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Molecules and Cells

Positive-Selection and Ligation-Independent Cloning Vectors for Large Scale *in Planta* Expression for Plant Functional Genomics

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Transient expression is an easy, rapid and powerful technique for producing proteins of interest in plants. Recombinational cloning is highly efficient but has disadvantages, including complicated, time consuming cloning procedures and expensive enzymes for large-scale gene cloning. To overcome these limitations, we developed new ligation-independent cloning (LIC) vectors derived from binary vectors including tobacco mosaic virus (pJL-TRBO), potato virus X (pGR106) and the pBI121 vector-based pMBP1. LIC vectors were modified to enable directional cloning of PCR products without restriction enzyme digestion or ligation reactions. In addition, the *ccdB* gene, which encodes a potent cell-killing protein, was introduced between the two LIC adapter sites in the pJL-LIC, pGR-LIC, and pMBP-LIC vectors for the efficient selection of recombinant clones. This new vector does not require restriction enzymes, alkaline phosphatase, or DNA ligase for cloning. To clone, the three LIC vectors are digested with *Sna*BI and treated with T4 DNA polymerase, which includes 3′ to 5′ exonuclease activity in the presence of only one dNTP (dGTP for the inserts and dCTP for the vector). To make recombinants, the vector plasmid and the insert PCR fragment were annealed at room temperature for 20 min prior to transformation into the host. Bacterial transformation was accomplished with 100% efficiency. To validate the new LIC vector systems, we were used to coexpressed the *Phytophthora* AVR and potato resistance (R) genes in *N. benthamiana* by infiltration of Agrobacterium. Co-expressed AVR and R genes in *N. benthamiana* induced the typical hypersensitive cell death resulting from *in vivo* interaction of the two proteins. These LIC vectors could be efficiently used for high-throughput cloning and laboratory-scale *in planta* expression. These vectors could provide a powerful tool for high-throughput transient expression assays for functional genomic studies in plants.

INTRODUCTION

Transient expression is a powerful technique for producing proteins of interest in plants. The technically simplest *in planta* transient expression systems take advantage of the ability of *Agrobacterium* to transfer DNA into plant cells. Because *Agrobacterium*-mediated transformation is so efficient, easy, and inexpensive to use, it has become a common method for producing proteins in plants (An, 1987; Bevan, 1984; Huitema et al., 2004). Recently, *in planta* transient expression methods were developed that combine *Agrobacterium*-derived T-DNA and plant viral genomes such as Potato virus X (PVX) and Tobacco mosaic virus (TMV) (Huitema et al., 2004; Lindbo, 2007). These virus-based vectors can produce higher agro-infection efficiency and recombinant protein expression levels than other, conventional agro-infiltration/transient expression systems in plants (Huitema et al., 2004; Lindbo, 2007). However, although this vector system has advantages, it also has some disadvantages. First, conventional methods use restriction enzymes and ligase to digest DNA and ligate the insert into the vector. This enzyme-mediated cloning technique requires sequence information for the restriction enzyme sites. It is also necessary to prevent the growth of non-recombinant transformants by treating the vector with alkaline phosphatase. Despite this treatment, a considerable fraction of the clones often lack inserts. Second, the PCR-based approach is now the method of choice for the rapid amplification and isolation of specific DNA sequences from genomic DNA (Saiki et al., 1988). In this case, single adenosines (A) are added to the 3′-ends of the amplified PCR products by *Taq* polymerase, and used to ligate the product into a vector containing the complementary 3′-ends thymidine (T) extensions (Marchuk et al., 1990). This method is much more commonly used for PCR product cloning than restriction enzyme -based methods. However, this method is not directional, and correctly oriented (Goda et al., 2004). Accordingly, restriction endonuclease sites may be introduced into the amplification primers at their 5′ ends to facilitate cloning, but the cleavage efficiency is variable (Clark, 1988). Third, the GATEWAY method has been successfully implemented for high-throughput cloning of PCR products. However, this system also has disadvantages, including time-consuming, complicated cloning procedures and a high cost for large-scale cloning (reviewed by Dong et al., 2007). Thus, the limitations of currently

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available plant expression vectors include not only the multiple steps for cloning but also the expensive price cloning.

To overcome these limitations, Aslanidis and de Jong (1990) developed a ligation-independent cloning (LIC) method for PCR products that does not involve restriction enzymes, alkaline phosphatase, T4 polynucleotide kinase, or DNA ligase (Dieckman et al., 2002). Many researchers have constructed improved LIC-plasmid vectors (Aslanidis et al., 1994), and these tools have been used for cloning and expression of various cDNAs (Bonsor et al., 2006; Hamel et al., 1993; Haun et al., 1992). Recently, Dong et al. (2007) developed a TRV-LIC vector using modified LIC system for the high-throughput cloning of tomato cDNA clones for gene silencing. LIC is much faster and more accurate than other conventional cloning systems as only a single transformation is required. This procedure uses the exonuclease activity of T4 DNA polymerase to generate 5′ extended complementary cohesive ends in both the vector and the PCR product (Aslanidis et al., 1994). However, a disadvantage of this method is the strict requirement for correct T4 DNA polymerase treatment of both insert and vector.

