promoter (camvP), GUS gene with His6 (GUSPlus-His6), and nopaline terminator (nosT) was isolated from pCambia1305.2. T-DNA left border (LB), right border (RB), overdrive (OD), *Eco* RV (EV), *Sph* I (Sp), *Hind* III (HIII), *Nco* I (Nc), *Xho* I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sac* I (Sa)

2.3.6. Tobacco Leaf Discs Assay and T_1 Progeny Analysis

The functional aspects of the new T-DNA borders and new plant selection marker genes were tested by tobacco transformation assay three times (Burow at al., 1990). Tobacco leaf discs were co-cultivated with *A. tumefaciens* for four days and transformed calli were selected for in the presence of 300 mg/L kanamycin or 100 mg/L hygromycin for four weeks. After four weeks, the fresh weights of old leaf discs were weighed. Although the fresh weight increases in tobacco leaf discs is putative but not direct evidence for plant transformation, it was demonstrated previously that the essentially almost all calli selected by 300 mg/L kanamycin were transformed based on DNA blot analysis of T-DNA and RNA and protein expression analyses of the transferred bean phaseolin gene (Burow et al., 1992). As shown in Tables 5 and 6, the fresh weight increases in leaf discs treated with our new binary vectors were higher than those with the positive control vectors. Higher number of leaf discs induced calli and the total fresh weight of leaf discs were larger than positive controls, although in hygromycin vectors including pCambia1305.2 did not performed well generally and the result was not as clear as kanamycin resistance vectors. Also, the standard deviation of each vectors were high because not all leaf disc induced calli and even for those leaf disc with calli, the numbers of calli induced and growth level of calli were very different.

Tobacco shoots were regenerated from kanamycin-resistant calli originated from independent transformed leaf discs. Genetic analysis of kanamycin resistance trait among T_1 progeny showed that the kanamycin resistance and sensitibity traits were segregated into the 3:1 ratio as described in Table 7. The results indicated that T-DNAs containing the kanamycin resistance gene were integrated stably into a locus or closely linked loci of the nuclear chromosomal DNA of the primary transgenic tobacco plants and inherited stably to the second generation.

Table 5. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens* LBA4404. pLSU-2C and pLSU-2H represent Co-directional or Head-on transcriptional orientation of ColE1 and VS1 replicons, respectively. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20^{o}C for 4 days, leaf disks were selected at 25^{o}C on shooting medium containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for an additional 2 weeks of selection. Co-cultivation was from 11/23/09 to 11/27, the first selection was 11/27 to 12/11 and the second selection was 12/11 to 12/25/2009. Each treatment has five plates with 10 leaf disks per plate. Negative control is from tobacco transformation without binary Ti vector. Numbers in parentheses indicate standard deviations. Superscript alphabets indicate significantly different values that were determined by the *t* test (P<0.05). The tobacco transformation experiment was repeated for three times.

pLSU binary Ti vectors	Average FW (g) of ten leaf discs per plate	Average FW (g) increased over negative control	% leaf discs with induced calli
Negative control	0.76 (0.07)	0	0
pCambia2301	8.48 (2.59)	7.68 (2.58) [a]	80
pLSU-2C	7.88 (2.65)	7.14 (2.65) [a]	66
pLSU-2H	7.74 (3.20)	7.00 (3.18) [a]	78
pLSU-3C	5.16 (2.66)	4.34 (2.66) [a]	92
pLSU-3H	8.12 (2.96)	7.33 (2.90) [a]	89

Table 6. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens* LBA4404. pLSU-4C and pLSU-4H represent Co-directional or Head-on transcriptional orientation of ColE1 and VS1 replicons, respectively. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20^{o}C for 4 days, leaf disks were selected at 25^{o}C on shooting medium containing 50 mg/L of hygromycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for an additional 2 weeks of selection. Co-cultivation was from 12/14/09 to 12/18/09, the first selection was 12/18/09 to 01/01/10 and the second selection was 01/01/10 to 01/15/2010. Each treatment has five plates with 10 leaf disks per plate. Negative control is from tobacco transformation without binary Ti vector. Numbers in parentheses indicate standard deviations. Superscript alphabets indicate significantly different values that were determined by the *t* test (P<0.05). The tobacco transformation experiment was repeated for three times.

pLSU binary Ti vectors	Average FW (g) of ten leaf discs	Average FW (g) increased over negative control	% leaf discs with induced calli
Negative control	1.08 (0.14)	0	0
pCambia1305.2	9.76 (2.17)	8.57 (2.19) [a]	98
pLSU-4C	4.54 (1.33)	3.33 (1.33) [b]	96
pLSU-4H	7.81 (2.70)	6.59 (2.69) [ab]	98
pLSU-5C	9.55 (3.09)	8.35 (3.05) [ab]	92
pLSU-5H	11.19 (3.89)	9.99 (3.88) [a]	100

Table 7. Genetic analysis of inheritance of the kanamycin resistance trait among T_1 progeny. Seeds were collected from three independent transgenic tobacco plants A, B, C, and planted on five MS media plates, respectively, under selection of kanamycin (300 μg/ml). The germinated seeds were grown at 25°C for four weeks under continuous illumination. Standard deviations are in parentheses. χ^2 test indicates the difference between values with asterisk mark are not significant and accepted (P=0.05).

T_1 tobacco plants		Number of kanamycin (300 μg/ml) resistant plants in T_1 generation		Segregation ration
		Resistant	Sensitive	
pLSU-3	A	79 (2.49)	26 (2.24)	3:1*
	B	74 (3.56)	28 (2.83)	3:1*
	C	75 (2.86)	25 (1.67)	3:1*
	Average	76 (3.5)	26.5 (2.4)	3:1
Wild type *N.t. Xanthi*		0	102	-

2.4. Discussion

I constructed smaller higher-yielding binary Ti vectors to increase the cloning efficiency and plasmid yield in *E. coli* and *A. tumefaciens*, and to improve the transformation efficiency of higher plants. To transfer a gene of interest to higher plants, I followed a three-step process from *E. coli* (to propagate/manipulate the gene) through *A. tumefaciens* to plant genomes. I constructed all four components of vectors, i.e. two plasmid replication regions, one for *E. coli* (ColE1 replicon) and another for *A. tumefaciens* (STA/REP replicon), a bacterial kanamycin resistance gene, and the T-DNA region which is transferred to plant genomes. Each component of the Ti vector was analyzed for the minimum size requirement and tested for the biological activities. I reduced the size of the basic skeleton of binary Ti vector pLSU-1 to 4,566 bp and introduced a number of mutations to increase the copy number and other functions.

One of the interesting observations in this study was the effect of orientation of two replicons on the transformation frequency of *E. coli* and *A. tumefaciens*. The transcriptional direction of STA/REP replicon can be the same as that of ColE1 replicon (co-directional transcription), or opposite (head-on transcription). The widely used conventional vectors (pPZP or pCambia) maintain the orientation of head-on transcription. New binary vectors with co-transcriptional orientation produced in *E. coli* up to four-fold higher transformation frequency than those with the head-on transcriptional orientation (Table 2). In *A. tumefaciens* the effect of co-transcriptional direction is still positive in 1.2 to 1.7-fold higher transformation frequency than that of head-on transcription although the effect is not as pronounced as in *E. coli* (Table 3). Transformation frequencies of the new vectors are more than ten-fold higher those of the conventional pCambia vector in *A. tumefaciens*. Plasmid DNA yields of the new binary Ti vectors pLSU were three- to four-fold higher than the conventional pCambia2301 even though the orientation of replicons did not affect DNA yield (Table 4). There are both theoretical and

practical implications of these observations. The STA/REP replicon was originally isolated from *P. aeruginosa* and found to replicate in a wide-range of gram-negative bacteria including other *Pseudomonas* species (*P. syringae, P. putida*) as well as in *A. tumefaciens* and *Rhizobium leguminosarun*, but not in *E. coli*. The results from these experiments suggest the STA/REP replicon may be functional in *E. coli* when it is combined in a proper orientation with the ColE1 replicon. Practically, new vectors are now much easier to propagate and manipulate in *E. coli* and *A. tumefaciens* than older commercial vectors.

The co-directional transcription of ColE1 and VS1 replicons resulted in higher transformation frequency of *E. co*li than the head-on transcription of the two replicons. This finding is in agreement with the previous studies on the interaction of replication and transcription. Mechanistic details of interplay between the replication and transcription machineries were studied *in vivo* by using two-dimensional agarose gel electrophoresis which resolves different sizes and shapes of replication intermediates on *E. coli* plasmids generating so-called bubble arc (Martin-Parras et al., 1991). The replication fork progression from ColE1 replicon was not affected by transcription proceeding through seven tRNA genes in the co-directional orientation, but is attenuated severely in the head-on collision orientation (Mirkin and Mirkin, 2005). It appears likely that the replisome progression is interfered by direct physical contact with the head-on RNA polymerase machinery. However, after temporally stalled by the head-on RNA polymerase the replication fork appeared not collapsing from plasmid DNA, staying highly stable, and eventually resuming replication and replacing the RNA polymerase from DNA (Pomerantz and O'Donnell, 2010). Replication fork movement was studied by electron microscope when the ColE1 replicon was inserted on either side of a highly transcribed ribosomal operon *rrnB* in the *E. coli* chromosome. The rate of replication fork movement was faster when replication and transcription were in co-directional orientation than when they were

in opposite orientation (French, 1992). In the genome of *Bacillus subtilis*, transcription of 75 % of genes is co-directionally oriented with replication, and the co-orientation appeared to reduce adverse effects of transcription on replication (Wang et al., 2007). In *E. coli*, when the direction of DNA polymerase on the replication folk is aligned to the co-directional direction of RNA polymerase in transcription, the speed of replication progress is 20 folds higher than when the DNA polymerase and RNA polymerase are in the head-on direction (Mirkin and Mirkin, 2005). About 55 % of protein-coding genes and 62 % of tRNA genes in genome are transcribed co-directionally to the DNA replication folk direction. Especially, all seven ribosomal operons, which are essential genes for protein production, face all co-directionally to the replication folk.

Here, I have reduced the size of the Sta/Rep region of the broad host-range replicon VS1 from 3.8 kb fragment to the 2,660 bp fragment and demonstrated the 98 % stability of binary vector with this replicon in *A. tumefaciens*. The 8 kb region of the VS1 replicon in pGV910 was shown to maintain stably in *A. tumefaciens* (Van den Eede et al., 1992). The binary Ti vectors pPZP and pCambia contain the 3,770 bp fragment of VS1 broad host-range replication origin and stability region. The origin of replication of pVS1 was defined to a 3.1 kb region for *sta*A, ORF3, *rep*A and *ori*V by sequence analysis and maintenance assay in *P. fluorescens* (Heeb et al., 2000). I have also reduced the size of ColE1 replicon to the 715 bp fragment and demonstrated the stability of binary vector with this replicon in *E. coli*. It was able to reduce to 4,543 bp the final size of skeleton of new binary vectors, to increase the plasmid yield in *E. coli*, and the transformation frequency in *E. coli* and *A. tumefaciens*.

CHAPTER 3. BINARY PLANT TRANSFORMATION VECTOR pLSU-TET

3.1. Introduction

Bacteriophage λ relies on the site-specific recombination reaction to integrate the phage DNA by the BP clonase into the bacterial chromosome and to excise it out by the LR clonase (Landy, 1989). 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 (Hartley et al., 2000). 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 in between two *att*B sites. Succession of four Gateway vectors, donor, entry, destination, and expression vectors are 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 (Landy, 1989). 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 *att*R (Cheo et al., 2004). Thus, while *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 (Karimi et al., 2005 and 2007a). 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 (Karimi et al., 2007b).

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. 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 (Karimi et al., 2007a). Becasue the Gateway entry vectors carry the kanamycin-resistance gene for bacterial selection, the pPZP-based destination vectors with the streptomycin/spectinomycin resistance gene (Karimi et al., 2002, 2006, 2007a, 2007b) were preferred over the pCambia-based vectors with the kanamycin resistance gene (Curtis and Grossniklaus, 2003; Earley et al., 2006). However, the pPZP-based destination vectors are not suited for transformation of *A. tumefaciens* since the most commonly used strain LBA4404 of *A. tumefaciens* contains the avirulent Ti plasmid with the streptomycin-resistance marker. Thus, the introduction of pPZP-based destination vectors to *A. tumefaciens* is not assured with simple antibiotic selection, and needs additional steps to circumvent the difficulty. The other widely used *A. tumefaciens* strain, EHA101 has kanamycin resistance gene in the avirulent Ti plasmid, which is another inconvenience for using kanamycin-resistant destination vector. 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 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 transient expression of the *β-glucuronidase* gene.

3.2. Materials and Methods

3.2.1. Chemicals, Enzymes, Bacterial Strains, and Plasmid DNA

3.2.1.1. 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. The DeoxyribonucleaseII, restriction endonucleases (*Bam* HI, *Bcl* I, *Bgl* II, *Bsr* GI, *Bst* EI, *Cla* I, *Eco* RI, *Eco* RV, *Hind* III, *Kpn* I, *Mfe* I, *Nhe* I, *Pst* I, *Sac* II, *Xba* I, and *Xho* I), Deep Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolab and/or Bethesda Research Laboratory. The lysozyme was purchased from Sigma-Aldrich and Ultra *Pfu* DNA polymerase was from Stratagene. The enzymes were treated as instructions provided by suppliers.

3.2.1.2. Bacterial Strains and Plasmid DNA

The XL1Blue-MR strain was purchased from Stratagene. 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 *E. coli* One shot® *ccd*B Survival™2 $T1^R$ and One Shots TOP10/P3 strains were purchased from Invitrogen. The genotype of One shot® *ccd*B Survival™2 T1 phage-resistant strain is as follows: F^- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*Δ139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L, (Str^R), *end*A1, *nup*G, *fhuA*::*IS2*. The genotype of TOP10/P3 strain is as follows: F^- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*Δ139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L, (Str^R), *end*A1, *nup*G, P3 [Kan^R, Amp^R (am), Tet^R (am)]. The *A. tumefaciens* strain LBA4404 has the TiAch5 chromosome which contains rifampicin resistance gene and the disarmed Ti plasmid

pAL4404 with spectinomycin- and streptomycin-resistance genes (Hokekma et al., 1983). The final concentrations of spectinomycin and streptomycin for selection are 100 μg/ml (Sp^{100}) and 50 μg/ml (St^{50}). *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 10X salt solution per liter. For the 10X 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 M KOH. After making a volume to one liter, the 10X salt solution was heated to boil and the precipitates were filtered through Whatman #1 filter paper. After the 10x salt solution was added the pH of the media was adjusted to 7.0 prior to autoclaving. Transformation of *A. tumefaciens* was conducted using either YEB media (5 g Sucrose, 5 g Bacto-Peptone, 5 g Beef Extract, 1 g Yeast Extract, 0.002 M $MgSO_4$ per liter), YEP media (10 g Bacto-Peptone, 10 g Yeast Extract, 5 g NaCl, 0.002 M $MgSO_4$ per liter) or TYNG media (10 g Bacto-Triptone, 5 g Yeast Extract, 5 g NaCl, 0.002 M $MgSO_4$ per liter). Magnesium ion was omitted from the media when tetracycline was used for bacterial selection. The transformation efficiency of *A. tumefaciens* was compared by using different media.

Plasmids used for the experiments were pBR322 (Bolivar, 1978; Sutcliffe, 1978A; Sutcliffe, 1978B), pUC19 (Chambers et al., 1988; Yanisch-Perron et al., 1985). pCambia1301 purchased from Cambia, pBluescriptII KS(+) from Stratagene and pUC4-KIXX and -KSAC from Pharmacia (Barany, 1985).

3.2.1.3. Oligodeoxyribonucleotides and Plasmid DNA Manipulation

Oligonucleotides used for PCR, mutagenesis or DNA sequencing were custom-ordered and synthesized by Sigma-Aldrich and listed in Supplementary Tables C1 and C2. Plasmid DNA was isolated by alkaline lysis method (Bimboim and Doly, 1979) and purified by $CsCl_2$-EtBr gradient centrifugation method (Hansen and Olsen, 1977). The GENECLEAN kit

purchased from BIO101 was used to extract DNA from agarose gel (Vogelstein and Gillespie, 1979). The QuickChange Multi Site-Directed Mutagenesis Kit was purchased from Stratagene. Other molecular cloning methods were according to Sambrook and Russell (2001).

3.2.2. Construction of Tetracycline-based Binary Ti Vectors pLSU-12 and pLSU-14

New binary Ti vectors pLSU-11 to pLSU-17 have the tetracycline resistance gene *tet*C as a bacterial selection marker. The *tet*C gene was amplified from pBRVS1 and modified to eliminate five restriction enzyme sites, two *Nhe* I, *Eco* RV, *Sph* I, and *Sal* I sites. The primers used for the mutagenesis are as follows:

primer TetNh1 (5'-catcgataagctag**G**tttaatgcggtagtttatcacagttaaattgc-3' : boxed capital letter G is the introduced single point mutation and the inactivated *Nhe* I site is underlined), primer TetE5 (5'-gcgggatAT**T**gtccattccgacagc-3' : Isoleucine codon is capital letters, boxed capital letter T is the single point mutation, and the inactivated *Eco* RV site is underlined), primer TetNhe2 (5'-gtgctgCT**T**gcgctatatgcgttgatg-3' : Leucine codon is capital letters, boxed capital letter T is the introduced single point mutation, and inactivated *Nhe* I site is underlined), primer TetSph (5'-atctccttgCA**C**gcaccattccttgc-3' : Histidine codon is capital letters, boxed capital letter C is the introduced point mutations, and inactivated *Sph* I site is underlined), primer TetSal (5'-agagcgtCG**G**ccgatgcccttg-3' : Arginine codon is capital letters, boxed capital letter G is the introduced point mutations, and inactivated *Sal* I site is underlined). The mutagenesis reactions yielded 5,429 bp of pBRVS2ΔNENSS (Supplementary Figure C1).

The modified *tet*C gene was amplified by PCR from pBRVS2ΔNENSS using two primers Tet-F2 and Tet-R1. The primer Tet-F2 (5'-aagccgcgccgtcg**cTaGC**tctcatgtttgacagcttatca-3') binds to pBRVS2ΔNENSS between 5,412 and 23 nt, and primer Tet-R1 (5'-cgtattacc**gcTAGC**gagtgagctgatacc-3') binds from 1,478 to 1,449 nt of pBRVS2ΔNENSS. Both primers have misplaced bases (capital letters) to introduce *Nhe* I sites (underlined) at both ends

of the amplified *tet*C gene (1,468 bp). The amplified *tet*C gene replaced the *npt*I gene in binary vectors pLSU-4 and pLSU-2 (6,173 and 5,937 bp, respectively). To remove the *npt*I gene from the binary vectors, two primers were designed to perform an inverted PCR. The primer ΔNPT1-F2 (5'-ggcggctttg**CCT**a**GG**caattgatcttcttgaaatcc-3') binds from 984 to 1,020 bp of pLSU-4. Primer ΔNPT1-R2 (5'-tgagacacaacgt**CCTAGG**aattgcaaacaaacaaatacag-3') binds to pLSU-4 between 19 and 6,551 bp, and introduces *Avr* II sites (underlined) at the end of *npt*I gene (Supplementary Figure C1). Both primers have misplaced bases (capital letters) to introduce *Avr* II sites at outside of *npt*I gene to be removed, and amplified PCR products, pLSU4ΔNPTI (5,180 bp) and pLSU2ΔNPTI (4,944 bp). The amplified 1,468 bp of *tet*C gene was ligated into the binary vectors without the *npt*I gene resulting in new binary vectors pLUS-14 and pLSU-12 with tetracycline resistance as bacterial selection marker (6,648 and 6,412 bp, respectively). The compositions of T-DNA region in the tetracycline-based binary Ti vectors are illustrated in Figure 5. The pLSU-11 is a basic vector skeleton with the twelve common restriction sites in T-DNA as shown in Figure 4. While the binary vector pLSU-12 and pLSU-13 have the kanamycin resistance *npt*II gene as a plant selection marker, pLSU-14 and pLSU-15 contain the hygromycin resistance *hph* gene as a plant selection marker (Figure 5). As illustrated in Supplementary Figure C4, pLSU-13 and pLSU-15 also have the *gus* reporter gene in addition to the plant selection marker.

The *β-glucuronidase* gene was amplified from pCambia1305.2 with primer 1305-1F2 and 1305-1R2. The 28 nt primer 1305-1F2 (5'-tgagcttggat**A**ag**C**ttgtcgtttcccg-3') was designed to bind from 11,260 to 11,287 bp of pCambia1305.2, just outside of CaMV35S promoter region (capital letters are misplaced bases). The 38 nt primer 1305-1R2 (5'-cgccaatata**AG**ct**T**tcaaacactgatagtttaattcc) binds from 2,371 to 2,334 bp of pCambia1305.2 at the end of *nopaline synthase* polyA sequence (capital letters are misplaced bases).

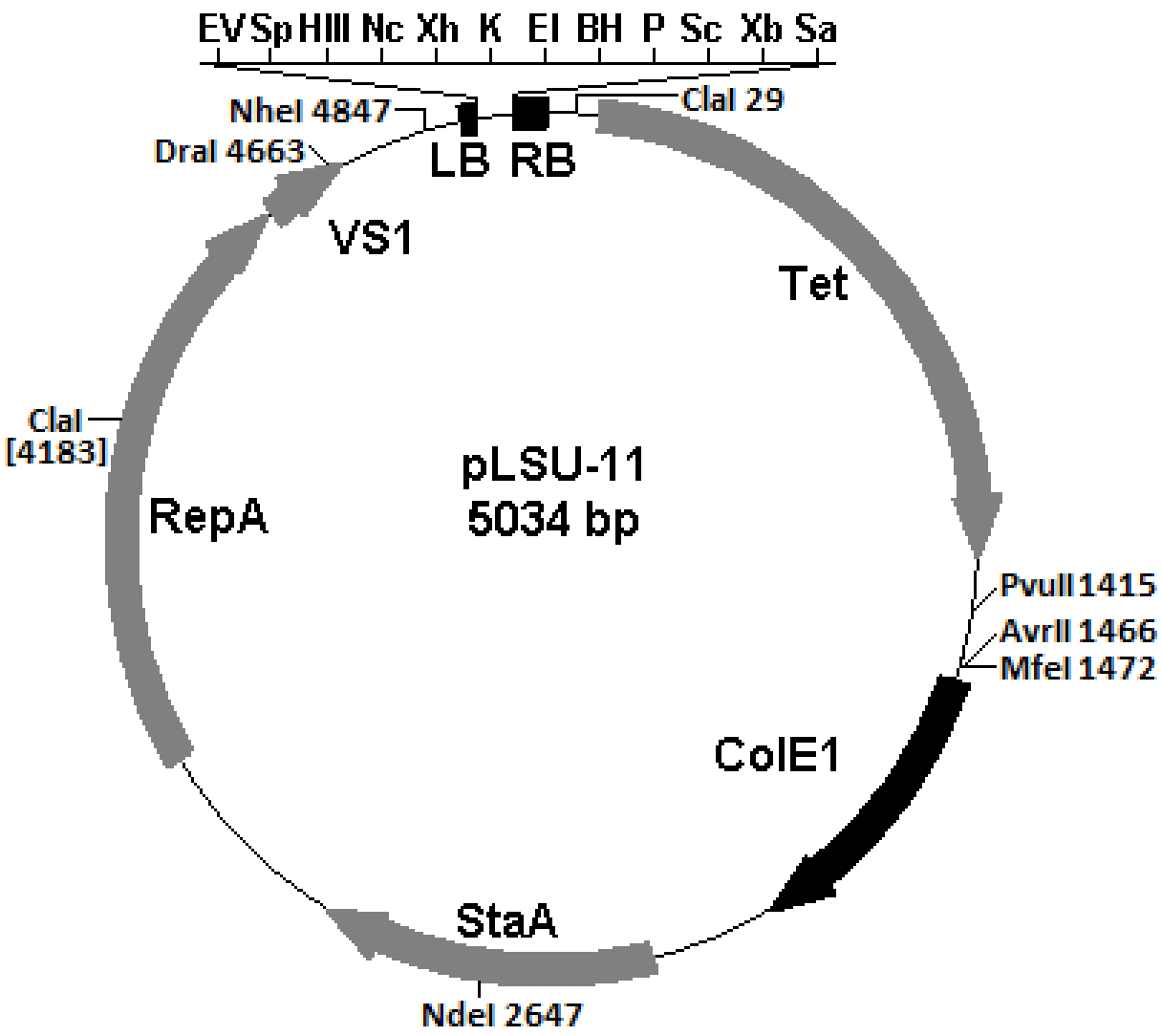

Figure 4. Schematic representation of backbone structure of tetracycline-based binary Ti vector pLSU-11 (5,034 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *Eco* RV (EV), *Sph* I (Sp), *Hind* III (HIII), *Nco* I (Nc), *Xho* I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sac* I (Sa). 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). The restriction enzyme sites: *Mfe* I (1,472 bp), *Cla* I (29 bp and 4,183 bp). DNA sequence of pLSU-11 was deposited to Genbank at accession number HQ608522.

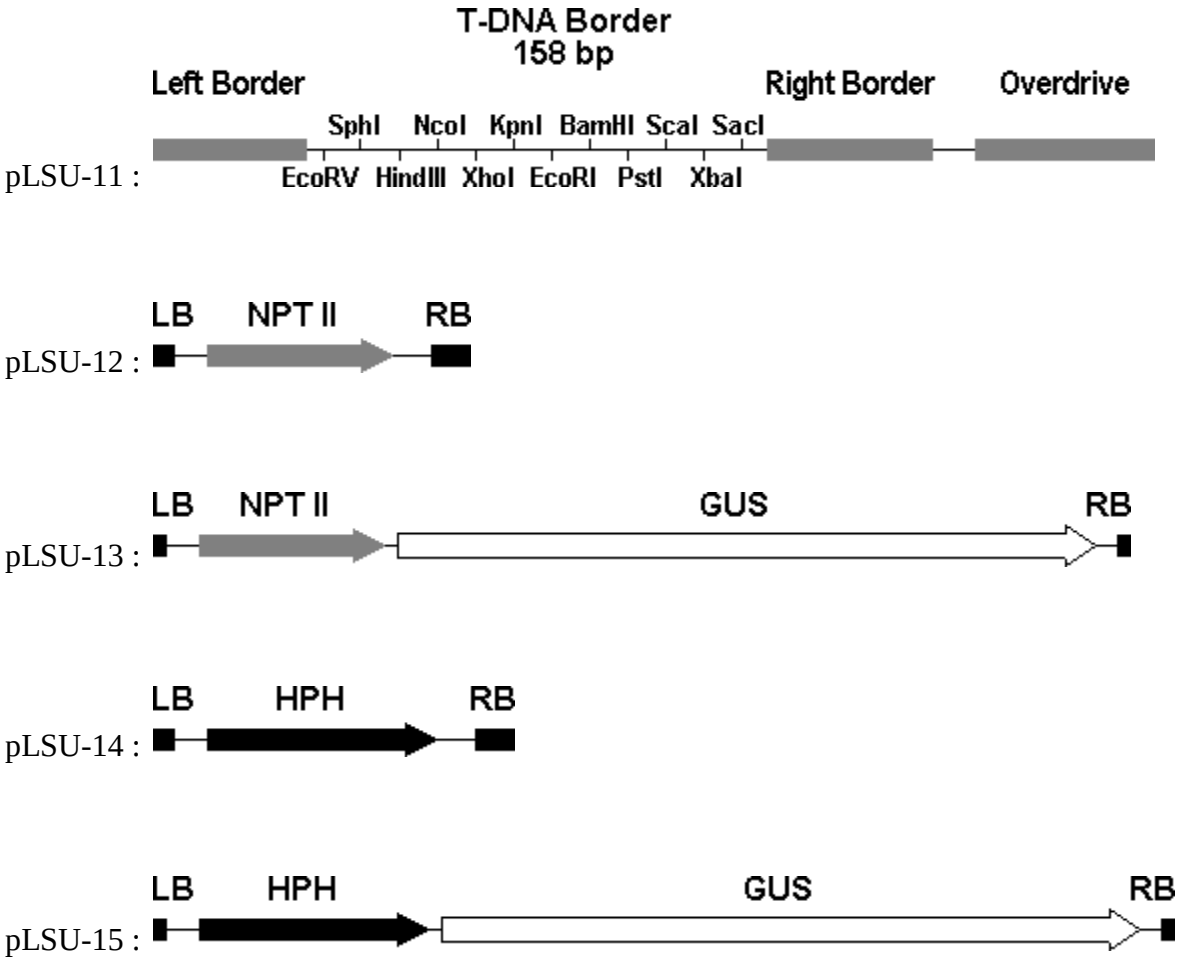

Figure 5. Schematic representation of the T-DNA region of tetracycline-based binary Ti vectors pLSU-11 to pLSU-15. pLSU-11 is a basic skeleton vector with the twelve common restriction sites in T-DNA. pLSU-12 and pLSU-13 have the *neomycin phosphotransferase* II gene (NPTII) adjacent to the left border as a plant selection marker for kanamycin resistance. pLSU-14 and pLSU-15 contain the *hygromycin B phosphotransferasae* gene (HPH) adjacent to the left border as a plant selection marker for hygromycin resistance. pLSU-13 and pLSU-15 also include the *β-glucuronidase* reporter gene (GUS) in addition to the plant selection marker in the T-DNA.

Both primers introduced new *Hind* III sites (underlined) at the ends of GUS cassette fragment including CaMV35S promoter, GUS gene with His6, glycine-rich protein signal peptide, catalase intron and nopaline terminator (Supplementary Figure C4). After *Hind* III digestion, the amplified 3,007 bp fragment was introduced into the expression vectors pLSU-14 and pLSU-12 at the *Hind* III site 3'-adjacent to the *hph* or *npt*II gene resulting in 9,655 bp of pLSU-15 and 9,419 bp of pLSU-13, respectively (Figure 6).

3.2.3. DNA Sequencing of pLSU-12

DNA sequences of two strands of the new binary vectors pLSU-12 were determined with an Applied Biosystems™ 3730xl DNA Analyzer at Eurofins MWG Operon (Huntsville, AL). Twenty eight sequencing primers were designed as listed in Supplementary Table 2, and synthesized by Sigma-Aldrich. The complete DNA sequence of tetracycline-based binary vector skeleton (pLSU-11) is submitted to GenBank (Accession number HQ608522). In the tetracycline resistance gene at 1,142 bp, the nucleotide C was confirmed as T, and in the termination region of Tet^R gene at 1,425 bp, GCGG were missing from the pBR322 sequence listed in GenBank. C was inserted at 1,467 bp, the ligation junction between Tet^R gene and ColE1 replicon. The unexpected G in the RepA region at 3,376 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 pLSU-4.

3.2.4. Mobilizable Tetracycline-based Binary Vector pLSU-16

The ColE1 replicon and tetracycline resistance gene of pBR322 (Sutcliffe, 1978) were amplified as a template for the mobilizable binary Ti vector pLSU-16. The ampicillin resistance and ROP genes were excluded by two separate amplification reactions of the pBR322 template, and unique *Hind* III and *Bam* HI sites of pBR322 were eliminated yielding 2,243 bp pORItet (Supplementary Figure C2). The unique *Hind* III site at 29 to 34 bp of pBR322 was knocked out

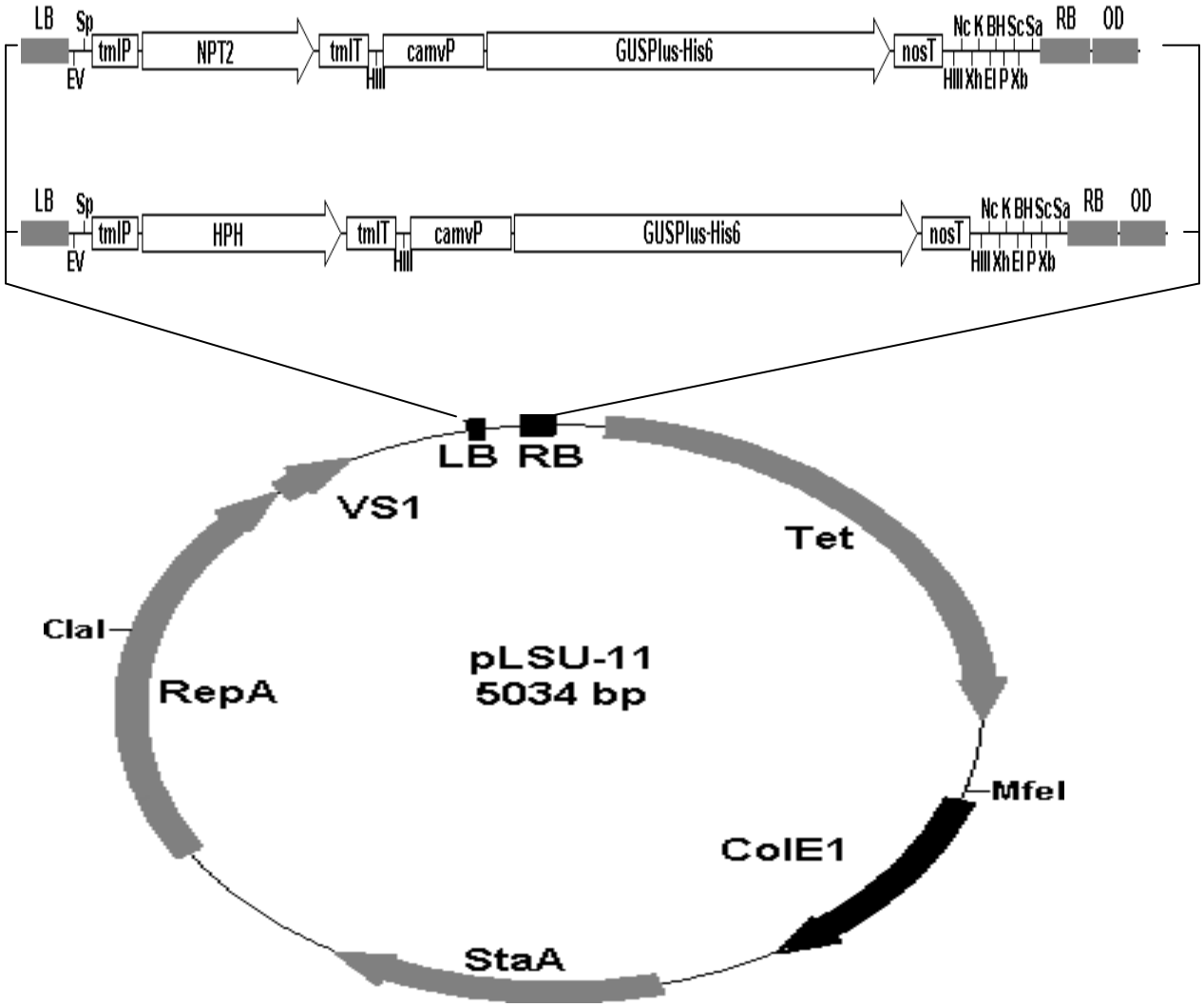

Figure 6. Detailed diagrams of the T-DNA regions of pLSU-13 and pLSU-15. The expression of the *npt*II and *hph* gene are under transcriptional control of the promoter (tmlP) and terminator region (tmlT) of *tumor morphlogy large* (*tml*) gene from pTi15955. The GUS reporter fragment including CaMV35S promoter (camvP), *gus* gene with His6 (GUSPlus-His6), and nopaline terminator (nosT) was isolated from pCambia1305.2. T-DNA left border (LB), right border (RB), overdrive (OD), *Eco* RV (EV), *Sph* I (Sp), *Hind* III (HIII), *Nco* I (Nc), *Xho* I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sac* I (Sa)

by *Hind* III digestion and filled-in by Klenow fragment producing pBR322-dHindIII. The region from the tetracycline resistance gene to ColE1 replicon was amplified with primers BR322-2 and BR322-1 producing a smaller 3,206 bp pBR-d1. BR322-1 is 5'- aaataggcgtatcacgaggccc-3', binding to pBR322-dHindIII between 4,329 and 4,351 nt, of and the primer BR322-2 is 5'-tccactgagcgtcagaccccg-3', binding from 3,169 to 3,148 nt of pBR322-dHindIII. Next, the unique *Bam* HI site (ggatcc→gga**T**c; 379 to 384 bp) in tetracycline resistance gene (not affecting amino acid codon in Tet^R gene; the underlined atc→atT is the same codon for Ile) was inactivated by making a single point mutation using primer BR322-5 and BR322-6, resulting in pBR-d1Bam. Primer BR322-5 (5'-cgtcctgtggat**T**ctctacgccgg-3') binds to pBR-d1 between 371 and 394 nt (the misplaced base is capital letter and the mutated *Bam* HI site is underlined), and primer BR322-6 (5'-ggtgtggtcgccatgatcgcg-3') binds from 370 to 349 nt of pBR-d1. Finally, in the primer BR322-3 and BR322-4, new *Pvu* II (cagcag→cagc**T**g) and *Bst* BI (ttcgagc→ttcg**AA**) sites were introduced by single point mutations on 1,408 to 1,413 bp and 2,396 to 2,401 bp of pBR-d1Bam, respectively. The ROP gene was also removed when amplified with primer BR322-3 and BR322-4, resulting in pORItet. Primer BR322-3 is 5'-ccgcgtgcggc**A**gctggagatggcgg-3' (new *Pvu*II site underlined), binding to pBR-d1Bam between 1,423 and 1,397 nt. Primer BR322-4 is 5'-gctcggtcgttcg**AA**tgcggcgagcg-3' (new *Bst*BI site underlined), binding from 2,387 to 2,413 bp of pBR-d1Bam.

The broad host range replication origin and stability region was amplified from a binary vector pGV941 using primers contained new restriction endonuclease sites *Eco* RI and *Mfe* I. The 3,188 bp STA/REP fragment was amplified with primer REPSTA3, 5'-ggcattgccgaattcgagcgttcc-3' (new *Eco* RI site underlined) and primer REPSTA4, 5'-ccggcggtcaattggcgacggcgc-3' (new *Mfe* I site underlined). The amplified fragment digested with both restriction enzymes was ligated into *Eco* RI site of pORItet resulting in 5,429 bp pBRVS1

(Supplementary Figure C2). The new *Mfe* I sites of the PCR fragment were inactivated by ligation to the *Eco* RI site of pORItet due to the compatible cohesive ends of *Mfe* I to *Eco* RI.

The T-DNA left and right border sequences originated from the octopine-type Ti plasmid pTi15955 were cloned from pKSLR (3,066 bp). 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 (*npt*II) gene of transposon Tn*5* (Beck et al., 1982). Plasmid pUC4-KIXX (4,200 bp, Pharmacia) was digested with *Hind* III and the 1,568 bp fragment containing the *npt*II gene was inserted into *Hind* III site of pKSLR (Supplementary Figure C2). This plasmid was named as pLR-KIXX and the colonies were double-selected for kanamycin and ampicillin resistance. After kanamycin resistance selection, the *Mfe* I-LB-Kan^{R}-MCS-RB-*Mfe* I fragment was cut with *Mfe* I and inserted into *Eco* RI site of pBRVS1. This kanamycin resistance gene was removed by *Hind* III after the insertion of single copy of T-DNA border into pBRVSI was confirmed. The final product was named as pLSU-16 (Figure 7).

3.2.5. Gateway Technology

The three reading frame cassettes flanked by *att*R1 and *att*R2 sites (1,711 bp of RfA, 1,713 bp of RfB, and 1,714 bp of RfC1) were purchased from Invitrogen. The binary vector pLSU-12 has *Eco* RV site in the multicloning site, adjacent to the T-DNA left border and the *Eco* RV site was used for cloning site of the Gateway® reading frame cassette. First, the *Eco* RV site in pLSU-12 was digested and dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Then the dephosphorylated vector was ligated with the Gateway® cassette RfA, RfB, and RfC1, respectively, producing pLSU-18A, -18B, and -18C1 (Figure 8). The ligation products were transformed into *E. coli* strain, One shot® *ccd*B Survival™2 T1^{R} phage-resistant competent cells,

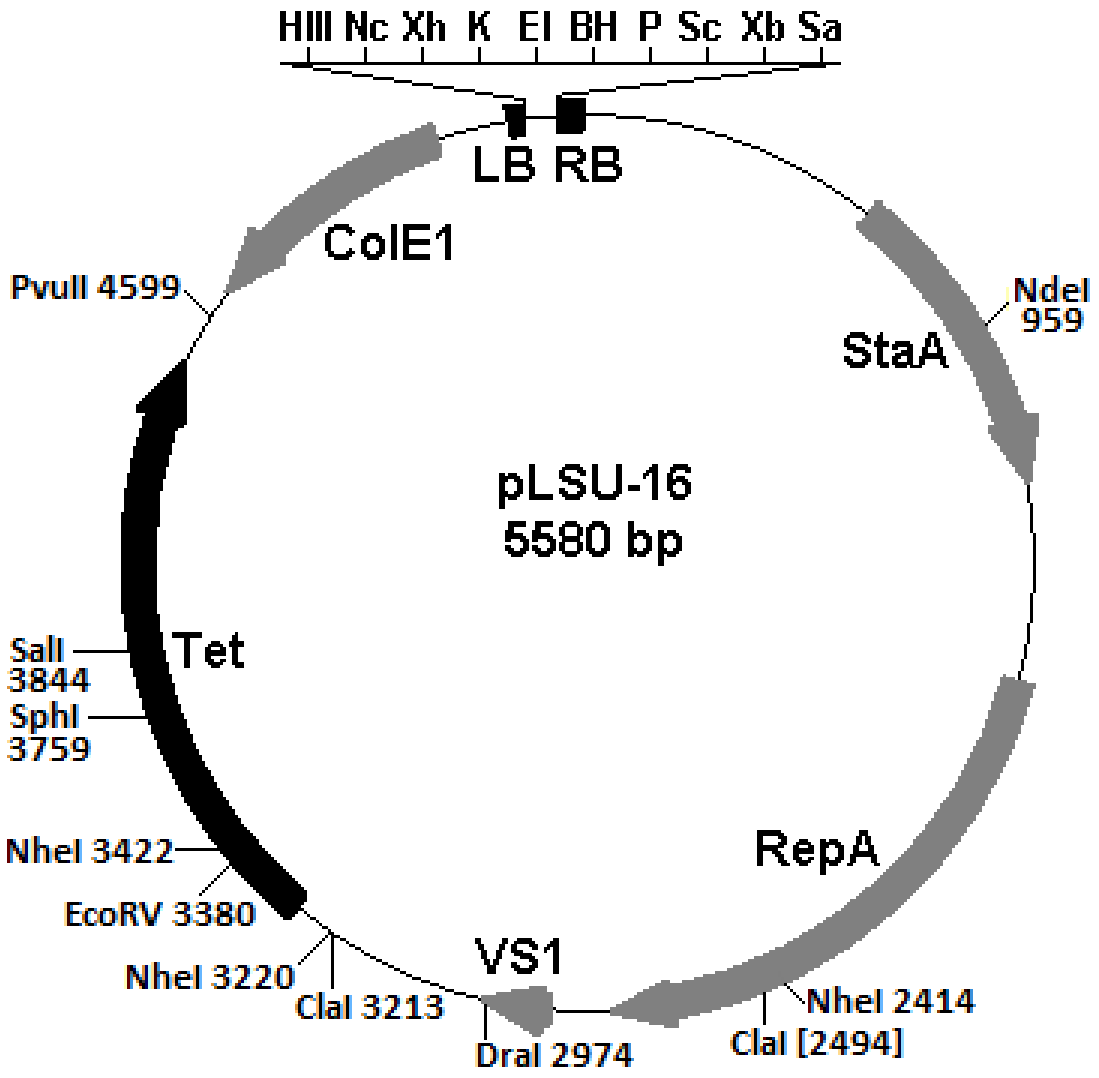

Figure 7. Schematic representation of mobilizable binary Ti vector pLSU-16 (5,580 bp). T-DNA is at the top of figure limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *Eco* RV (EV), *Sph* I (Sp), *Hind* III (HIII), *Nco* I (Nc), *Xho* I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sal* I (Sa). 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).

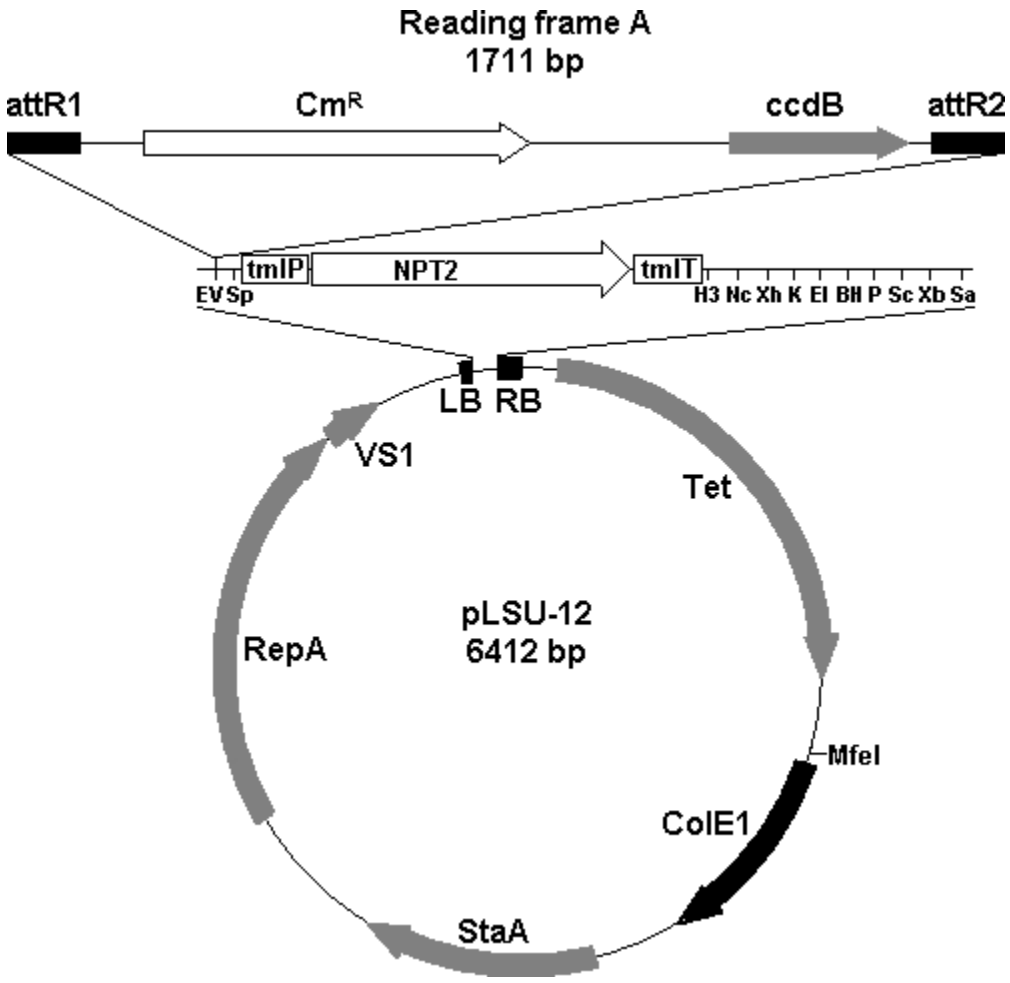

Figure 8. Schematic representation of Gateway destination vector pLSU-18A. At the top is the reading frame A fragment with two clonase recognition sites (*att*R1 and *att*R2), chloramphenicol resistance gene (Cm^R), and *ccd*B gene that inhibits the DNA gyrase (topoisomerase II). T-DNA is limited by the right (RB) and left border (LB) with 12 common restriction endonuclease sites, *Eco* RV (EV), *Sph* I (Sp), *Hin d*III (HIII), *Nco* I (Nc), *Xh o*I (Xh), *Kpn* I (K), *Eco* RI (EI), *Bam* HI (BH), *Pst* I (P), *Sca* I (Sc), *Xba* I (Xb), and *Sac* I (Sa). The kanamycin resistance gene *npt*II is located between the *Sph* I and *Hind* III 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).

and selected on LB agar plates containing 30 μg/ml chloramphenicol and 10 μg/ml tetracycline under dark condition. The insertion of the Gateway® cassette was determined by *Bsr* GI restriction enzyme whose recognition site is located in the *att*R1 and *att*R2 sites, and the direction of the reading frame were determined by *Eco* RI sites which are located in Cm^R gene and next to *tml* terminator of *npt*II gene. The activity of Gateway® LR Clonase™ II enzyme mix was also tested with pENTR™-gus vector.

The destination binary vectors were mixed with pENTR™-gus and Gateway® LR Clonase™ II enzyme mix, and incubated for overnight at 25°C. After the LR clonase reaction, the Proteinase K solution was added and incubated for 10 min at 37°C. The reaction was transformed in *E. coli* strain, One Shots TOP10/P3 from Invitrogen and selected for tetracycline 10 μg/ml so that only the transformed *E. coli* with recombined Expression vector survives due to the removal of ccdB gene. To make sure the site-specific recombination happened between *att*R1/R2 and *att*L1/L2 sites, colonies were picked and transferred to a replica plate under selection of chloramphenicol 30 μg/ml and tetracycline 10 μg/ml. The replica colonies were sensitive to chloramphenicol selection because of the Cm^R gene was removed by recombination, and therefor, did not survive under chloramphenicol selection.

3.2.6. 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 (1988) and as modified as follow. Ten mililiter 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 3,000 *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 microgram of DNA was mixed with 100 μl of cells, and frozen in an ethanol bath at -80^{o}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.

3.2.7. Tobacco Leaf Disc Transformation Mediated by *A. tumefaciens*

Agrobacterium-mediated transformation of tobacco leaf disc was performed as described previously (Appendix D, Su, 2010).

3.3. Results

3.3.1. Tetracycline-based Binary Ti Vectors pLSU-11

I constructed new tetracycline-based binary Ti vectors by replacing the bacterial kanamycin resistance gene of the binary vectors pLSU-2 and pLSU-4 with the tetracycline resistance gene. 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*. The overall scheme of the construction of the tetracycline-based binary Ti vector pLSU-14 is illustrated in Supplementary Figure C1.

3.3.2. Bacterial Tetracycline Rresistance Gene

The tetracycline resistance gene *tet*C from pBR322 was used for tetracycline selection of bacteria (Levy et al., 1999). Although there are some inconvenience in use of tetracycline because tetracycline is light-sensitive, and is inhibited by magnesium ion included in commonly used media, tetracycline is still a very effective antibiotics since the optimal concentrations for *E. coli* and *A. tumefaicnes* are 10 and 2 mg/L, respectively.

Five restriction enzyme sites for two *Nhe* I, *Eco* RV, *Sph* I, and *Sal* I were eliminated from the *tet*C 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 *tet*C gene, I 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 as described in Supplementary Figure C3. The new truncated *tet*C gene of 1,468 bp contains 1,191 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 tetracycline gene in *E. coli*. The truncated *tet*C gene was used to replace the bacterial kanamycin resistance *npt*I gene from binary vectors pLSU-2 and pLSU-4, generating pLSU-12 and pLSU-14 as shown in Supplementary Figure C1.

DNA sequence analysis of pLSU-12 indicated that all five single point mutations introduced to the tetracycline resistance gene Tet^R were confirmed as expected. However, I found one and 16 bp insertions at the junctions of ligation reactions and 4 bp deletions in the terminator region of tetracycline resistance gene as noted in Material and Methods. DNA sequence of basic vector skeleton, pLSU-11 was deposited to GenBank at accession number HQ608522.

3.3.3. Mobilizable Tetracycline-based Binary Vector pLSU-16

We also constructed a mobilizable version of tetracyline-based binary Ti vectors pLSU-16 in which the mob function of ColE1 replicon was maintained for mobilization by tri-parental mating (Figure 7). The final size of vector skeleton pLSU-16 is 5,580 bp long with the Tet^R gene, ColE1 and VS1 replicons, and T-DNA. The overall scheme of construction is illustrated in Supplementary Figure C2.

3.3.4. *A. tumefaciens*-mediated Transformation of Tobacco Leaf Discs

The new tetracycline-based binary Ti vector, pLSU-12 was used for *A. tumefaciencs*-mediated transformation of tobacco leaf discs in comparison of the kanamycin-based binary

vector, pLSU-2. After four-day co-cultivation with *A. tumefaciencs* LBA4404, leaf disc induced calli were selected at 25 ^{o}C on shooting medium in the presence of 300 mg/L of kanamycin and 500 mg/L of carbenicilin for two weeks, and then two more weeks under kanamycin selection. As shown in Table 8, a stable expression of introduced kanamycin-resistance gene was evident by up to 10-fold increase in fresh weight yield of kanamycin-resistant calli over vectorless control.

3.3.5. Gateway Technology Expression Vector with GUS

The tetracycline-based binary vector pLSU-12 (6,412 bp) was used to generate Gateway destination/expression vectors. Three different reading frame cassettes flanked by *att*R1 and *att*R2 sites (1,711 bp of RfA, 1,713 bp of RfB, and 1,714 bp of RfC1) were ligated to *Eco* RV site of pLSU-12, producing Gateway destination vectors, pLSU-18A, -18B, and -18C1 (Figure 8). 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 ccdB-resistant *E. coli* strain containing *gyr*A mutation, One shot® *ccd*B Survival™2 $T1^R$, and destination vectors were isolated by simple selection for tetracycline and chloramphenicol resistance. The correct size of destination vectors were confirmed on agarose gel electrophoresis with the plasmid DNA extracted by alkaline lysis miniprep. The insertion of the Gateway® cassette was determined by three *Bsr* GI restriction enzyme sites located in the *att*R1, *att*R2, and the *ccd*B gene. The direction of the reading frame was determined by *Eco* RI sites which are in Cm^R gene and next to *tml* terminator of *npt*II gene.

A *β-glucuronidase* (*gus*) gene in an entry vector was used to replace the Cm^R and *ccd*B gene in the destination vector using the LR clonase-catalized recombination reaction of the *att*L1/*att*L2 sites of the entry vector with the *att*R1/*att*R2 sites of the destination vector. The recombined expression vectors were transformed in ccdB-sensitive *E. coli* strain, TOP10/P3 and

Table 8. Tetracycline-based binary Ti vector pLSU-12 was compared with kanamycin-based binary vectors pLSU-2 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-2, pLSU-12. Leaf disks were selected at 25 ^{o}C on shooting 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. The experiment was performed once. Numbers in parentheses indicate standard deviations. Asterisk marks indicate significantly different values that were determined by the *t* test (P<0.05).

pLSU binary Ti vectors	Plate average of FW yield in g	Increase in FW in g per plate over vectorless control	% leaf discs with increased FW
Vectorless control	0.91 (0.20)	0	0
pLSU-2	12.06 (5.57)	10.96 (5.54)*	92
pLSU-12	8.21 (1.48)	7.09 (1.48)*	98

were selected for *ccd*B sensitivity. The site-specific recombination between *att*L1/L2 and *att*R1/R2 sites in the expression vector was confirmed by simple selection for tetracycline resistance and chloramphenicol sensitivity. A pLSU expression vector with the *gus* gene was isolated by alkaline lysis miniprep, and the proper insertion of *gus* gene and the removal of Cm^R/*ccd*B gene were confirmed on agarose gel electrophoresis. The presence of *gus* gene was determined by four *Bsr* GI restriction enzyme sites located between the *att*L1 and *att*L2 sites, and the direction of the *gus* gene were determined by *Mfe* I sites which are located in *gus* gene and pLSU-12.

3.3.6. Improved Freeze-thaw Transformation of *A. tumefaciens*

The freeze-thaw transformation of *A. tumefaciens* was preferred to electroporation or tri-parental mating. The freeze thaw procedure is technically more reliable than electroporation, and is less time-consuming than tri-parental mating. Evaluation of the transformation efficiency of *A. tumefaciens* was more effective in YEB media than other two commonly used media, YEP and TYNG media (Table 9A). Addition of 20 mM $CaCl_2$ improved the transformation efficiency of YEP and TYNG media without any positive effect on YEB media, presumably beef extracts in the YEB media already contain a high concentration of Ca ions from beef muscle. Two-fold increase in the sucrose concentration of YEB media also improved the transformation frequency of *A. tumefaciens* (Table 9B). YEB media is also preferred over YEP or TYNG media since the cultured or resuspended cells from YEB media did not aggregate while many clusters were noticed from YEP or TYNG media. The aggregation of *A. tumefacien* cells causes technical difficulties in accurately assessing the bacterial concentration by optical density, in distributing even amount of cells for each transformation, and in spreading evenly on the solid selection media after transformation. These cell clusters also let many untransformed cells survive around the cluster on the solid selection media.

Table 9. (A) Transformation frequency of *A. tumefaciens* was compared among the three commonly used media, YEB, YEP, and TYNG with or without the effect of addition of 20 mM $CaCl_2$. (B) Increased concentration of sucrose by twice improved the transformation frequency of *A. tumefaciens*. The colonies were counted per 1 μg of DNA. Standard deviations are in parentheses.

A.

Growth media	Resuspension solution	Average colony per 1 μg DNA
YEB	YEB	2740 (60)
	20 mM $CaCl_2$	2202 (78)
YEP	YEP	334 (34)
	20 mM $CaCl_2$	2296 (218)
TYNG	TYNG	176 (37)
	20 mM $CaCl_2$	1170 (160)

B.

Growth media	Increased composition	Average colony per 1 μg DNA
YEB	-	3044 (113)
	2x Beef Extract	970 (67)
	2x Sucrose	5486 (295)
	2x Beef Extract + 2x Sucrose	1206 (76)

3.4. Discussion

I previously constructed a series of kanamycin-based binary Ti vectors pLSU-1 to pLSU-5 to improve the transformation frequency and plasmid yield in *E. coli* and *A. tumefaciens* for *Agrobacterium*-mediated transformation of higher plants. 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). The new binary vectors pLSU-2 to pLSU-5 with co-directional transcription yielded in *E. coli* up to four-fold higher transformation frequency than those with the head-on transcription. Here I converted these kanamycin-based vectors to the tetracycline-based binary vectors pLSU-11 to pLSU-16 to exploit the user-friendly features of the Gateway® Technology for efficient cloning.

The Gateway Technology relies on the use of four kanamycin-based plasmid vectors in quick succession, from the donor, entry, destination to expression vectors. Many destination vectors for the transformation of higher plants used as a vector skeleton pCambia, pGreen, or pBin19 which has a bacterial kanamycin resistance gene (Curtis and Grossniklaus, 2003; Brand et al., 2006; Earley et al., 2006; Chen et al., 2009). The use of the same kanamycin-based vectors made it impossible for 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 *ccd*B gene in destination binary vector (Xu and Li, 2008). 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, by this method, the plasmid DNA of expression binary vector cannot be recovered and reused for another plant transformation since it is very 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 (Karimi et al., 2002; 2005; 2007a; 2007b; Dafny-Yelin and Tzfira, 2007; Himmelbach et al. 2007; Fernandez et al., 2009). The streptomycin selection is often not suitable for *Agrobacteria*-mediated transformation because the widely used *A. tumefaciens* strain LBA4404 has 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 *Agrobacterium*-mediated transformation using other *A. tumefaciens* kanamycin-resistant strain, such as 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, 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 functional analysis of plant genes.

CHAPTER 4. CONCLUSION

In this dissertation, I described the development of small, high-yielding binary Ti vectors pLSU with user-friendly features for *Agrobacterium tumefaciens*-mediated transformation of higher plants. In the first part of this dissertation, kanamycin-based binary Ti vectors were constructed to increase the cloning efficiency and plasmid yield in *Escherichia coli* and *A. tumefaciens*, and to improve the transformation efficiency of higher plants. Four basic components of binary Ti vectors, ColE1 replicon, VS1 replicon, a bacterial kanamycin resistance gene, and the T-DNA region were analyzed for the minimum size requirement and a number of mutations were introduced to increase the copy number and to maximize biological activities. The broad host-range replicon VS1 was reduced its size to 2.7 kb, 1.1 kb smaller than the currently used replicon with the 98 % stability in *A. tumefaciens*. The final size of the basic skeleton of binary Ti vector pLSU-1 was 4,566 bp. The results from *A. tumefaciens*-mediated transformation of tobacco leaf discs and T_1 progeny analysis indicated that the kanamycin resistance gene in T-DNA of pLSU vector is integrated into the plant nuclear chromosome and stably inherited to the next generation.

One of the interesting results was the effect of orientation of two replicons, ColE1 and VS1, on the transformation frequency of *E. coli* and *A. tumefaciens*. Current conventional vectors such as pPZP or pCambia maintain the transcriptional direction of VS1 replicon to be opposite (head-on transcription) to ColE1 replicon. The new pLSU binary vectors with co-directional transcription resulted in higher transformation frequency than the head-on direction in *E. coli* up to four-fold higher, and in *A. tumefaciens* up to 1.2 to 1.7-fold higher. Comparison of pLSU vectors with the conventional pCambia vector showed even more than ten-fold higher transformation frequency in *A. tumefaciens*. This result suggests the VS1 replicon may be functional in *E. coli* when it is combined in a proper orientation with the ColE1 replicon. In the

previous studies of mechanical interaction of replication and transcription machinery in *E. coli*, the replication fork progression is severely interfered by direct physical contact with the head-on RNA polymerase machinery in transcription. Ater temporally stalled, the replisome progression resumes the replication and replaces the head-on RNA polymerase machinery from DNA. This may explain why in this study, the co-directional binary vectors did not affect the DNA yield compared to the head-on vectors, while had very high transformation frequency. Unlike the transformation, the DNA is extracted after long-time cultivation. Thus, while the plasmid copy number "boom" due to the fast replication speed in the early stage can be critical for the enhancement of transformation frequency, the DNA yield by the slower but eventually resuming, head-on directional replisome progression may not be much different than by co-directional replication after overnight culture.

In the second part, the kanamycin-based binary Ti vectors were converted to the tetracycline-based binary vectors pLSU-11 to pLSU-16 to exploit the user-friendly features of the Gateway® Technology for efficient cloning. Because the Gateway donor/entry vectors currently available are kanamycin-resistant, the same kanamycin-based destination vectors from pCambia, pGreen, or pBin19 make it impossible for the simple antibiotic selection of colonies to distinguish the destination vectors from donor/entry vectors after LR clonase reaction. The other destination vectors are based on pPZP200 vector which has streptomycin/spectinomycin resistance gene and the streptomycin selection is also often not suitable for *Agrobacterium*-mediated transformation because the widely used *A. tumefaciens* strain LBA4404 has streptomycin/spectinomycin resistance gene in the avirulent Ti plasmid.

The new tetracycline-based, Gateway-compatible binary Ti vectors pLSU are more user-friendly than the currently available binary vectors. 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 the T-DNA region of pLSU. The high-throughput modular assembly of promoter, terminator, and coding region, and the reading frame-specific assembly of reporter coding region will enhance the functional analysis of plant genes.

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Zupan JR, Zambryski P (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol **107:** 1041-1047

APPENDIX A. PROVISIONAL PATENT APPLICATION 2010

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Document Description: Provisional Cover Sheet (SB16)

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Provisional Application for Patent Cover Sheet

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

Inventor(s)

Inventor 1 — Remove

Given Name	Middle Name	Family Name	City	State	Country
Norimoto		Murai	Baton Rouge	LA	US

Inventor 2 — Remove

Given Name	Middle Name	Family Name	City	State	Country
Seokhyun		Lee	Baton Rouge	LA	US

All Inventors Must Be Listed – Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. — Add

Title of Invention	Binary Ti Vectors with Co-Directional Replicons for Transformation of Eukaryotes and Prokaryotes
Attorney Docket Number (if applicable)	Murai 10A12

Correspondence Address

Direct all correspondence to (select one):

◉ The address corresponding to Customer Number	○ Firm or Individual Name
Customer Number	25547

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

◉ No.

○ Yes, the name of the U.S. Government agency and the Government contract number are:

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PTO/SB/16 (11-08)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Entity Status

Applicant claims small entity status under 37 CFR 1.27

- (•) Yes, applicant qualifies for small entity status under 37 CFR 1.27
- ( ) No

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Signature	/John H. Runnels/			Date (YYYY-MM-DD)	2010-04-27
First Name	John H.	Last Name	Runnels	Registration Number (If appropriate)	33451

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APPENDIX B. SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 2

Supplementary Table B1. List of Names and sequences of custom-designed oligonucleotide primers used for this research. The positions indicate the first nucleotide of template plasmid DNA to be amplified. Bold Capital letters indicate the sites of mutations to be introduced. The restriction endonuclease sites created or inactivated by the introduced mutations are described.

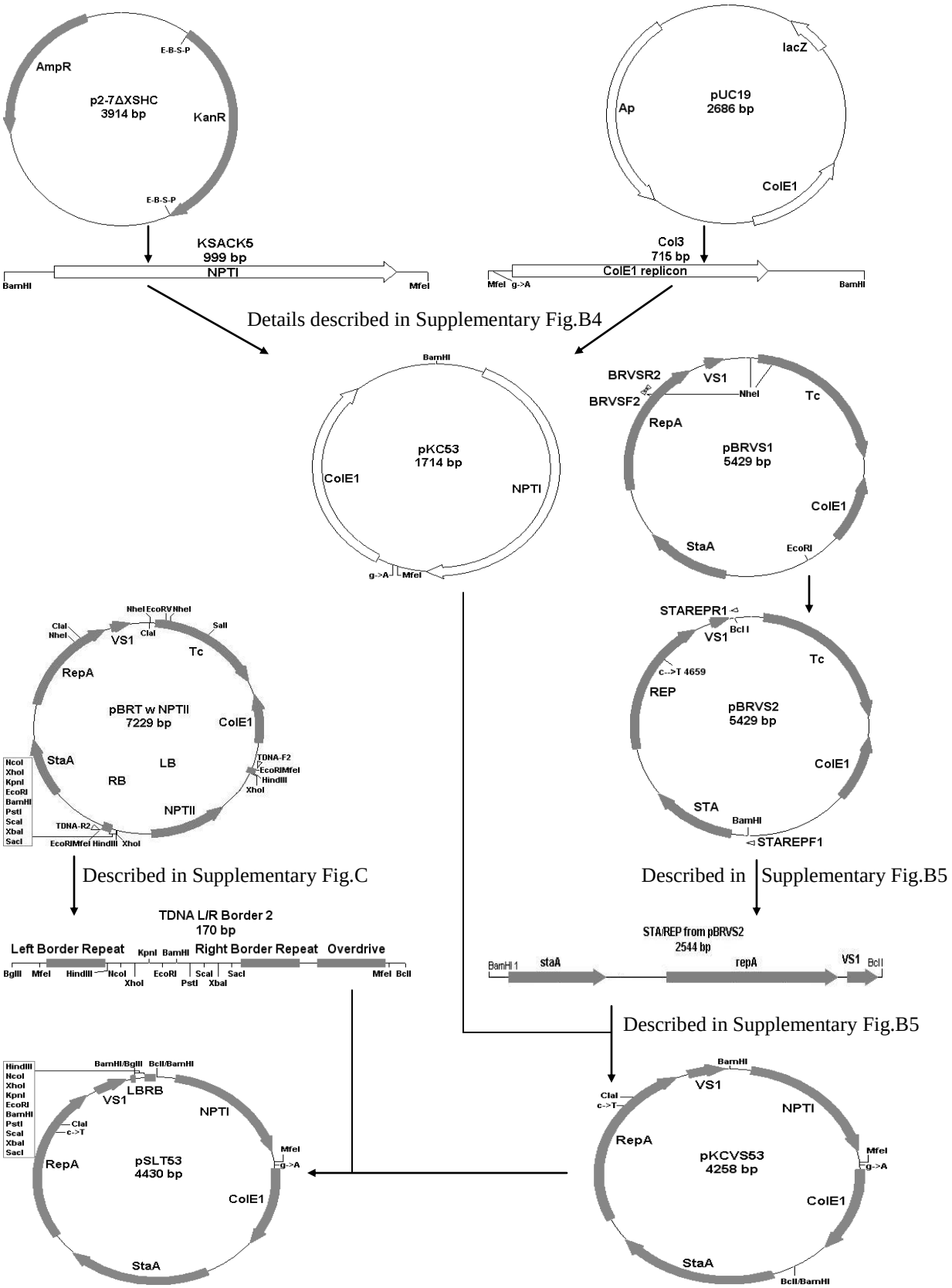
Primer	Sequence (5'→3')	Location at template plasmid DNA	Modified enzyme sites
T-DNA1-revised	gat ccg cgg caa ttg caa aca	pT-DNA, 656bp→	
T-DNA2-revised	gat atc gat ctt gat caa ttg gca gga	pT-DNA, ←837bp	MfeI & ΔHindIII
KSACK-F1	cga ttt att caa c**GG** a**T**c cac gtt gtg tct c	pKSAC, 1039bp→	BamHI site
KSACK-R1	tca gca aaa gtt c**A**a tt**G** att caa caa agc cgc c	pKSAC, ←2068bp	MfeI
KSACK-F2	aag ttc gat **GG**a t**C**c aac **TG**a **T**c**A** acg ttg tgt ctc	pKSAC, 1034bp→	BamHI & BclI
KSACK-R2	ctt tgc cac g**C**a a**TT** gtc tgc gtt gtc g	pKSAC, ←2123bp	MfeI
Col-F1	aag gcc agg a**T**c cgt aaa aag gcc g	pUC19, 829bp→	BamHI
Col-R1	aag agc tac caa c**AA** tt**G** ttc cga agg taa ctg g	pUC19, ←1373bp	MfeI
Col-F2	gag cgg tat c**G**g **A**tc **C**ct caa agg cgg	pUC19, 737bp→	BamHI
Col-F3	cca acg cg**G ATC C**ag agg cgg ttt gc	pUC19,648bp→	BamHI
Col-R3	aga aaa gat caa **TT**g atc ttc ttg a**A**a tcc ttt ttt tct gcg taa ctg g	pUC19, ←1476bp	MfeI & ΔMboI
BRVS-F1	agc tag **T**tg att gga tgt acc gcg aga tca cag aag gc	pBRVS1, 4653bp→	ΔNheI
BRVS-R1	cca atc a**A**c tag ctc gat ctc gat gta ctc cgg c	pBRVS1, ←4666bp	ΔNheI
BRVS-F2	cga gat cac aga agg caa gaa ccc	pBRVS1, 4675bp→	
BRVS-R2	cgg tac atc caa tca **A**ct agc tcg atc t	pBRVS1, ←4674bp	ΔNheI
BRVS-F4	cga tta ctt ttt gat **T**ga tcc cgg cat cgg c	pKCVS53,3696bp→	ΔClaI
BRVS-R4	ggg tga acc gtc agc acg tcc ggg ttc ttg c	pKCVS53,←3695bp	
STAREPF1	ggc cag ctt g**AT** c**A**c tga aga aac cga gc	pBRVS2, 2731bp→	BclI
STAREPR1	gcg ctt ttt g**G**a **T**c**C** ctt cgg ctg tgc	pBRVS2, ←5297bp	BamHI
STAREPF2	ggc cag ctt g**AT** c**A**c tga aga aac cga gc	pBRVS2, 2732bp→	BclI
STAREPR2	gcg ctt ttt g**G**a **T**c**C** ctt cgg ctg tgc	pBRVS2, ←5297bp	BamHI
SRL-F1	agg cgt gaa gtt tgg c**TG ATC A**cc tac cct cac ccc g	pBRVS2, 2303bp→	BclI
SRL-R1	ctt gtc cgc gc**G GAT CC**a gat tgc ctg gcc gta gg	pBRVS2, ←5414bp	BamHI
KSRL-F1	agc tcc ac**A** g**AT CT**a att gca aac	pKSLR, 654bp→	BglII & ΔMfeI
KSRL–R1	cgg tat cga tct **A**ga tc**T** att ggc agg	pKSLR, ←839bp	BglII & ΔMfeI
SMGPst	ctg aat gaa ct**C** cag gac gag gca gc	pSMG, 712bp→	ΔPstI
SMGSph	caa ggc gcg **G**at gcc cga cg	pSMG, 1065bp→	ΔSphI
SMGNco	cgt cgt gac **A**ca tgg cga tgc ctg c	pSMG, 1095bp→	ΔNcoI

(Table continued)

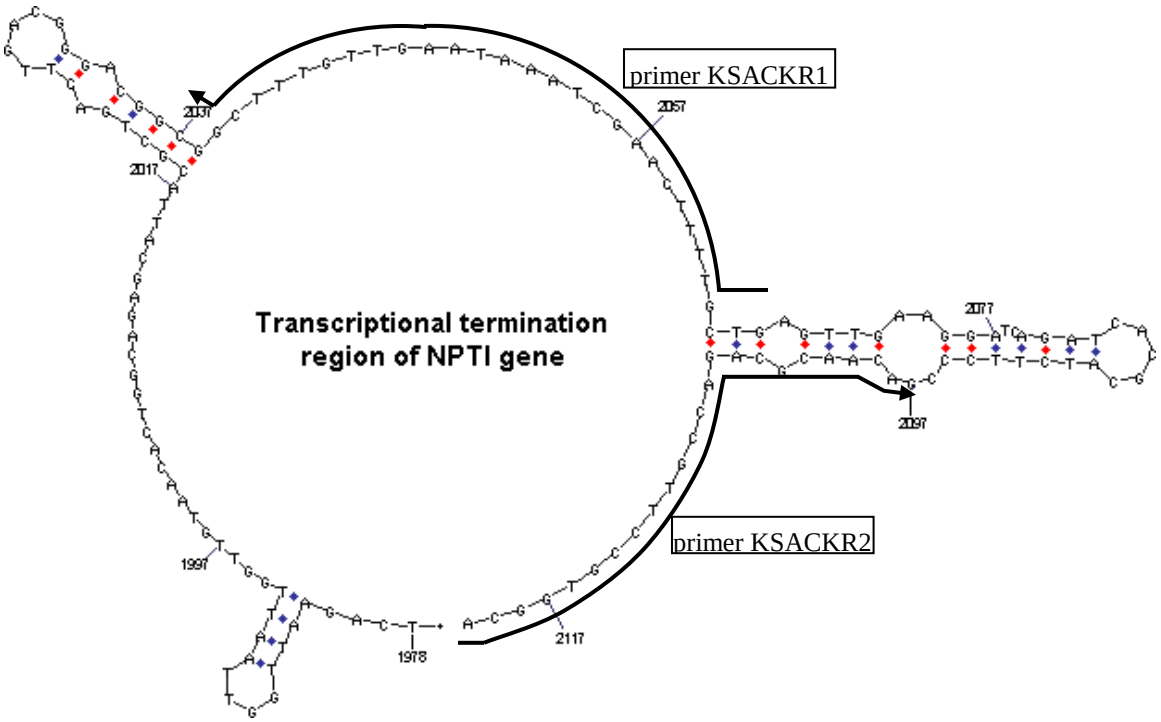
NPT2BglF1	tgc gcc Aga tcT tcg cat gat tg	pSMG, 529bp→	BglII & ΔBamHI
NPT2BglR1	ggt cat ttc gaG ATc TGg agt ccc gct c	pSMG, ←1364bp	BglII
TRANco	cgg agg c**G**a tgg atg cga	pTRA151,1144bp→	ΔNcoI
TRASca	tgt gta gaa gt**T** ctc gcc gat agt gg	pTRA151,1758bp→	ΔScaI
TRANhe	agt gtt cta agc **GG**g cct ggt aat aat cta tac gag c	pTRA151, 2043bp→	ΔNheI
hphBgl-F2	ctc tag agg atc cc**A** g**AT CT**c aat gag ata tga aaa agc ctg aac tca cc	pTRAΔNSN, 1809bp→	BglII
hphBgl-R2	gga tcc ggt cg**A G**at cta ctc tat tcc ttt gcc ctc	pTRAΔNSN, ←735bp	BglII
TmlBcl-F2	tcc gca t**G**a t**CA** ccg tcg ccg aca cct aat aaa gtc	pSMGΔPSNN, 1528bp→	BclI
TmlBcl-R2	tgt tca atc atg cga **T**ga tc**A** ggg cag tgt tgg atg tac tac	pSMGΔPSNN, ←554bp	BclI & ΔBamHI
tmlP-H3II	agc tcg gta ccc **A**ag **CT**t tat gca gca ttt ttt tgg tgt atg	pTmlH, 237bp→	HindIII
tmlT-H3II	ccg ggt acc **A**ag ct**T** g**C**t cgt ggt gat att aaa gag ag	pTmlH, ←1876bp	HindIII &ΔSacI
LRSphF1	aag ggg aaa agg gg**G CAT GCG ATA TC**a ttt aca act gaa tat atc ctg c	pLRH, 2407bp→	EcoRV & SphI
LRSphR1	gga aaa ggg g**GC ATG C**ta tgc agc att ttt ttg gtg tat gtg ggc	pLRH, ←2401bp	SphI
1305-1F2	tga gct tgg at**A** ag**C** ttg tcg ttt ccc g	pCambia1305.2, 11260bp→	HindIII
1305-1R2	cgc caa tat a**AG** ct**T** tca aac act gat agt tta att cc	pCambia1305.2, ←2371bp	HindIII
CH-F1	cac agc cga ag**C CTA GG**a ttg gca gga tat att cag ttg	pCVH, 1679bp→	AvrII
CH-R1	cgg ttt ctt cag **CCT AGG** ctc aaa ggc ggt aat acg	pCVH, ←4286bp	AvrII
CH-F7	ccg gcc agc t**A**g **AT**c **T**ct gaa gaa acc gag	pCVHS, 2125bp→	BglII
CH-R6	tga ggg tag g**TG ATC A**ct caa agg cgg taa tac ggt tat cc	pCVHS, ←5414bp	BclI

Supplementary Table B2. List of Names and sequences of custom-designed oligonucleotide primers used for DNA sequencing of two strands of pLSU-4.

Primer	Sequence (5'→3')	Location at template plasmid DNA
C-1F	tac aag ggg tgt tatgagcc	pLSU-4, 98bp→
C-1R	act gaa tcc ggt gag aat gg	pLSU-4, ←688bp
C-3F	ctc agg cgc aat cac g	pLSU-4, 559bp→
C-3R	tgc gct ctg ctg aag c	pLSU-4, ←1128bp
C-4F	tgc gcg taa tct gct gc	pLSU-4, 1029bp→
C-4R	aag gcc gcg ttg ctg g	pLSU-4, ←1621bp
C-5F	gcc acc tct gac ttg agc	pLSU-4, 1539bp→
C-5R	cgt cga tca cta cga agt cg	pLSU-4, ←2088bp
C-6F	acc gct aac cgt tgt cg	pLSU-4, 2003bp→
C-6R	acg ttg cag cct tgc	pLSU-4, ←2558bp
C-7F	cgc tgg ccg ctg aaa t	pLSU-4, 2437bp→
C-7R	tgc ctc gat gcg ttg c	pLSU-4, ←3009bp
C-8F	acg gtc gca aac cat c	pLSU-4, 2903bp→
C-8R	tcg tac gct gca tgg c	pLSU-4, ←3520bp
C-9F	acg ttg cgg acg tac tc	pLSU-4, 3407bp→
C-9R	tgc cct agc atc tgc tc	pLSU-4, ←3995bp
C-10F	gca agc tga tcg ggt c	pLSU-4, 3848bp→
C-10R	tgc tgc ata gca tgc g	pLSU-4, ←4427bp
C-11F	aaa tgg ctg gcc tac g	pLSU-4, 4346bp→
C-11R	tgt aga aac cat cgg cg	pLSU-4, ←4887bp
C-12F	tcg tgc ttt cag ctt cg	pLSU-4, 4814bp→
C-12R	tcg ctc cag tca atg acc	pLSU-4, ←5368bp
C-13F	tcg gct cca aca atg tc	pLSU-4, 5308bp→
C-13R	atg ggt ggc gat gtt tg	pLSU-4, ←5899bp
C-14F	tgt gat tga gtg tgt ctt gac	pLSU-4, 5782bp→
C-14R	ccg aca tta tcg cga g	pLSU-4, ←211bp



Supplementary Figure B1. Overall scheme of the construction of the binary vector pLSU1



NPT I gene (coding sequence (CDS) = 1162bp → 1977bp)

-Promoter region : Tn903 next to 5′CDS (←1163)

-35 -10

5′-aaagccacg ttgtgtctca aaatctctga tgttacaTTG CACAagataa aaataTATCA

Tcatgaacaa taaaactgtc tgcttacata aacagtaata caaggggtgt t NPTI CDS→

-Transcription termination : Tn903 next to 3′CDS (1978→)

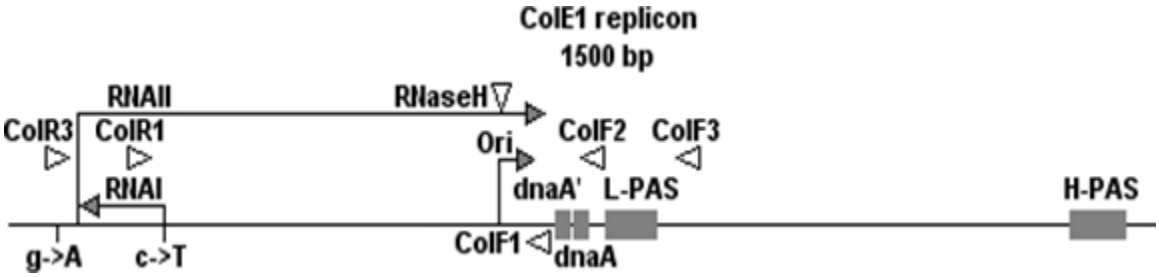
5′-NPTI CDS end tc agaattggtt aattggttgt aacactggca ggcattacgc

tgacttgacg ggacggcggc tttgttgaat aaatcgaact tttgctgagt tgaaggatca

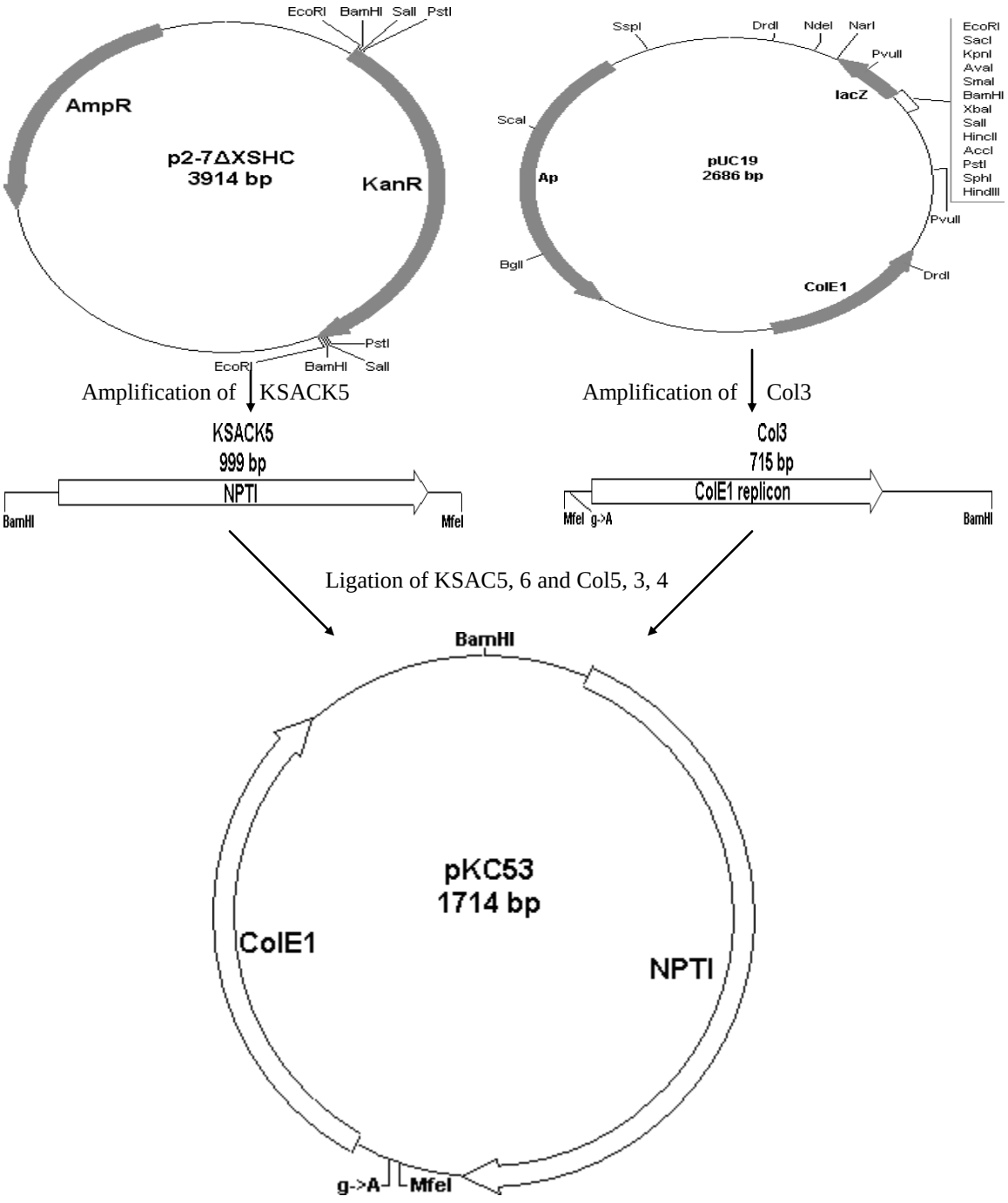
gatcacgcat cttcccgaca acgcagaccg ttccgtggca aagcaaaagt tcaaaatcac

caactggtcc

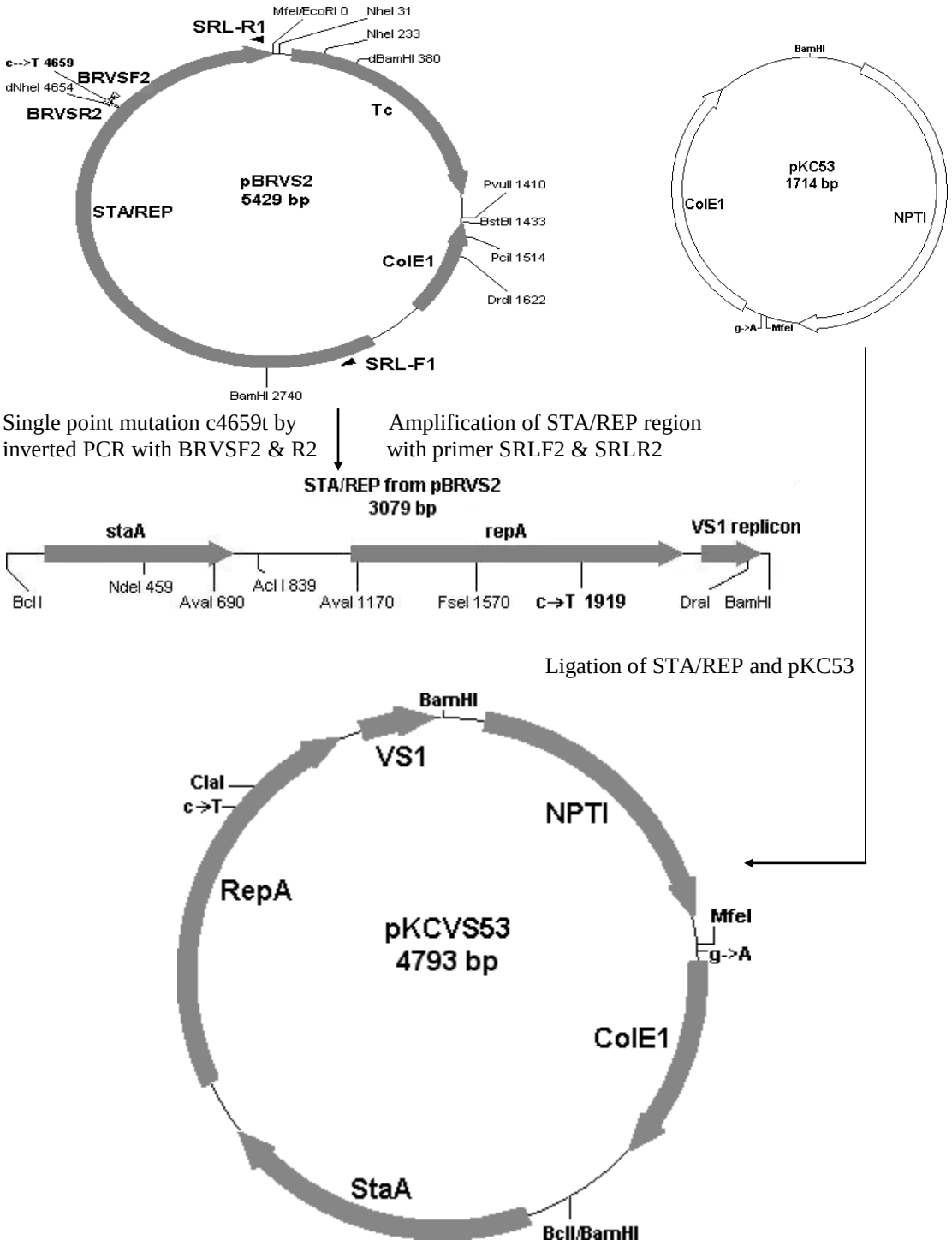
Supplementary Figure B2. Stem-loop structures of transcriptional termination region of NPTI gene in pUC4-KSAC and the location of primers for amplification. The figure includes the sequence of 1978-2120bp of pUC4-KSAC. The transcription initiation elements, -35 and -10 regions, are boxed letters and the sequences for secondary structures in the transcription termination region are italic and underlined.



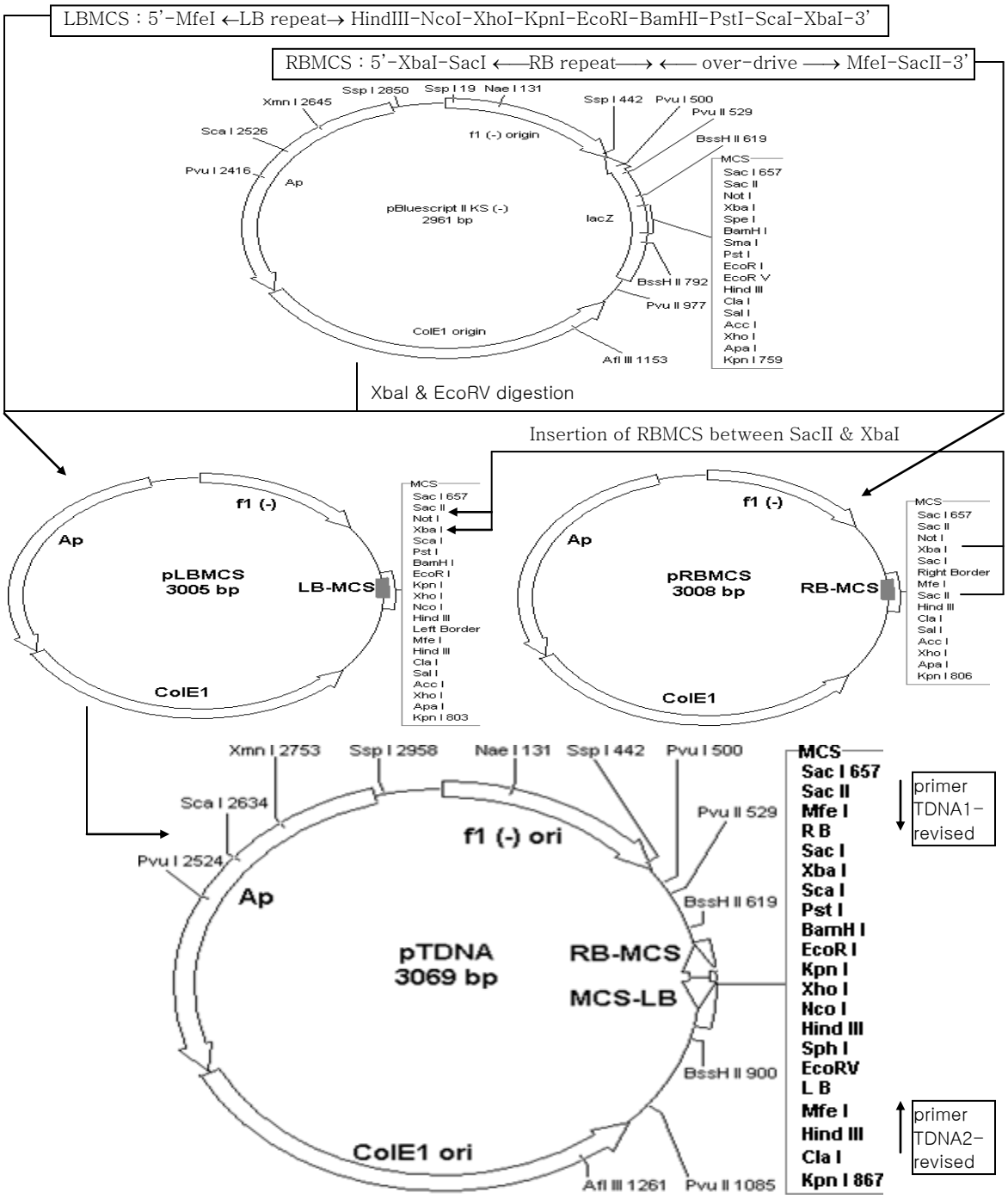
Supplementary Figure B3. Location of genetic elements of ColE1 replicon to be required for plasmid replication and the primers for amplification of modified ColE1 replicons Col3 and Col4 (715, 807bp). Both replicons include two single point mutations, g->A in -35 promoter region of RNAII and c->T immediately proceeding RNAI transcript. Replicon Col3 only includes up to dnaA boxes in the downstream of replication origin (Ori) and Col4 includes up to primosome assembly site for lagging strand.



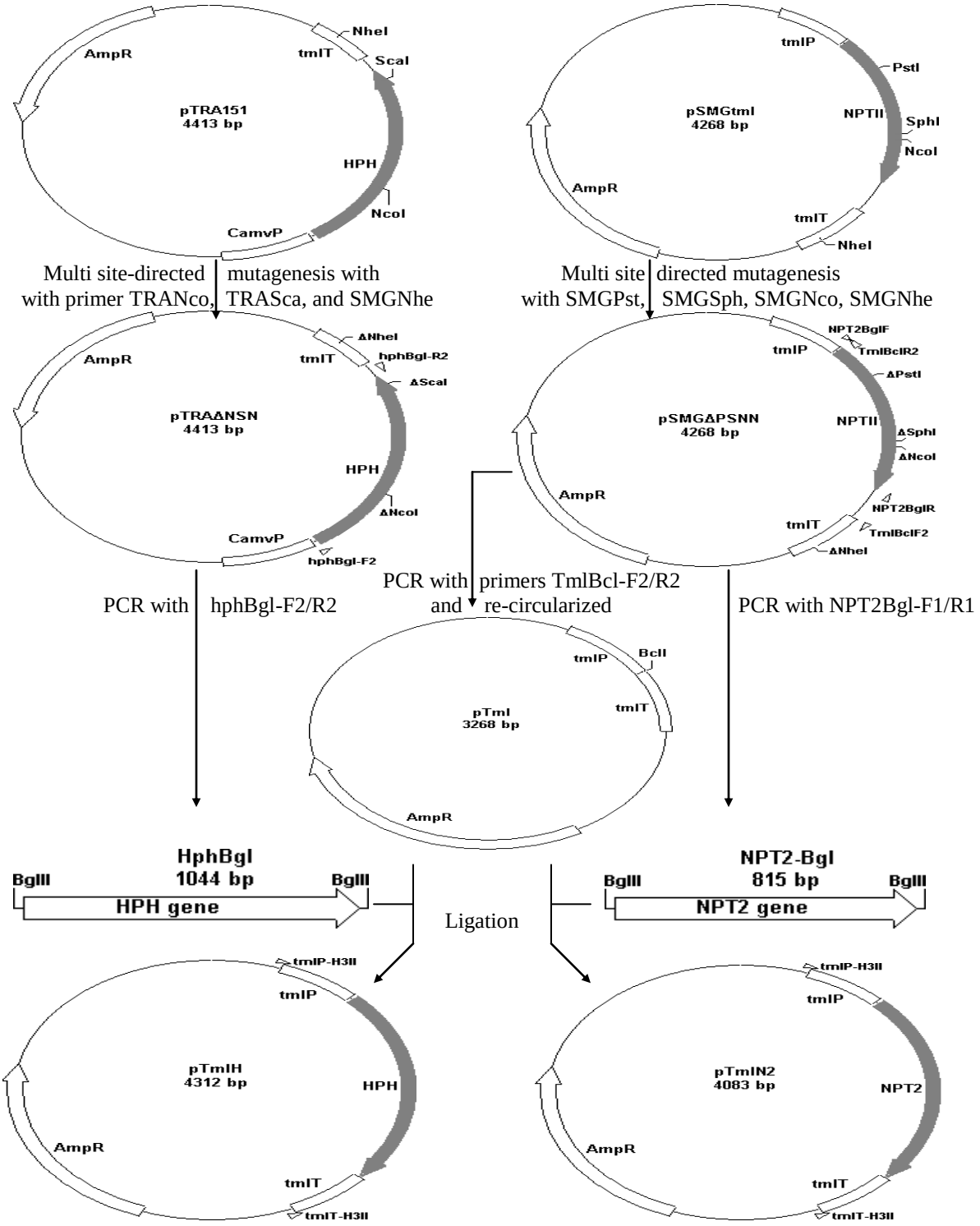
Supplementary Figure B4. Amplification of site-direct mutagenized Tn903 NPTI gene KSACK5, 6 (999, 1,056bp) and ColE1 replicon Col5, 3, 4 (626, 715, 807bp) to construct pKC55, 53, 54, 65, 63, 64 (1,625, 1,714, 1,806, 1682, 1771, and 1863), restectively. AmpR, ampicillin resistance gene; KanR, kanamycin resistance gene (NPTI); ColE1, ColE1 replicon.



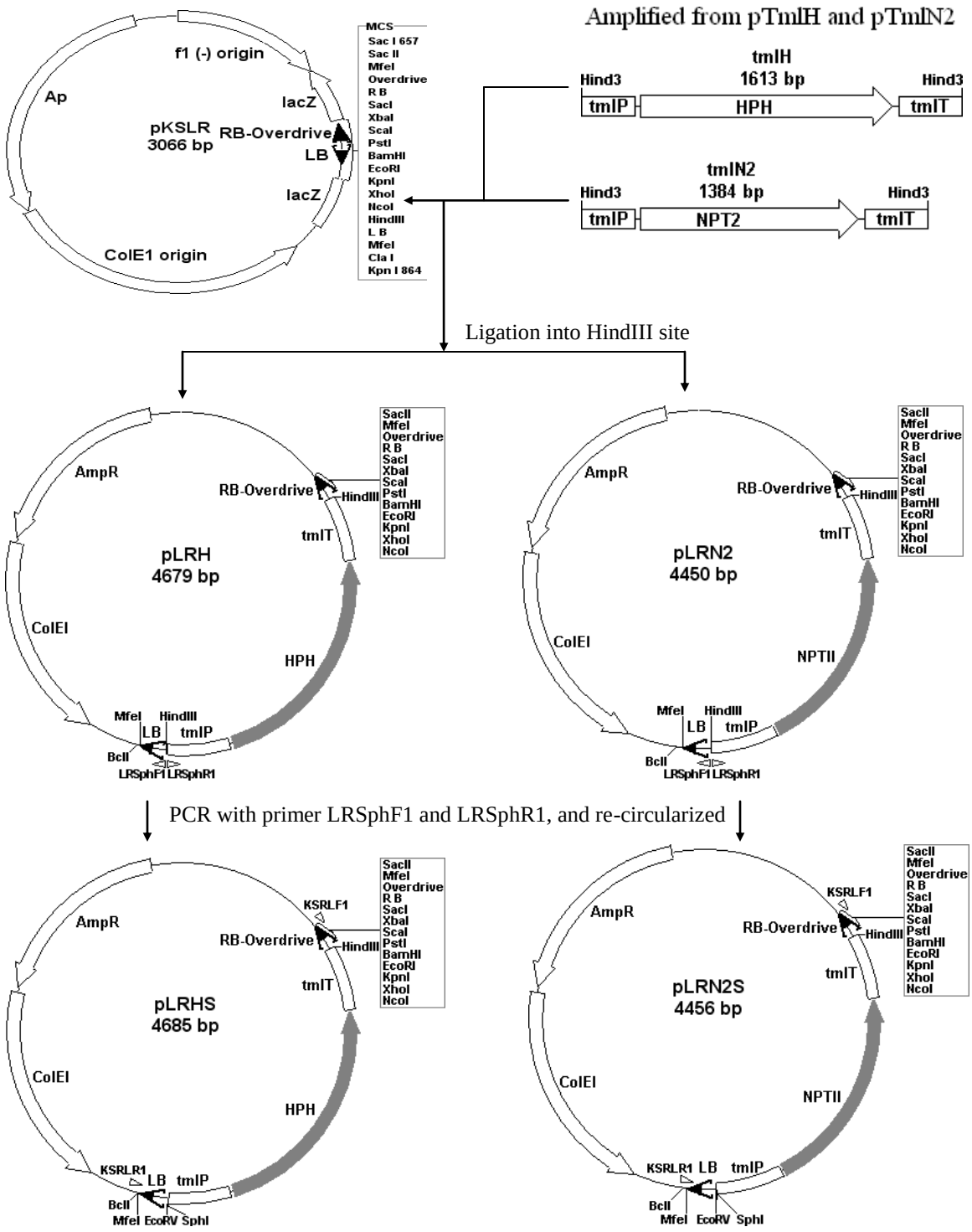
Supplementary Figure B5. Modifications of VS1 broad-rang replicon. A single point mutation c4659t was introduced to replace alanine with valine to increase the copy number by reverse PCR of pBRVS1 (5,429bp), producing pBRVS2. Amplification of mutated STA/REP fragment (3,079bp) and ligation with pKC53 and 54, producing pKCVS53 and 54 (4,793 and 4,887bp). StaA, stability A gene; RepA, Replication A gene; NPTI, kanamycin resistance gene.



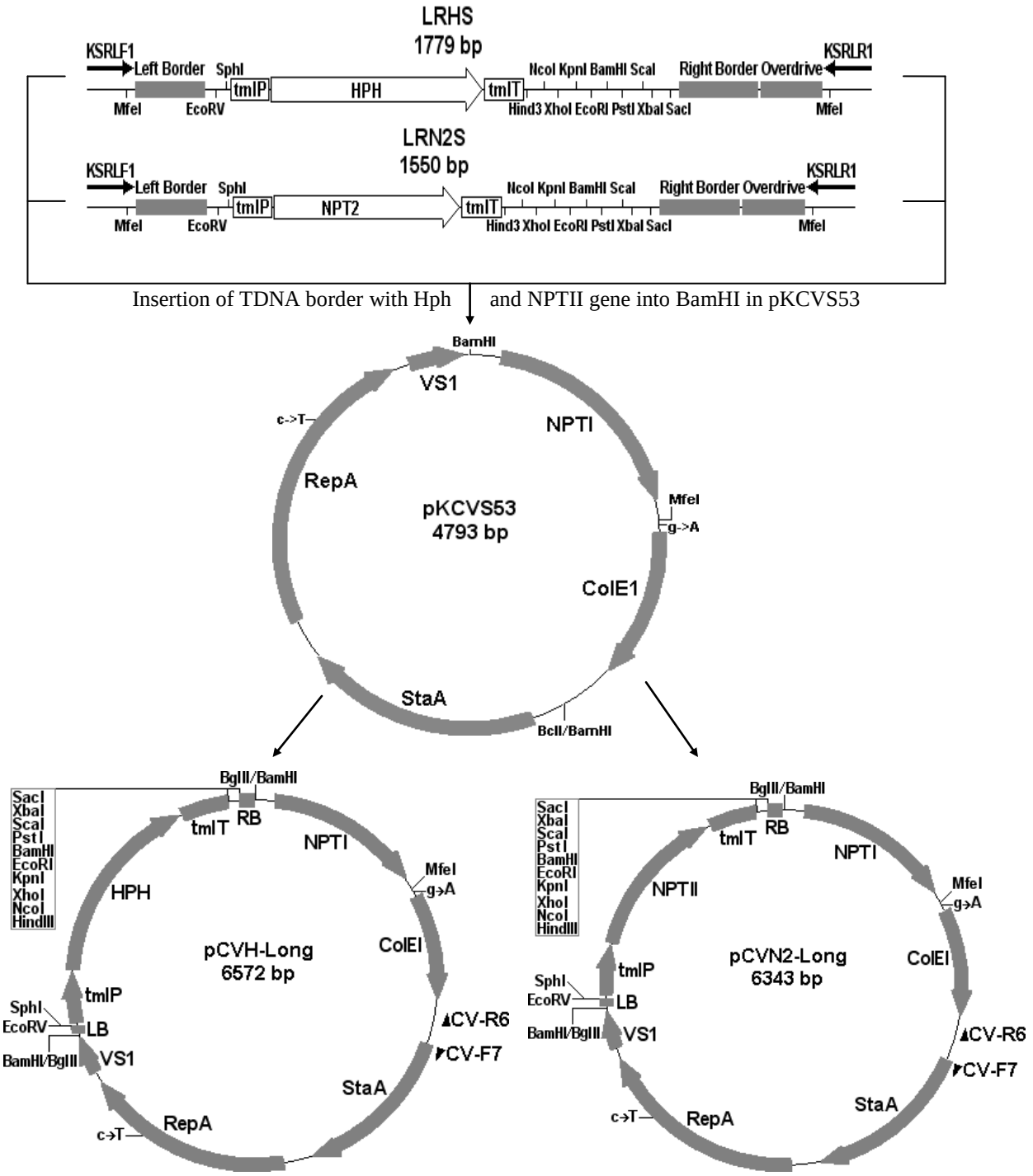
Supplementary Figure B6. Construction of T-DNA border sequences from pTi15955 with multi-cloning sites. A pair of oligonucleotides containing the left border (LB) sequence and multi-cloning site (MCS), and another pair of the right border (RB) sequence, overdrive and MCS was cloned into pBluescriptII KS(-) between XbaI and EcoRV among MCS, respectively. The right border-overdrive sequence of T-DNA and MCS, and the left border-MCS cloned separately into pBluescript were combined in pTDNA. AP, ampicillin resistance gene.



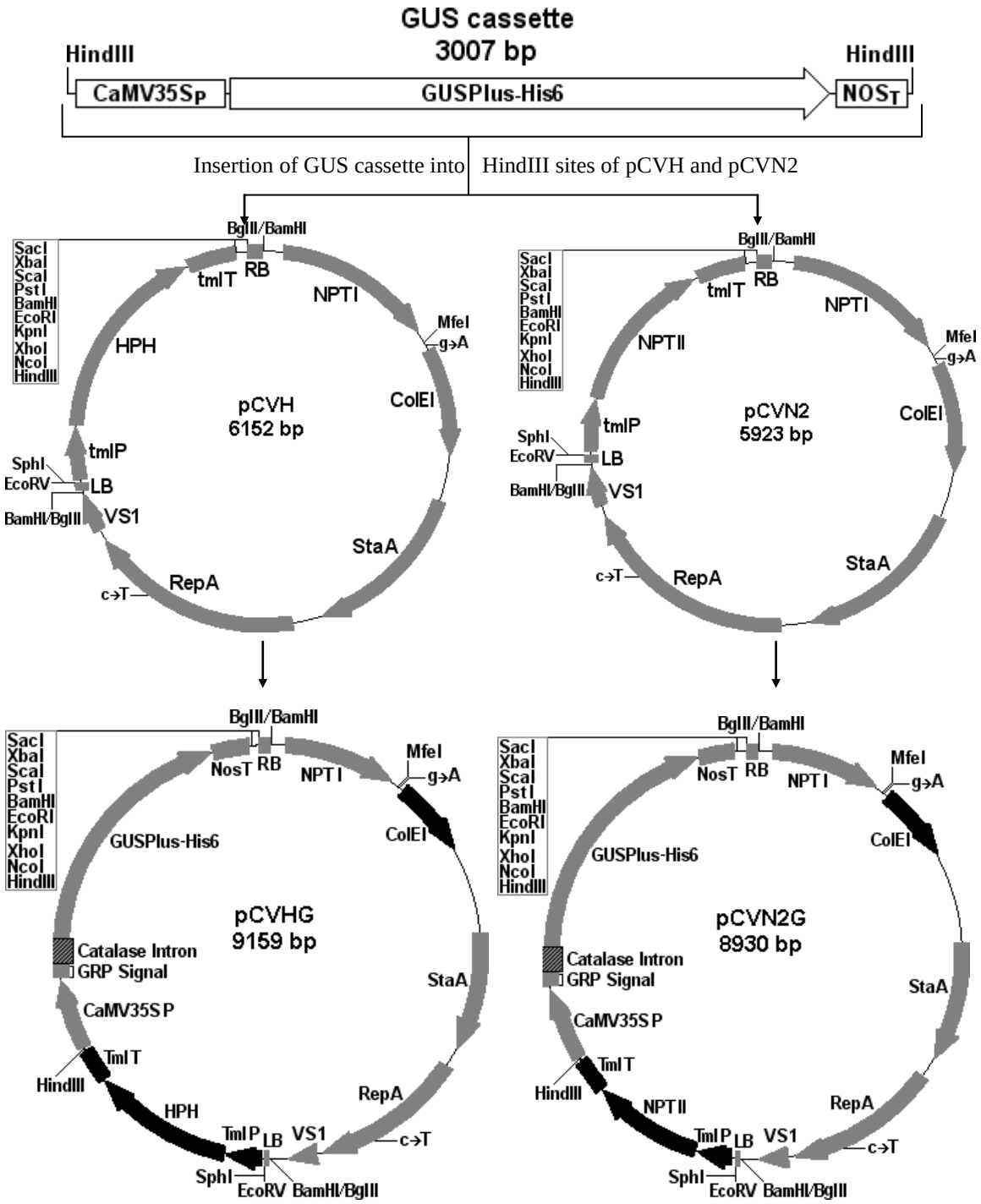
Supplementary Figure B7. Construction of plant selection markers, hygromycin resistance gene in pTmlH and kanamycin resistance gene in pTmlN2 (4,312bp and 4,083bp). Hph gene and NPTII gene were introduced between promoter and terminator regions of *tumor morphology large* gene (*tml*) from pTi15955.



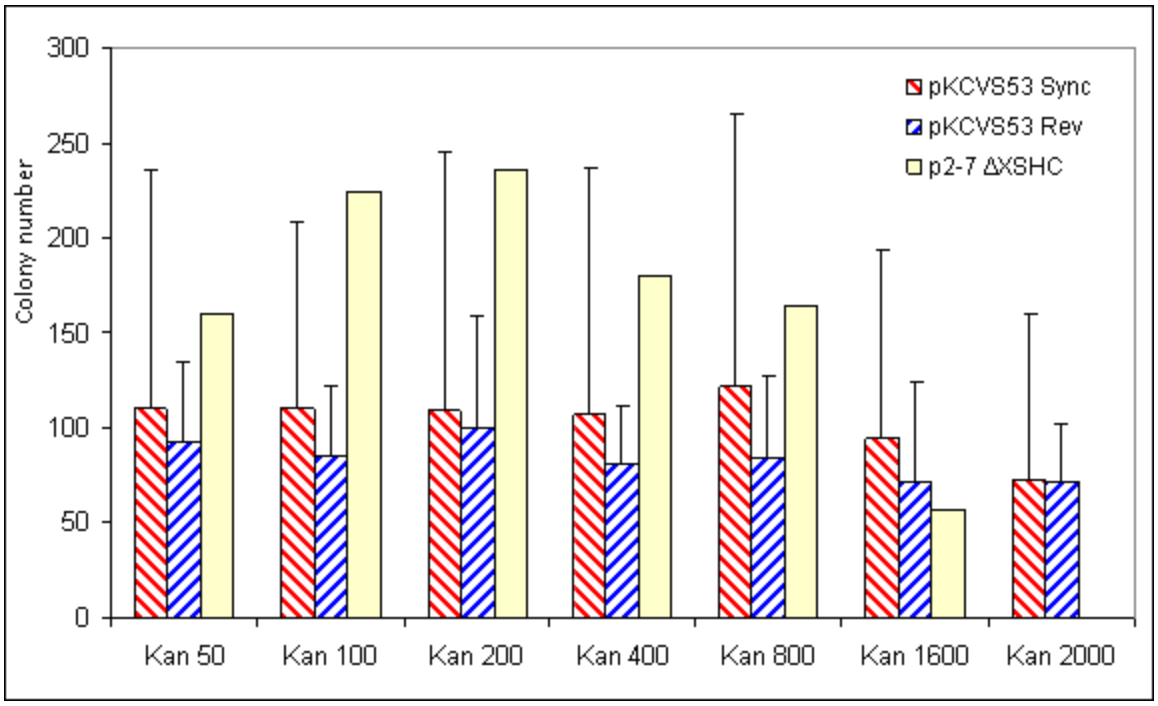
Supplementary Figure B8. Plant selection marker genes introduced to the T-DNA regions in pBluescript. Construction of pLRH, pLRN2 (4,679bp, 4,450bp). Plasmid pLRH and pLRN2 have hph and NPT2 gene inserts between HindIII sites. The new EcoRV and SphI sites were created by amplification with primer LRSphF1/R1.



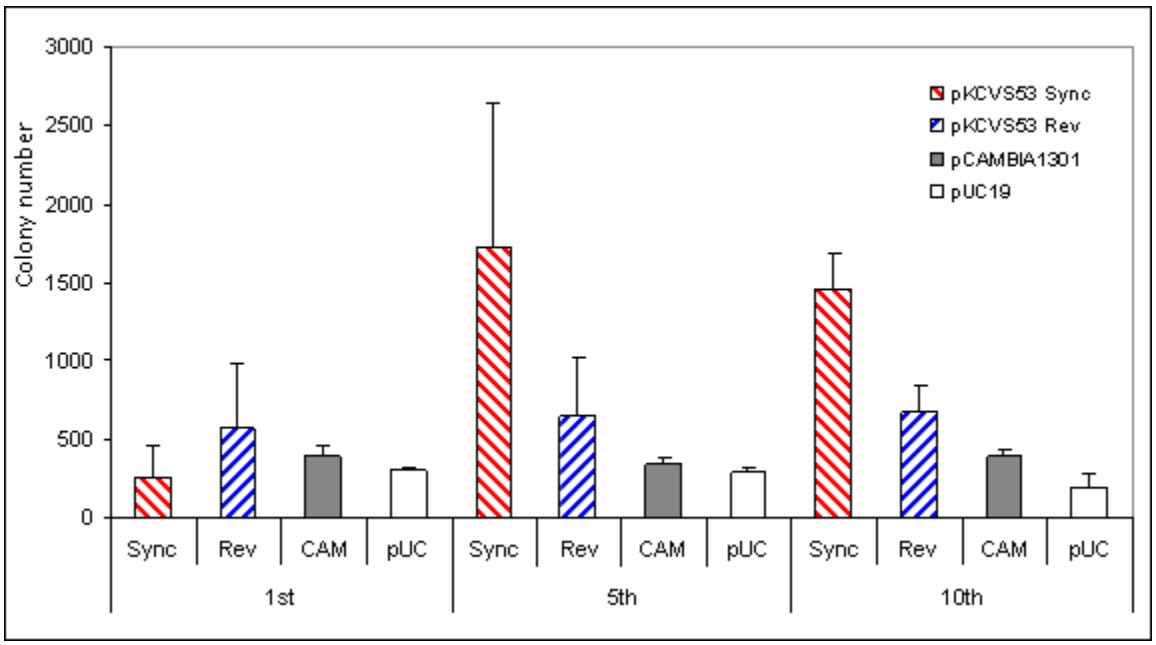
Supplementary Figure B9. Plant selection marker genes introduced to the T-DNA regions of binary vector pLSU. Construction of pCVH-Long and pCVN2-Long (6,572bp, 6,343bp). The binary vector pCVH-L has Hph gene as an eukaryotic selection marker and pCVN2-L has NPT II gene. tmlP/tmlT, *tumor morphology large* gene (*tml*) promoter/terminator.



Supplementary Figure B10. GUS reporter gene introduced to the T-DNA of pLSU vector. Construction of pCVHG and pCVN2G (9,159bp, 8,930bp). The GUS cassette was introduced to the binary vector pCVH and pCVN2 as a reporter gene. tmlP/tmlT, *tumor morphology large* gene (*tml*) promoter/terminator; CaMV35SP, cauliflower mosaic virus 35S RNA promoter; NosT,



Supplementary Figure B11. Kanamycin resistance test of two pKCVS53 constructs determined by the insertion direction of STA/REP fragment. Overnight cultures of pKCVS53-Sync, pKCVS53–Rev, p2-7ΔXSHC, and pCambia1301 grown until the optical density of 1/10 dilution reaches to 0.2 at 600 nm were plated on seven LB agar plates containing kanamycin 50, 100, 200, 400, 800, 1600, and 2000 μg/ml, respectively.



Supplementary Figure B12. Effects of transcriptional directions between the ColE1 and VS1 replicons (Co-directional sync or Head-on rev) were tested by plasmid maintenance experiments in *E. coli*. Nine single colonies of pKCVS53-Sync and pKCVS53-Rev, and three single colonies of pCambia1301 and pUC19 were picked and incubated in 20 ml of LB-Kan^{50} media (LB-Amp^{100} for pUC19) overnight at 37°C with 250 rpm. On the next day, 10 µl each from every overnight culture were put into 10ml LB with appropriate antibiotics and grown for 14 hrs at 37°C with 250rpm. This 14hrs incubation was repeated for ten consecutive times and after first, fifth, and tenth consecutive growth, each culture was diluted to 10^{-6}. 100 µl of each dilution were plated on the LB agar plate in the presence of appropriate antibiotics (Kan^{50} and Amp^{100}). All four plasmids were introduced in the same *E.coli* strain XL1Blue-MR.

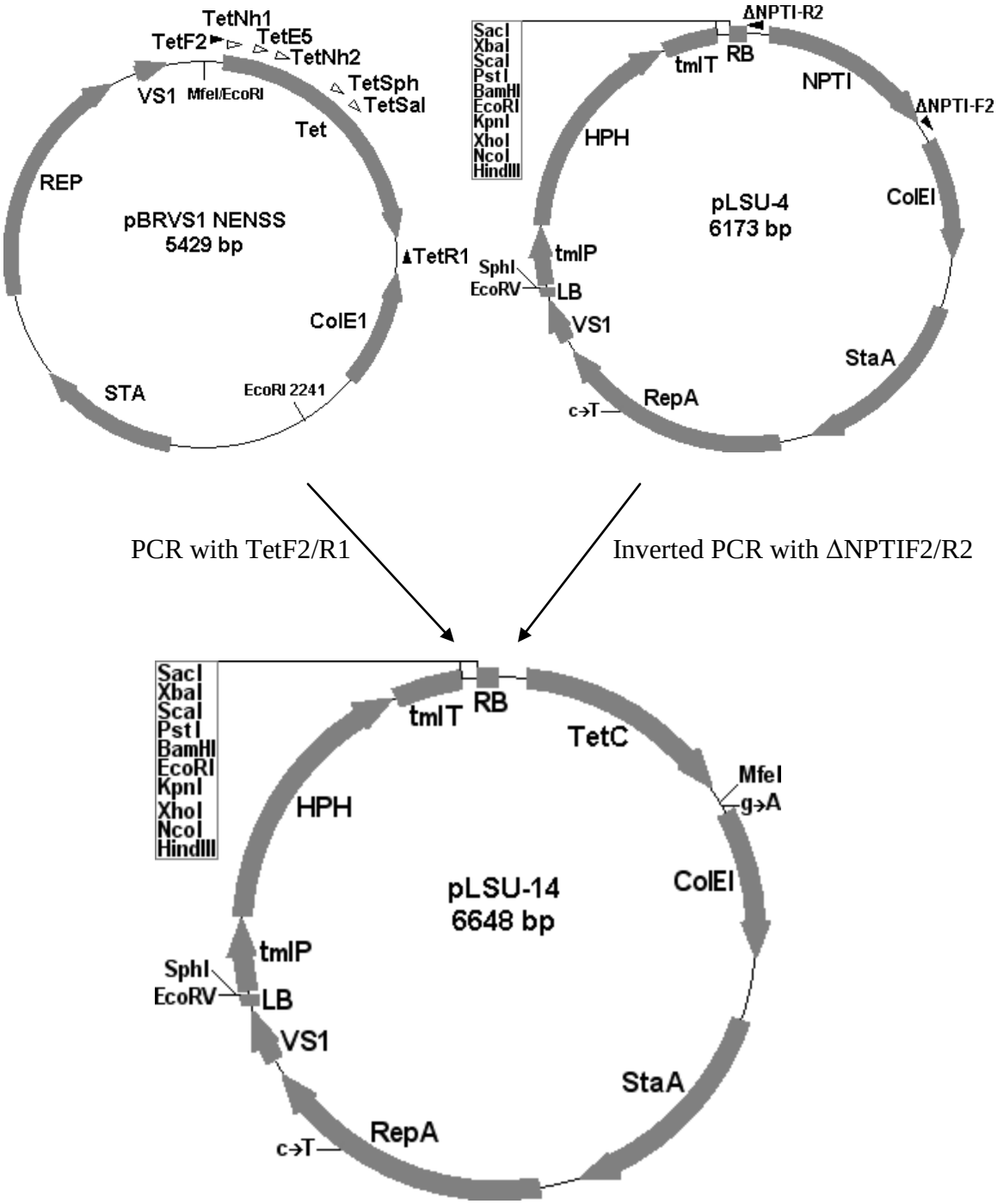
APPENDIX C. SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 3

Supplementary table C1. List of names and sequences of custom-designed oligonucleotide primers used for amplification or restriction enzyme site mutations. Locations at template plasmid DNA indicate the positions of first nucleotide of template plasmid DNA to be amplified. Bold Capital letters indicate the sites of mutations to be introduced. Underlined sequences are new restriction endonuclease recognition sites introduced or inactivated by the mutagenesis reactions.

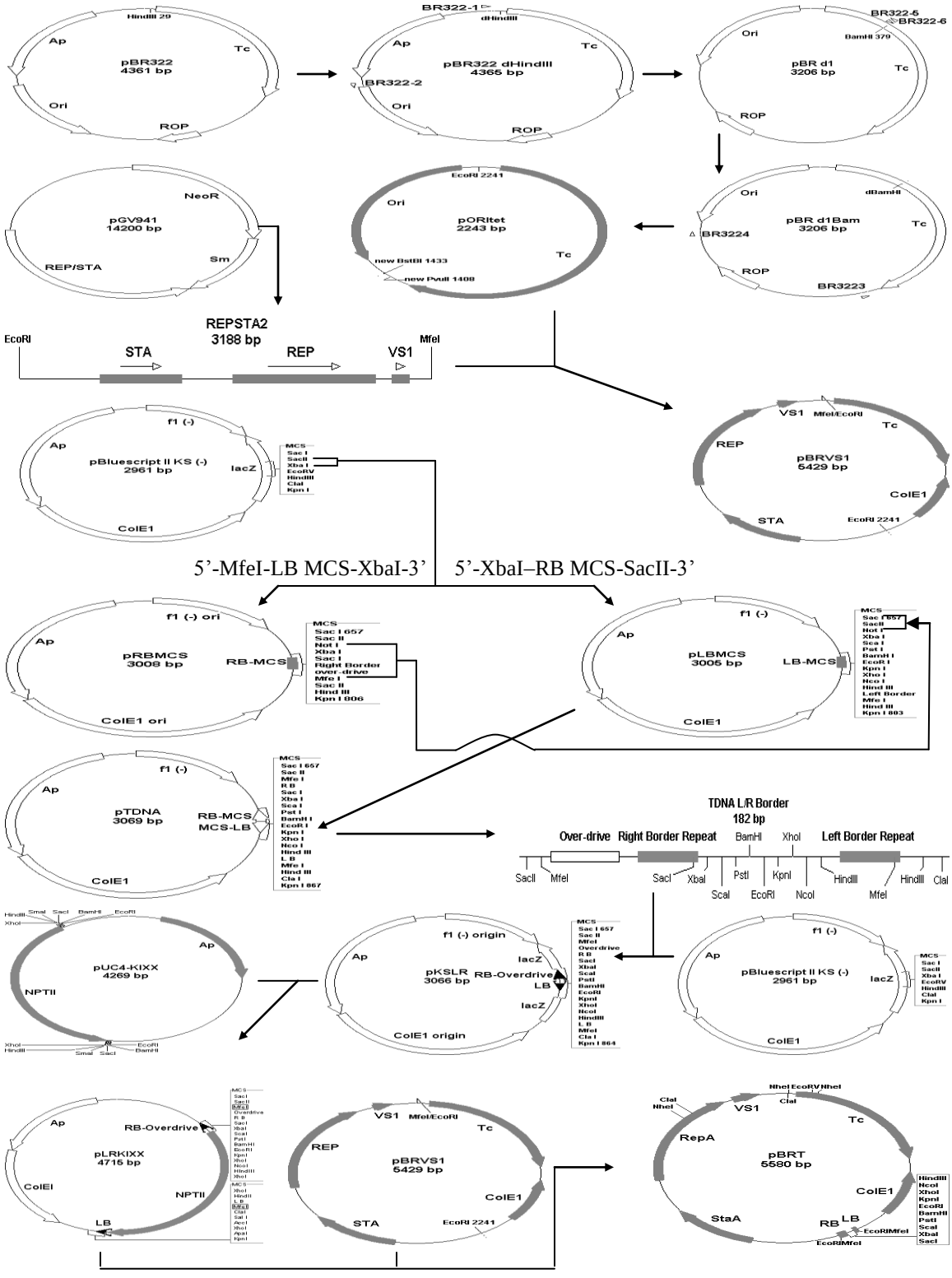
Primer	Sequence (5'→3')	Location at template plasmid DNA	Modified enzyme sites
BR322-1	aaa tag gcg tat cac gag gcc c	pBR322 dHindIII, 4329bp→	
BR322-2	tcc act gag cgt cag acc ccg	pBR322 dHindIII, ←3169bp	
BR322-3	ccg cgt gcg gc**A** gct gga gat ggc gg	pBR d1Bam, ←1423bp	PvuII
BR322-4	gct cgg tcg ttc g**AA** tgc ggc gag cg	pBR d1Bam, 2387bp→	BstBI
BR322-5	cgt cct gtg gat **T**ct cta cgc cgg	pBR d1, 371bp→	ΔBamHI
BR322-6	ggt gtg gtc gcc atg atc gcg	pBR d1, ←370bp	
TDNA-F1	ttg gag ct**T G**a**T** c**A**c ggc aat tgc a	pKSLR, 650bp→	BclI
TDNA-R1	tcg acg gta t**A**g atc ttg atc aat tgg cag g	pKSLR, ←843bp	BglII
TDNA-F2	gcc ctt tcg t**GA** tca aga att gca aac	pBRT, 2225bp→	BclI
TDNA-R2	agg gaa cgc t**A**g a**TC** tgg cag gat ata ttc	pBRT, ←2408bp	BglII
TetNh1	cat cga taa gct ag**G** ttt aat gcg gta gtt tat cac agt taa att gc	pBRVS2, 22bp→	ΔNheI
TetNh2	gtg ctg ct**T** gcg cta tat gcg ttg atg	pBRVS2, 228bp→	ΔNheI
TetE5	gcg gga tat **T**gt cca ttc cga cag c	pBRVS2, 185bp→	ΔEcoRV
TetSph	atc tcc ttg ca**C** gca cca ttc ctt gc	pBRVS2, 558bp→	ΔSphI
TetSal	aga gcg tcg **G**cc gat gcc ctt g	pBRVS2, 650bp→	ΔSalI
Tet-F2	aag ccg cgc cgt cgc **T**a**G C**tc tca tgt ttg aca gct tat ca	pBRVS2ΔNENSS, 5412bp→	NheI
Tet-R1	cgt att acc gc**T AGC** gag tga gct gat acc	pBRVS2ΔNENSS, ←1478bp	NheI
ΔNPT1-F2	ggc ggc ttt g**CC T**a**G G**ca att gat ctt ctt gaa atc c	pCVHS, 984bp→	AvrII
ΔNPT1-R2	tga gac aca acg t**CC TAG G**aa ttg caa aca aac aaa tac ag	pCVHS, ←19bp	AvrII

Supplementary Table C2. List of names and sequences of custom-designed oligonucleotide primers used for DNA sequencing of pLSU-12.

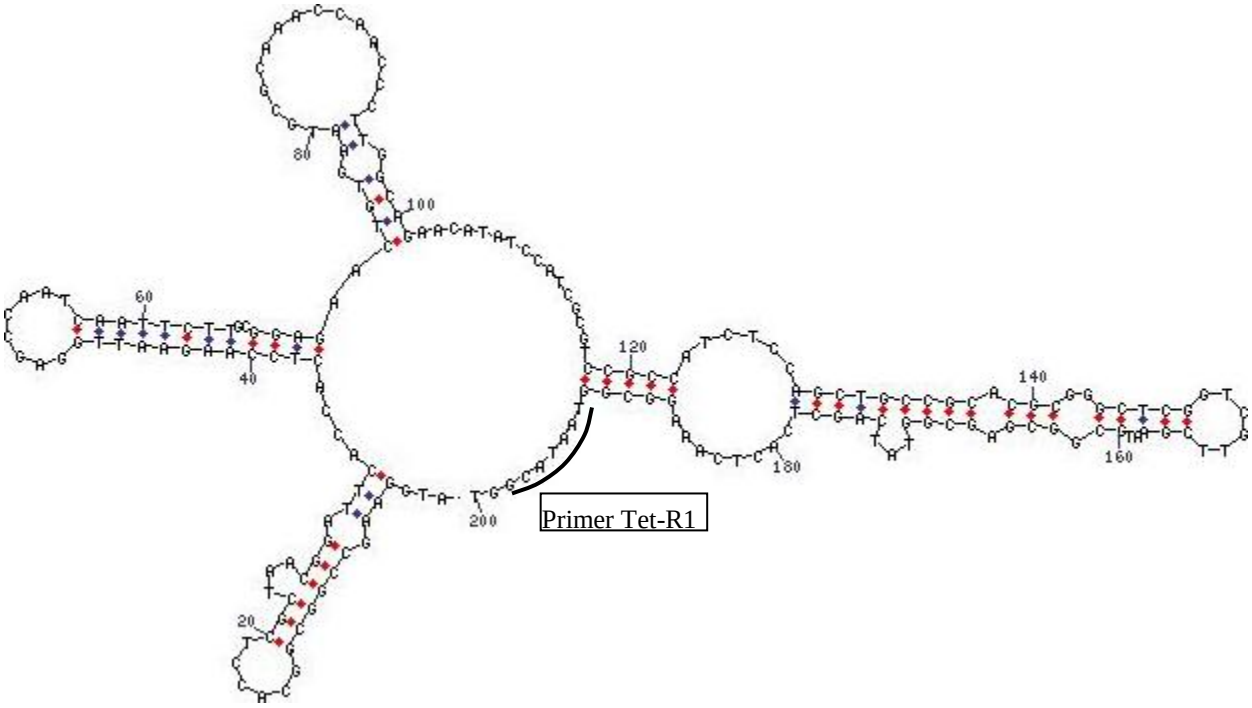
Primer	Sequence (5'→3')	Location at template plasmid DNA
T-1F	tgctaacgca gtcaggc	pLSU-12, 71bp→
T-1R	tgcgactcct gcattagg	pLSU-12, ←648bp
T-2F	actgttgggc gccatc	pLSU-12, 550bp→
T-2R	tgaagctgtc cctgatgg	pLSU-12, ←1104bp
T-3F	tgctgtccag gcaggtag	pLSU-12, 1062bp→
C-3R	tgcgctctgc tgaagc	pLSU-12, ←1615bp
C-4F	tgcgcgtaat ctgctgc	pLSU-12, 1501bp→
C-4R	aaggccgcgt tgctgg	pLSU-12, ←2093bp
C-5F	gccacctctg acttgagc	pLSU-12, 2011bp→
C-5R	cgtcgatcac tacgaagtcg	pLSU-12, ←2560bp
C-6F	accgctaacc gttgtcg	pLSU-12, 2475bp→
C-6R	acgttgcagc cttgc	pLSU-12, ←3030bp
C-7F	cgctggccgc tgaaat	pLSU-12, 2909bp→
C-7R	tgcctcgatg cgttgc	pLSU-12, ←3482bp
C-8F	acggtcgcaa accatc	pLSU-12, 3375bp→
C-8R	tcgtacgctg catggc	pLSU-12, ←3973bp
C-9F	acgttgcgga cgtactc	pLSU-12, 3880bp→
C-9R	tgccctagca tctgctc	pLSU-12, ←4468bp
C-10F	gcaagctgat cgggtc	pLSU-12, 4321bp→
C-10R	tgctgcatag catgcg	pLSU-12, ←4914bp
C-11F	aaatggctgg cctacg	pLSU-12, 4819bp→
T-11R	agccgattgt ctgttgtg	pLSU-12, ←5287bp
T-12F	ggattgcacg caggttc	pLSU-12, 5212bp→
T-12R	tccagatcat cctgatcgac	pLSU-12, ←5672bp
T-13F	acgtactcgg atggaagc	pLSU-12, 5628bp→
C-13R	atgggtggcg atgtttg	pLSU-12, ←6159bp
C-14F	tgtgattgag tgtgtcttga c	pLSU-12, 6042bp→
T-14R	tgactggcga tgctgtc	pLSU-12, ←225bp



Supplementary Figure C1. Construction of pLSU-14 (6,648bp). NPTI gene was eliminated by reverse PCR of pLSU-4 and the amplified fragment was ligated with Tet gene fragment amplified from pBRVS1ΔNENSS.



Supplementary Fig C2. Overall scheme of the construction of a mobilizable tetracycline-based binary vector pLSU-16



TetC gene

-Promoter region

-35

5′-ttctcatgtt tgacagctta tcatcgataa gctagctTTA ATGcggtagt ttatcacagt

-10

taaattgcta acgcagtcag gcaccgtgt TetC CDS start codon→

-Transcription termination region

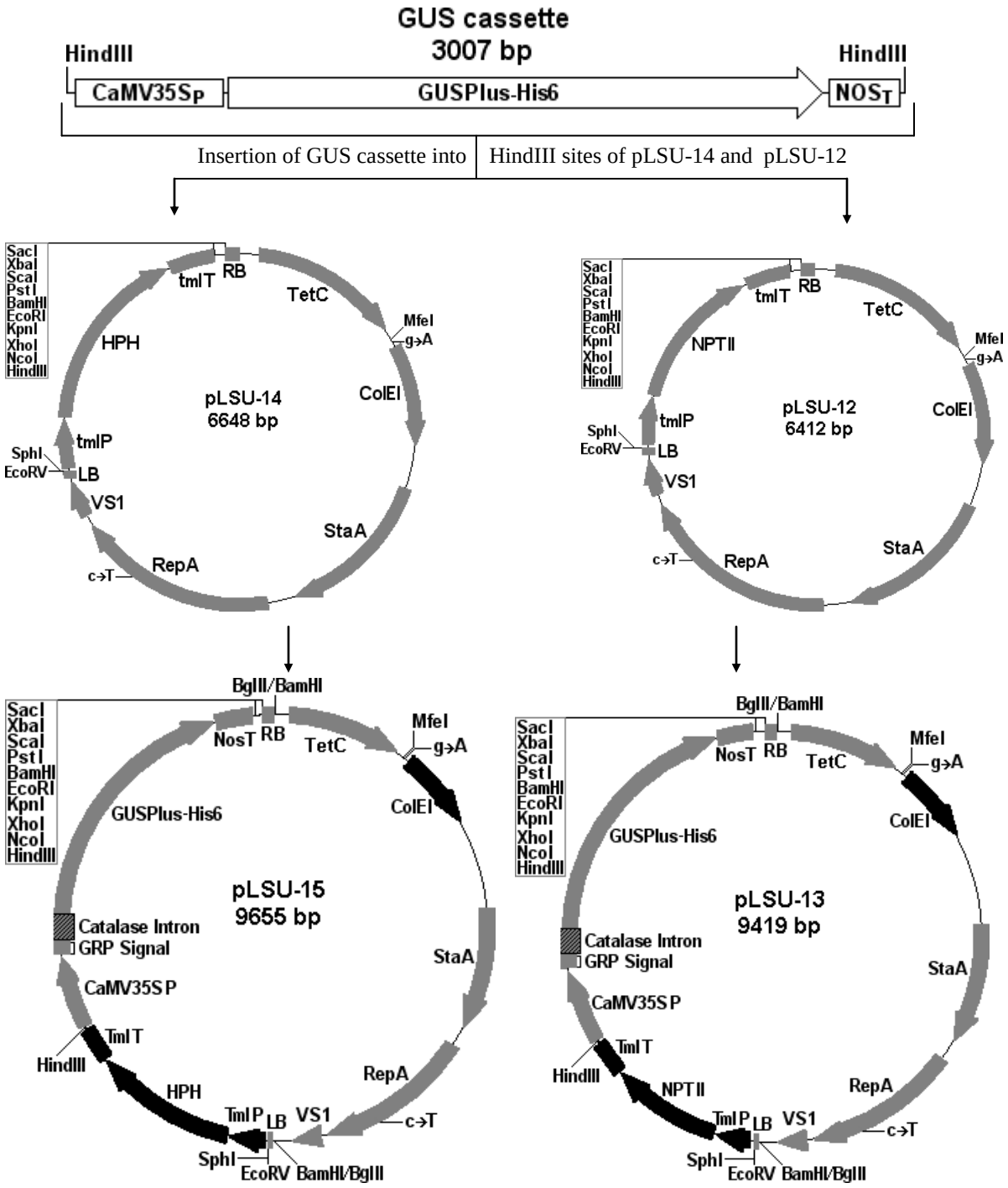
5′-TetC CDS end codon atg*gaagccg gcg*gcacct*c gctaacggat tc*acca*ctcc*

*aagaattg*ga gccaat*caat tcttgcggag* aactgtgaat gcgcaaacca acccttggca

gaacatatcc atcgcgtccg ccatctccag ctgccgcacg cgggctcggt cgttcgaatg

cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt

Supplementary Fig C3. Stem-loop structures of transcriptional termination region of TetC gene in pBRVS2 and the location of primers for amplification. The transcription initiation elements, -35 and -10 regions, are boxed letters and the sequences for secondary structures in the transcription termination region are italic and underlined.



Supplementary Fig C4. Construction of pLSU-13 and pLSU-15 (9,419bp and 9,655bp, respectively). The GUS cassette was insered in the *Hind*III site of the binary vector pLSU-12 and pLSU-14 as a reporter gene.

APPENDIX D. LOW CO-CULTIVATION TEMPERATURE AT 20^{O}C IMPROVED *A. TUMEFICEINT*-MEDIATED TRANSFORMATION OF TOBACCO LEAF DISKS

Authors: Guiying Su*, Sunjung Park*, Seokhyun Lee, and Norimoto Murai

* The first two authors contributed equally to this work and are both considered to be senior author.

Affiliation and address of the authors:

Department of Plant Pathology and Crop Physiology, Louisiana State University and LSU Agricultural Center, Baton Rouge, Louisiana 70803-1720, U.S.A.

Corresponding author: Norimoto Murai
E-mail address: nmurai@lsu.edu
Telephone: 1-225-578-1380
Fax: 1-225-578-1415

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Abstract

We were interested in evaluating the importance of temperature during the four-days co-cultivation period under the most physiologically relevant conditions that might be easily applied for plant transformation experiments. To reproduce the near-identical temperature and light conditions we selected Tissue Culture Chambers of Model CU-36L5 of Percival Scientific. The temperatures during the four-day co-cultivation period were from 15, 18, 20, 22 to 25^{o}C with the range of 0.7^{o}C under constant light (56 μE m^{-2} s^{-1}). We compared the effect of co-cultivation temperatures on the expression of β-glucuronidase (GUS) activity, and on the increase in the fresh weight yield of kanamycin-resistant calli.

Initially we chose to measure the expression of GUS activity to set up optimal experimental conditions for *A. tumefaciens*-mediated transformation of leaf disks of tobacco (*Nicotiana tabacum* L. cv. Xanthi (nn, Smith). Five experimental conditions were compared singly or in combination among the range of concentrations or of time durations, and the most optimal conditions were selected. Those are co-cultivation days, bacteria concentrations, surfactant Silwet L-77 concentrations, vacuum infiltration duration, and acetosyringone concentrations.

We concluded that temperature at 20 ℃ during co-cultivation of leaf disks with *A. tumefacines* is the most critical factor on the reproducible enhancement of the fresh weight yield and GUS reporter gene expression.

Key words *Agrobacterium tumeficeint*, co-cultivation temperature, fresh weight increase, GUS expression, tobacco leaf disks, transformation

Abbreviations

GUS *β*-glucuronidase

Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium and plant pathogen causing crown gall disease in angiosperms and gymnosperms (Smith and Townsend 1907). *Agrobacterium*-plant interaction was one of the first model systems in which the molecular mechanism for plant pathogenicity has been elucidated in details (Zaenen et al. 1974; Chilton et al. 1977). About 20 kb segment of DNA (T-DNA) in a tumor-inducing plasmid (ca. 200 kb Ti plasmid) is transferred from the bacterium to the host plant genome by a molecular machinery closely resembling to a bacterial conjugal transfer (Zupan and Zambriski 1985; Sheng and Citovsky 1996; Gelvin 2003). The disease phenotype is a manifestation of expression of bacterial T-DNA genes in plant cells that is over-production of plant growth hormones cytokinin and auxin.

This natural DNA transfer system has been exploited to introduce genes of agronomic interest into plants resulted in the production of genetically modified crops by commercial plant biotechnology industries. Initial approaches of gene transfer were to introduce a target gene into the T-DNA region of Ti plasmid after either a single- (co-integration) or double-homologous recombination between an intermediate vector (pRK290) and Ti plasmid (Zambriski et al. 1983; Murai et al. 1983). A binary plant vector strategy was designed to separate the T-DNA region in a small plasmid from the virulence genes in avirulent T-DNA-less Ti plasmid (Hoekema et al. 1983). The small plant vectors with the T-DNA region have been simply now called binary Ti vectors (Hellens et al. 2000; Komori et al. 2007).

A. tumefaciens-mediated transformation has been generally used for genetic transformation of higher plants since 1983. In a model plant *Arabidopsis thaliana* gene transfer became a routine using floral dip procedure (Clough et al. 1998). However, floral dip procedure does not work well for most other plants, and there are major technical barriers in transformation

of major crops such as soybean, maize, sugarcane and wheat. We had learned hard lessons when we attempted to generate a large number of transgenic tobacco plants to study the effect of 5'-deletion mutation on the promoter activity of the bean seed storage protein phaseolin gene (Burow at al. 1990 and 1992). We intended to generate a minimum of ten independent transgenic plants for each of seven deletion constructs, and ended up repeating tobacco transformation experiments eight times since we found out only two worked successfully to generate sufficient number of transgenic plants. At the moment we had no clue as to why we had to waste so much time and effort. Based on the results from our current experiments we now know why.

The importance of controlled temperatures during the co-cultivation period was not taken seriously at all in the above-mentioned transformation experiments in 1989. The small culture room (160 cm wide x 142.4 cm deep x 215 cm high) housed one metal shelf rack with four shelves (45 cm wide x120 cm deep) spaced 40 cm apart. Two fluorescent lamp fixtures each holding two 48 inch-long fluorescent lamps (Philip Westinghouse Lamps F40CW Cool White 40W) were hung from each shelve for constant light. The fluorescent lamps generated the heat in the small culture room resulting in the temperature gradient from the top to bottom shelves. While the room temperature of the building was maintained at around 24.5^{o}C, the temperature of the top shelve in the culture room could have been over 30^{o}C. Accordingly, we observed that the transformation of tobacco leaf disks were most effective at the bottom shelf, and least effective at the top shelf.

Here, we found that a co-cultivation temperature at 20℃ was one of the most critical factors on the reproducibility and consistency of kanamycin-resistance fresh weight increase and GUS activity expression after *A. tumefaciens*-mediated transformation of tobacco leaf disks. We believe the controlled temperature environment at 20^{o}C during the co-cultivation period might be one of the most critical conditions for efficient transformation of other higher plants.

Materials and methods

Tobacco seeds

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) seeds were obtained from E. Ted Woodlief, Department of Crop Science, North Carolina State University, Raleigh, NC. Two genotypes, nn (Smith) and NN with regard to the resistance to infection by tobacco mosaic virus were identified.

Tobacco plantlets grown *in vitro*

Tobacco seeds were sterilized by 50% (v/v) Clorox and planted per petridish on MS-media (Murashige and Skoog, 1962) (per L 4.4 g Sigma MS salts, 0.1g *myo*-inositol, 0.4mg thiamine⊕HCl, 30g sucrose, pH 5.6, 10g agar for solid medium). Seeds were germinated and plantlets were grown in the growth room under constant light with 61 μE m^{-2} s^{-1} at room temperature. Plantlets were subcultured at four week intervals.

Binary Ti vectors

New binary Ti vectors pLSU were constructed by Seokhyun Lee in this laboratory (Lee 2010). The pLSU-N2SGS vector contained a plant-expressible kanamycin-resistance gene and β-glucuronidase gene from pCambia1305.02. Commercial binary TI vectors pCambia1305.02 were purchased from Cambia (Canberra, Australia).

Agrobacterium tumefaciens

A. tumefaciens LBA4404 strain was purchased from Invitrogen, Carlsbad, CA. The bacteria was maintained at 28^{o}C in Agrobacterium media (A-media) (per liter 5.0 g yeast extract, 2.0 g mannitol, 2.0 g $(NH_4)_2SO_4$, 8.570 g K_2HPO_4, 4.192 g KH_2PO_4, 160 mg $MgSO_4.7H_2O$, 5.0 mg $FeSO_4.7H_2O$, 11.0 mg $CaCl_2.2H_2O$, and 2.0 mg $MnCl_2.4H_2O$).

Co-cultivation of tobacco leaf disks with *A. tumefaciens*

A. tumefaciens-mediated transformation of tobacco leaf disc is according to Burow et al. (1990) as modified by Park (2006). *A. tumefaciens* LBA4404 strains were grown overnight in the A medium at 28℃, 250rpm with appropriate antibiotics. Cells were harvested by centrifugation and resuspended in liquid MS media (per L 4.4 g MS salts, 30 g sucrose, pH 5.5) at the concentration of $3x10^7$ cells/ml (0.3 A_{600} units/ml). Leaf disks were cut from mature leaf of four week-old tobacco plantlets using a cork borer with 1cm inner diameter, mechanically wounded with multiple-needle devise, Kenzan from Stone Lantern (Passumpsic, VT) and soaked in 10 ml of *A. tumefaciens* inoculum containing 200 μM acetosyringone and 0.005 %(v/v) Silwet L-77 for 60 minutes. Inoculated leaf disks were blotted thoroughly on sterilized brown paper towels. Ten leaf disks were plated per petridish on the cocultivation media (per L 4.4 g MS basal salts, 1 mg nicotinic acid, 1 mg pyridoxine·HCl, 0.1 mg thiamine-HCl, 100 mg *myo*-inositol, 1mg 6-benzylaminopurine, 0.1 mg α-naphtalene-3-acetic acid, 30 g sucrose, 10 g agar at pH 5.6) containing 200 μM acetosyringone without any antibiotics.

Tobacco leaf disk transformation mediated by *A. tumefaciens*

After leaf disks were co-cultivated with *A. tumefaciens* at 20°C for four days under constant light with 56μE m-2 s-1, they were transferred to the shoot selection medium (the same media composition as cocultivation media) containing 500 mg/L carbenicillin and 300 mg/L kanamycin or 50 mg/L hygromycin. Leaf disks were incubated at 25 ± 0.5℃ under constant light for 14 days and then transferred to the fresh shoot selection media for another 14 days. At the end of 28 days selection the digital images of cultures were taken, the fresh weights were measured, and the calli and shoots were stored at -80^{o}C for β-glucuronidase assay.

Quantitative β-Glucuronidase Assay

β-Glucuronidase activity was measured by colorimetric assays (Jefferson 1987) as modified by Park (2006). GUS activity was measured with Jasco FP-6300 Spectrofluorometer (Jasco Co.,

Great Dunmow, UK) in the laboratory of professor Marcia Newcomer, Department of Biological Science, LSU. The wavelength was set to 365 nm for excitation and 455 nm for emission. The spectrofluorometer was calibrated with GUS stop buffer using quarz cuvettes. A standard curve was prepared using 0.005, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 μM 4-methylumbelliferone solution (MU). Relative intensity of MU light emission in sample solutions was read in order of 1, 15, 30, and 60 min. The amount of the GUS was calculated based on the established standard curve.

Protein concentration was determined by colorimetric assay using the *DC* protein assay kit (Bio Rad). Absorbance was read by an ELISA reader at 630 nm after 15 min of reaction at room temperature.

Statistical analysis

Each treatment has five plates with 10 leaf disks per plate. The data were subjected to one-way ANOVA program of Statistical Analysis System (SAS) software, SAS Online Doc 9.1.3 from SAS Institute (Cary, NC). Means were compared using either Fisher's least significant difference (LSD) test (α =0.05) (Figure 1) or Tukey's Studentized Range (HSD) test (α=0.05) (Figure 2).

Results

We were interested in evaluating the importance of temperature during the four-days co-cultivation period under the most physiologically relevant conditions that might be easily applied for most plant transformation experiments. To reproduce the near-identical temperature and light conditions we selected Tissue Culture Chambers of Model CU-36L5 of Percival Scientific. The temperatures during the four-day co-cultivation period were from 15, 18, 20, 22 to 25^{o}C with the range of 0.7^{o}C under constant light (56 μE m^{-2} s^{-1}). We compared the effect of co-cultivation temperatures on the expression of β-glucuronidase (GUS) activity (Figure 1), and on the increase

in the fresh weight yield of kanamycin-resistant calli (Figure 2).

β-Glucuronidase (GUS) activity assays for optimal conditions

Initially we chose to measure the expression of GUS activity to set up optimal experimental conditions for *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf disks. The details of methodology have been described by Burow et al. (1990) as modified by Park (2006). Unless otherwise specified, *Agrobacterium tumefaciens* strain LBA4404 containing pCambia1305.01 was grown overnight at 28^{o}C in a liquid media and the concentration was adjusted to 3x10^{7} cells/ml (0.3 A_{600} units/mL). Tobacco leaf disks were inoculated with bacteria under 50 mmHg vacuum infiltration for 20 min in the presence of the 0.001% (w/v) Silwet L-77. Leaf disks were co-cultivated in the presence of 200 mM of acetosyringone for four days under constant light at 20°C, transferred to selection media containing 50 mg/L hygromycin and 500 mg/L carbenicillin, and grown for 14 days under constant light at 25°C. The β−glucuronidase activity was measured at the end of growth period by quantitative GUS assay and GUS histochemical staining to compare the effect of experimental variables.

Five important experimental conditions were compared singly or in combination among the range of concentrations or of time durations, and the most optimal conditions were selected and shown by bold (Park, 2006). Co-cultivation days ranged from 2, 4 to 6 days; bacteria concentrations from 3x10^{5}, 3x10^{6}, 3x10^{7}to 10^{8} cells/ mL; surfactant Silwet L-77 concentrations from 0.001, 0.005, 0.05 to 0.1% (v/v); vacuum infiltration duration, from 1.5, 4, 20 to 40 min; and acetosyringone concentrations from 100, 200 to 400 μM.

After optimization of the five important experimental conditions we decided to compare the effect of temperatures during four day co-cultivation period, ranging from 15, 18, 20, 22 to 25°C to find the optimal temperature for maximum GUS expression. All GUS activity values were pooled from four separate experiments and plotted as dots to show the range of activity at

each temperature (Figure 1). Values were distributed from 20 to 2300 (pmol/mg protein/h). The highest average of GUS activity was obtained at 18 and 20°C among the five temperatures although the difference between the 18 and 20°C treatment was not statistically significant. The lowest average of GUS activity was observed at 25°C co-cultivation treatment. The GUS activity in 15 and 22°C treatment was an intermediate between these highest averages at 18 and 20 °C and the lowest average at 25°C, and was not statistically significantly different. However histological GUS assay showed that callus formation is more pronounced at 20°C than at 18°C (Park 2006). We concluded that co-cultivation at 20°C is the most effective condition for the enhanced GUS activity after transformation of tobacco leaf disks.

One limitation of the above results was that no statistically significant difference was obtained among the five co-cultivation temperatures tested, partly due to a wide distribution of GUS activities ranging form 20 to 2,300 pmo/mg protein/h. We observed during the vacuum infiltration period that significant portions of leaf disks were destroyed under the low vacuum and unable to produce calli resulting in undetable GUS activity. We rectified this limitation by substituting the vacuum infiltration with mechanical wounding using multiple-needles device Kensan routinely used in flower arrangement. This improvement appeared to reduce the statistical variables in the subsequent experiments.

Growth comparison by fresh weight increases

Our primary objective was to valuate the importance of temperature during the four-days co-cultivation period under the most physiologically relevant conditions that might be easily applied for most plant transformation experiments. We decided to use growth comparison assay of rigorously selected calli by antibiotic selection agent. The concentrations of kanamycin was tested ranging from 100, 250, 500, to 1,000 mg/L (Su 2010). The lowest concentration of kanamycin at 100 mg/L was sufficient enough to inhibit fully the growth of leaf discs of tobacco

cultivar Xanthi (nn Smith). We also tested the concentrations of a second selection agent hygromycin ranging from 25, 50, 100, to 200 mg/L. Hygromycin was more toxic than kanamycin and inhibited completely the growth of leaf disks at the lowest concentration of 25 mg/L and turned the color of leaf disks from green to yellow. On the basis of this result we chose the kanamycin concentration of 300 mg/L and the hygromycin concentration of 50 mg/L to provide a rigorous selection scheme that only transformed cells are selected for and survived. To show the effectiveness of the kanamycin concentration at 300 mg/L for rigorous selection we selected kanamycin-resistant calli and regenerated over 100 transgenic tobacco plants (Burow et al. 1992). The universal expression among transgenic plants of transferred bean seed storage protein β-phaseolin gene was demonstrated by RNA dot blot assay and protein expression by Enzyme-Linked Immunosorbent Assay.

The experimental conditions were essentially the same as described above except for the following two conditions; Leaf disks were cut from mature leaves of four week-old tobacco plantlets grown *in vitro*, and mechanically wounded using a multiple-needle device. Wounded leaf disks were first co-cultured in liquid for 60 min with *A. tumefaciens* containing a new binary Ti vector pLSU suspended at the concentration of $3x10^7$ cells/ml (0.3 A_{600} units/ml) in 10 ml of MS salts containing 30 g/L of sucrose, 200 μM acetosyringone and 0.005 %(v/v) Silwet L-77. Co-cultured leaf disks were transferred to the shoot selection medium containing 500 mg/L carbenicillin and 300 mg/L kanamycin. Leaf disks were incubated at 25 ± 0.7℃ under constant light with 56μE m-2 s-1 for 14 days and then transferred to the fresh shoot selection media for additional 14 days. At the end of 28 days growth period digital images of leaf disk growth were taken, the fresh weight yields were measured in mg, and leaf disks were stored at -80^{o}C for subsequent assay for β-glucuronidase (GUS) activity.

The temperatures during the four-day co-cultivation period were from 15, 18, 20, 22 to 25^{o}C with the range of 0.7^{o}C under constant light (56 μE m^{-2} s^{-1}). The results from the temperature experiments were summarized as shown in Table 1, and Figures 2 and 3. We found the four-days co-cultivation at 20^{o}C resulted in the highest increase in the plate average of fresh weight yields. The 20^{o}C treatment was statistically significantly better than the 15 and 18^{o}C treatments. The 20^{o}C co-cultivation provided higher FW yield than 22 and 25^{o}C even though the differences were not statistically significant. The percentage of leaf disks with the increased FW yield indicated the proportion of leaf discs with presumably transformed calli and shoots. The 20^{o}C treatment showed 82% of leaf disks with the increased FW yield which is significantly higher than 50 and 68% by the 15 and 18^{o}C treatment, respectively (Table 1). The 22 and 25^{o}C treatment gave 78 and 97.9 %, respectively, of leaf disks with the increased FW yield.

Discussion

We found that co-cultivation temperature at 20℃ is the most critical environmental factor to achieve the reproducibility and consistency of the increase in the fresh weight yield and GUS gene activity after *A. tumefaciens*-mediated transformation of tobacco leaf disks. Among the five temperatures from 15, 18, 20, 22 to 25℃ tested, co-cultivation at 20℃ resulted in statistically greater growth than that at 15 and 18℃. Statistically distinct and higher growth was observed after co-cultivation at 20℃ than at 22 and 25℃. The results demonstrated that four days co-cultivation with different temperature treatments determined the outcome of 28 days growth of transformed tissues after selection. Temperature profile of fresh weight yield increase is in agreement with thermosensitivity profile of plasmid transfer during transconjugation and of stability of pili that is a channel structure responsible for plasmid transfer and probably T-strands. Although 18 and 20 °C co-cultivation condition is the best condition for bacterial pili formation,

but is not for favorable condition for the tobacco leaf disks. Tumor induction studies have been routinely performed in the range 25 to 28 °C because it has been reported that tumor are mostly formed over 25 °C in *Nicotiana* (Ricker et al., 1941).

The temperature profile during the co-cultivation period in our experiments is different from those reported for GUS activity previously. Transient expression of quantitative GUS activity in *Phaseolus acutifolius* embryogenic callus had a peak at 22℃ in a range of co-cultivation temperature from 15, 19, 22, 25, 27 to 29^{o}C. Histochemical staining of GUS activity was also the highest at 22^{o}C for both *Phaseolus* calli and *Nicotiana tobacum* (Dillen et al., 1997). For hypocotyls explants of cauliflower transformation, 22℃ co-cultivation resulted in the highest GUS expression, and no GUS expression was detected at 28℃ (Chakrabarty et al., 2002). GUS expression in *Phaseolus acutifolius* transformation experiment also showed 22℃ co-cultivation is optimal condition to increase transformation efficiency (De Clercq et al., 2002). In an agreement with this study, all these previous experiments reported that GUS expression was reduced dramatically at co-cultivation temperatures over 25°C.

The lower optimal co-cultivation temperature in this study may be partly accounted for by the differences between stable vs transient expression, and also the difference in co-cultivation and selection conditions employed. We measured the increase in fresh weight yield of stable transformants, and have applied the most physiologically relevant conditions during the co-cultivation and selection period using identical tissue culture chambers of Model CU-36L5 of Percival Scientific. Leaf disks were placed for four days on MS shoot media in a Petri dish under constant light (56 μE m^{-2} s^{-1}) with the temperature range of $\pm$0.7^{o}C. Selection for transformed cells were conducted for 28 days on MS shoot media containing kanamycin and carbenicillin in a Petri dish under constant light at 25^{o}C $\pm$0.7^{o}C. In comparison, Dillen at al. (1997) had used less than physiologically appropriate conditions during the co-cultivation, no selection of tissues and

shorter culture period before GUS assay. Leaves of *Nicotiana tabacum* cv SR1 Petit Havana were infiltrated with 0.8 A_{600} unit of *Agrobacterium tumefaciens* under reduced pressure for 20 min, placed on a filter paper in a Petri dish which was sealed in a plastic bag, and submerged for three days under water in a water bath. Calli of *Phaseolus acutifolius* were placed in a glass jar submereged under water in a water bath. After two days co-cultivation, treated calli were cultured on non-selected medium for four days prior to GUS assay. Consequently, the reported GUS activity was significantly lower than this study and had no standard deviation indicated. We assumed a minimum effect exerted by the difference in binary vectors, helper plasmids or strains of *Agrobacterium tumefaciens* used in this and previous experiments.

Riker (1926) made an initial observation of important role of temperature on the formation of crown gall tumors in tomato and other plants. The size of tumors was largest at the optimal temperature of 22^{o}C and was gradually decreased as the incubation temperature increased to below 28^{o}C. No tumor was formed at or above 30^{o}C. Braun (1947) used this thermo-sensitivity to distinguish two phases of crown gall tumor formation between the temperature-sensitive inception phase and the temperature-insensitive development phase. The autonomous neoplastic growth of transformed cells was achieved only after plant cells reached to the second development phase. The inception phase was further divided to two processes, the thermo-sensitive induction process and the thermo-insensitive conditioning process. The conditioning of plant cells was initiated by wounding, took places either below or above 30^{o}C, and improved during the 48 hr period after wounding up to the time the first cell division was observed. Thus, Braun identified the thermo-sensitivity of the induction process in which Tumor Inducing Principal (TIP) transfers from bacteria to plant cells. The nature of Braun's TIP was later elucidated as T-strand of transfer DNA (T-DNA) of tumor-inducing plasmid (Ti-plasmid), and the components of T-DNA transfer machinery was further identified.

The thermo-sensitivity of conjugative transfer of Ti plasmid from the virulent to cured avirulent strains of *A. tumefaciens* was noted by Tempe et al. (1977). The frequency of plasmid transfer was 4.0 and 3.5 x 10^{-2} at 23^{o}C and 27^{o}C, respectively, reduced by four-fold to 0.9 x 10^{-2} at 30^{o}C, and went below 10^{-6} at 33 and 36^{o}C. IncQ group plasmid pML122 is a non-conjugative plasmid but can utilizes a transfer machinery of conjugative Ti plasmid of *A. tumefaciens*. Conjugal transfer of the IncQ plasmid mediated by *A. tumefaciens* was found thermosensitive (Fullenr, Nester 1996b). The frequency of conjugal transfer was the highest 2.6 x 10^{-3} at the optimal temperature of 19^{o}C, gradually reduced to 1.1 x 10^{-3} at 22^{o}C, 5.3 x 10^{-4} at 25^{o}C, and 2.0 x 10^{-4} at 15^{o}C, and then drastically decreased by over 1,000-fold at 28 and 31^{o}C. The temperature profile of conjugal transfer of IncQ plasmid is more similar to that of crown gall tumor formation (Riker, 1926) than that of conjugal transfer of Ti plasmid (Tempe at al., 1977). The similarity of thermo-sensitivity profile between crown gall formation and conjugal transfer of IncQ plasmids suggests that the same machinery may involve in transfer of T-strands from the bacteria to plant cells, and in conjugal transfer of these plasmids between bacteria.

The thermo-sensitivity of bacteria pilus assembly was observed by Fullner and Nester. (1996a and b). The induction by 200 μM of acetosyringone of *vir* genes was essential for the pilus assembly. The pilated cells constituted 10 to 20% of cells observed under electron microscope at 19^{o}C and were over ten-fold more abundant than those at 28^{o}C. The loss- and gain-of-function experiments demonstrated that the same collection of *vir* genes, *vir*A, *vir*G, *vir*B1 through *vir*B11, *vir*D4 were required for the pilus production and conjugal transfer of plasmid. The additional *vir* gene, *vir*E1 required for transport of *vir*E2 was necessary for tumor induction on leaves of *Kalanchoe daigremontiana*. The results suggest that the assembly of pilus on *A. tumefaciens* is essential for both processes, transfer of T-strands from the bacteria to plant cells and conjugal transfer of plasmid between bacteria.

Distinct profiles of temperature-dependence were evident between the expression of *vir* B genes and conjugal transfer of plasmid. The induction of *vir*B genes was found optimal at 25^{o}C and the conjugal transfer of plasmid optimal at 19^{o}C. Baron et al., (2001) reported that highest amount of pili and pili component VirB were detected at 20 °C, and small amount of pili was detected at 26 °C while pili formation at 28°C was strongly inhibited. They also reported that tumor formation was induced at 20 °C at wounded *K. diagremontiana*. It can be concluded that 20 °C is optimal temperature to induce vigorous pili formation, and to maintain plant tissue viability.

Table 1. Co-cultivation temperature at 15, 18, 20, 22 and 25°C were compared in its effect on the final fresh weight yield of tobacco leaf disks. Leaf disks were co-cultivated for four days with *Agrobacterium tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate. Numbers in parentheses indicate standard deviations.

Co-cultivation temperatures	Plate Average of FW yield in g	Increase in FW in g per plate over vectorless control	% of leaf discs with increased FW
15 °C pLSU	2.62 (1.30)	1.99 (1.19)	50
18°C pLSU	3.31 (1.87)	2.59 (1.85)	68
20 °C pLSU	10.48 (5.91)	9.74 (5.91)	82
22 °C pLSU	6.97 (2.76)	6.24 (2.74)	78
25 °C pLSU	7.23 (3.85)	6.48 (3.88)	98
20°C control	0.58 (0.21)	0	0

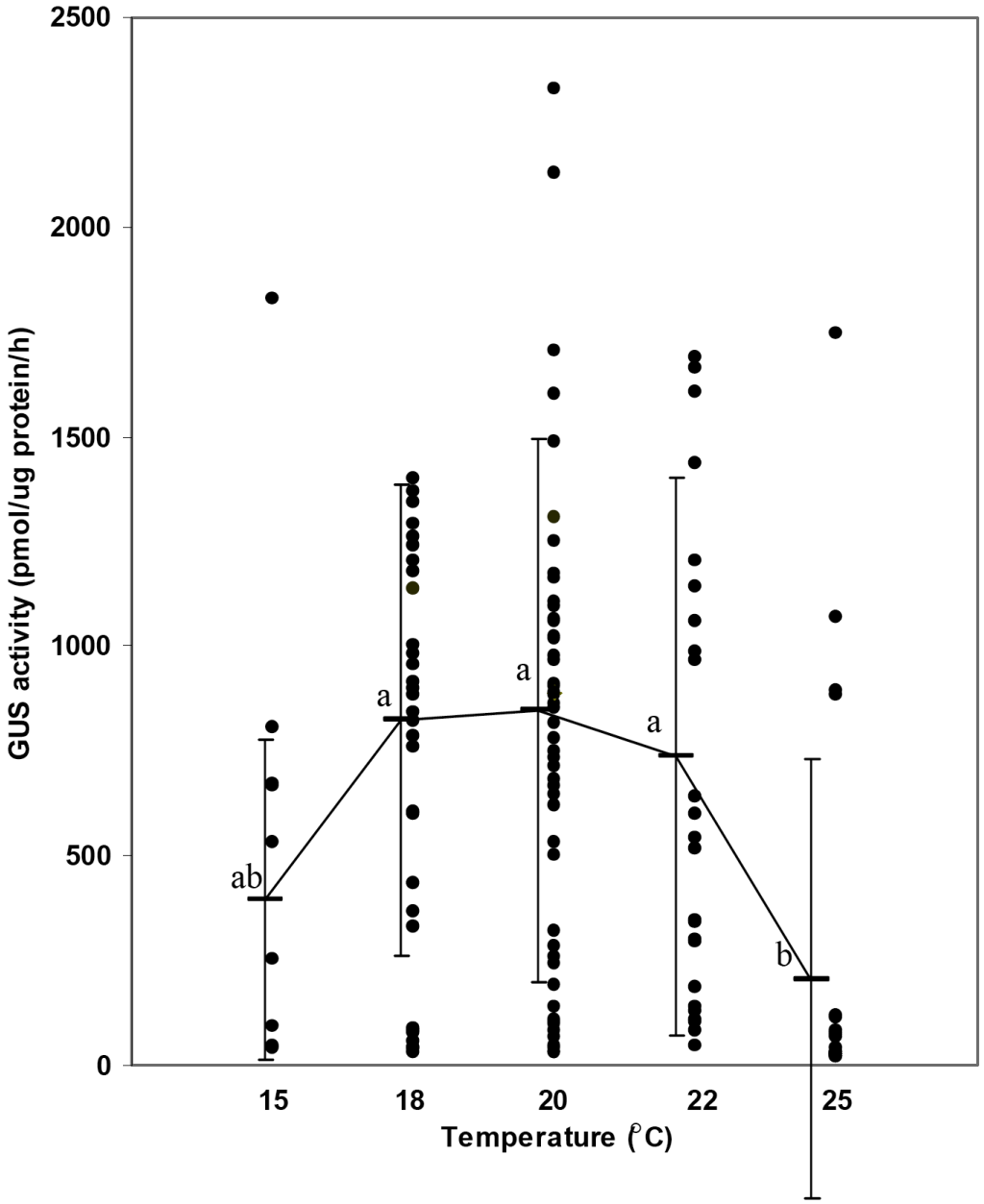

Figure 1. Co-cultivation temperature at 15, 18, 20, 22 and 25°C were compared in its effect on GUS activity measured after four day co-cultivation at 20 ℃ and 14 day hygromycin selection at 25°C. All values from four different experiments were pooled and plotted as dots. Averages of GUS activity at each temperature were represented as horizontal bar and vertical bar represents standard deviation. Leaf disks were inoculated with bacteria (0.3 A_{600} units) under 50 mmHg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. Means followed by the same letter are not significantly different at a =0.05 level using Fisher's least significant difference (LSD) test. Experiment was conducted from 10/18/05 to 11/25/05.

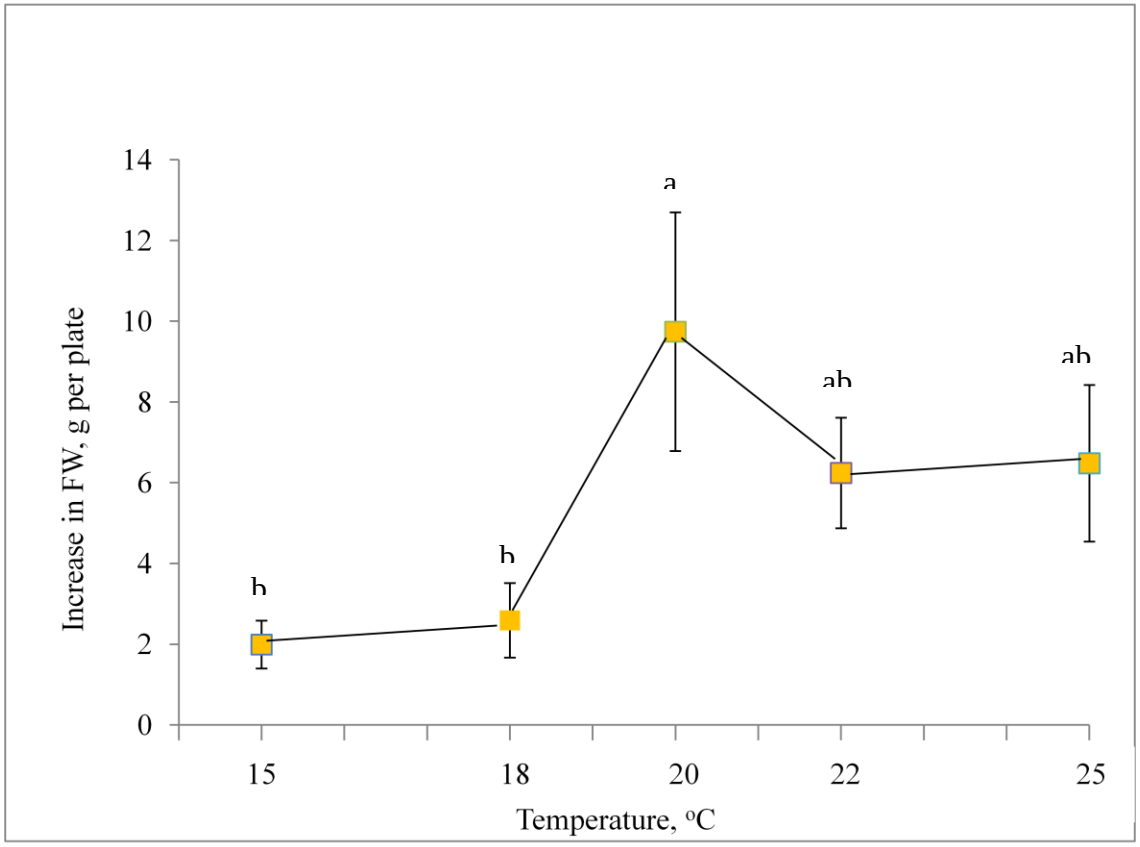

Figure 2**.** Co-cultivation temperature at 15, 18, 20, 22 and 25°C were compared in its effect on the final fresh weight yield of tobacco leaf disks after four days co-cultivation and 28 day kanamycin selection at 25°C. Leaf disks were co-cultivated for four days with *Agrobacterium tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Leaf discs were selected at 25 °C on shooting media containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for two weeks. Fresh media were prepared for additional two weeks of selection. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate. Vertical bars at the average FW yields indicate standard deviations. Means followed by the same letter are not significantly different at the α =0.05 level using Tukey's Studentized Range (HSD) test (α=0.05).

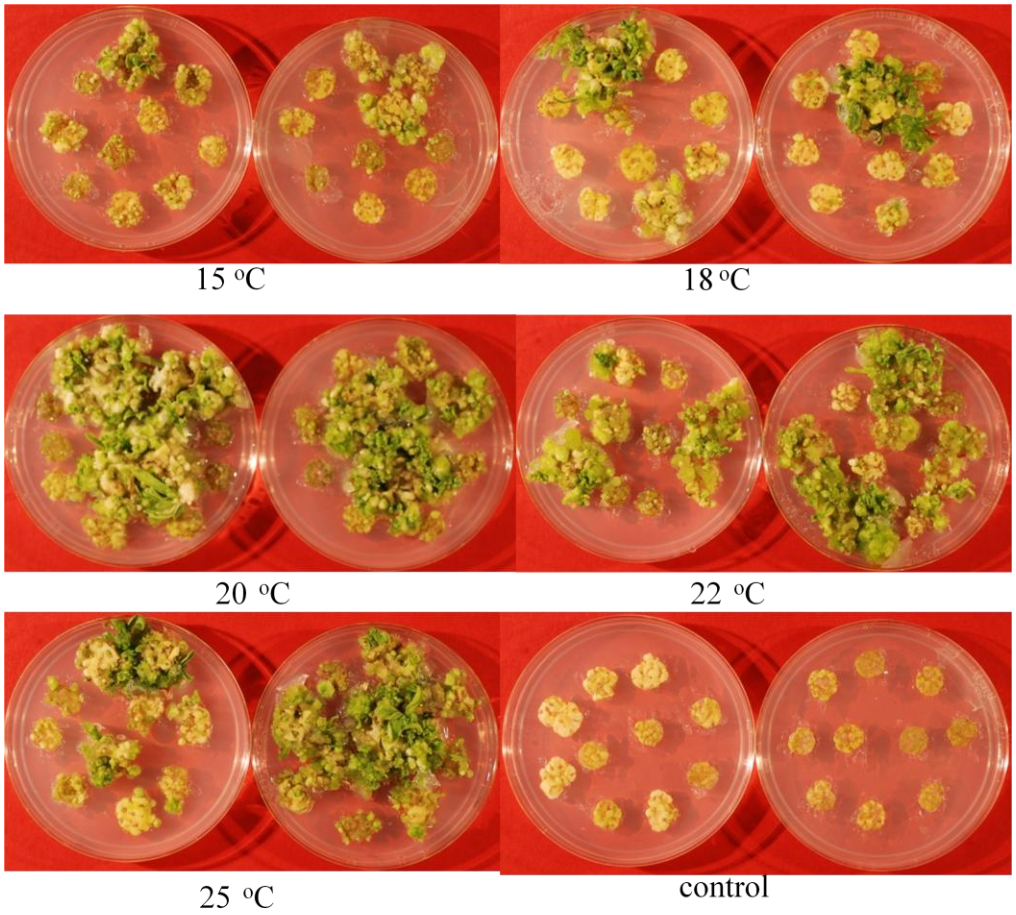

Figure 3**.** Co-cultivation temperature at 15, 18, 20, 22 and 25°C were compared in its effect on the final fresh weight yield of tobacco leaf disks after four days co-cultivation and 28 day kanamycin selection at 25°C. Leaf disks were co-cultivated for four days with *Agrobacterium tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Leaf discs were selected at 25 °C on shooting media containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for two weeks. Fresh media were prepared for additional two weeks of selection. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate.

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VITA

Seokhyun was born on June, 9, 1969 in Inchon, Korea. His father, Dr. Choo Hie Lee, is a professor of Microelectronics in KyoungHee University in Korea and his mother, Dr. Young-Ja Lim, is a pediatrician still practicing in her clinic. Seokhyun attended Ajou University, Suwon, Korea, majoring in biological science from 1989 to 1996. While he was in college, he served for three years in the Korean Army for the military duty as a sergeant. After graduation, he decided to pursue for academic career in the graduate school and received a master degree in biology at Ajou University, studying a plant transformation system in rice chloroplast. While he was in graduate school, he met Yoonjeong Kim and married her.

Seokhyun entered the graduate program in the department of Plant Pathology and Crop Physiology at Lousiana State University in 2002. He received C. W. EDGERTON HONOR AWARD in 2009. Currently, he is a candidate for the Degree of Doctor of Philosophy in plant pathology and crop physiology.