On the other hand, positive-selection cloning vectors are efficient tools that simplify *in vitro* DNA recombination procedures by reducing or eliminating recombinants with plasmids lacking inserts (Bernard and Couturier, 1992). The *ccdB1* gene encodes a potent cell-killing protein that acts by trapping DNA gyrase (Bernard and Couturier, 1992). DNA gyrase (or topoisomerase) is a unique class of ubiquitous enzymes that are able to control supercoiling by breaking and rejoining the phosphodiester backbone of one or both DNA strands (Type I and II topoisomerase, respectively). Therefore, positive-selection can be made in F$^-$strain bacteria with almost no trace of background and without any special biochemicals (Bernard and Couturier, 1992). To date, many expression vector systems have been developed, but none of them are LIC or positive-selection expression vectors for plants.

In this study, we constructed three positive-selection LIC vectors, pGR106-PSLIC, pJL-PSLIC and pMBP1-PSLIC, which rely on the replacement of the cytotoxic *ccdB1* gene of the target plasmid vector for positive selection. These vectors could provide a powerful tool for high-throughput cloning and transient expression assays for functional genomics in plants.

MATERIALS AND METHODS

Bacteria and reagents

All restriction enzymes, T4 DNA polymerase and corresponding buffers were purchased from New England Biolabs (USA). *E. coli* strain DH5α was purchased from Real-Biotech (Korea). Other strains, including *E. coli* strain DB3.1 and DH10B, were purchased from Invitrogen (USA). Oligonucleotides were synthesized and HPLC-purified by Bioneer Biotech (Korea). Amplified or digested reaction products were column purified using the DNA clean up and gel purification kit (Zymo Research, USA) to remove unincorporated dNTPs and primers or other products. For plate selection, kanamycin was used at 50 μg/ml. *Pfu* and *Taq* polymerases were purchased from SolGent Ins. (Korea).

Positive selection-LIC vector construction

The plant expression vectors pMBP1, pJL-TRBO, and pGR106 were used as the basis for the positive selection and ligation independent cloning (PSLIC) vectors (Baulcombe, 1999; Lindbo, 2007; Oh et al., 2005). First, the plasmid pMBP1-LIC was constructed by cloning two annealed oligonucleotides that included *Sna*BI restriction sites (to serve as adaptors) into the *Bam*HI and *Kpn*I sites of pMBP1. The DNA sequences of these primers were: pLIC-*Sna*BI-For, 5′-GGATCCTTAATTAAAGCCAATCCCTC**TACGTA**GGAGGATACCCATACGATGTTCCAGATTACGCTTGATAGGGTACC-3′ and pLIC-*Sna*BI-Rev, 5′-GGTACCCTATCAAGCGTAATCTGGAACATCGTATGGGTATCCTCC**TACGTA**GAGGGATTGGCTTTAATTAAGGATCC-3′. Second, to enable positive selection, the *ccdB* gene was amplified by PCR from the plasmid TRV-LIC (Dong et al., 2007). The primer sequences for this reaction were: ccdB-For, 5′-TACGTAAATTCTCGACTAAGTTGGCAGCATCACCCGACG-3′ and ccdB-Rev, 5′-TACGTACTCGAGCAGACTGGCTGTGTATAAGGGAGCC TG-3′. The *ccdB*-containing PCR product was then cloned into the pMBP1 plasmid via the *Sna*BI sites (Fig. 1) to

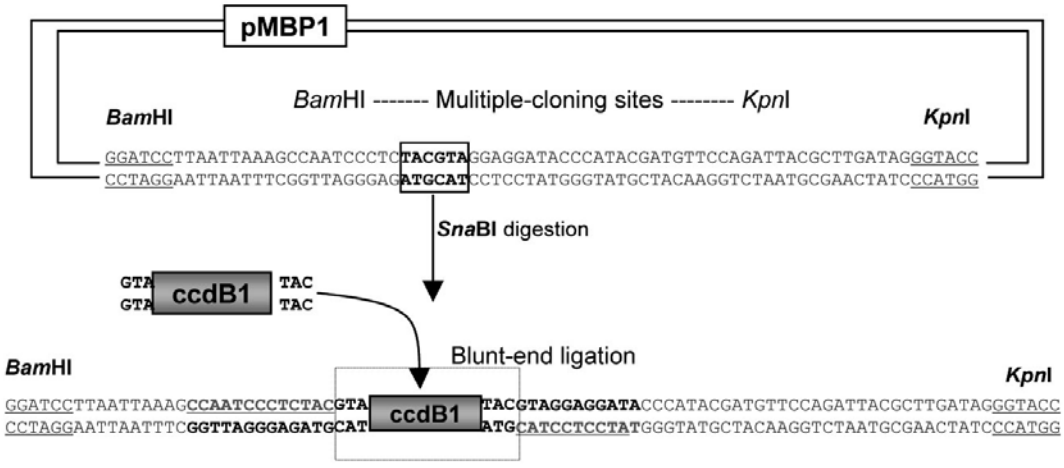

Fig. 1. PSLIC sequence of the cloning sites for the positive-selection, LIC-based vectors. The plant expression vector pMBP1 was used as the basis for the PSLIC vectors. The plasmid pMBP1-LIC was obtained by cloning two annealed oligonucleotides into the *Bam*HI and *Kpn*I sites of pMBP1 to produce the pMBP-LIC plasmid. These annealed oligonucleotides included two adaptors with *Sna*BI restriction sites. The pMBP-LIC plasmid was then further modified by adding a *ccdB* gene for positive selection. The *ccdB* gene was amplified by PCR with *Sna*BI sites in the primers, and then digested by *Sna*BI. The *Sna*BI-digested *ccdB* PCR fragment was then inserted into pMBP-LIC at the *Sna*BI site to create the pMBP-PSLIC vector.

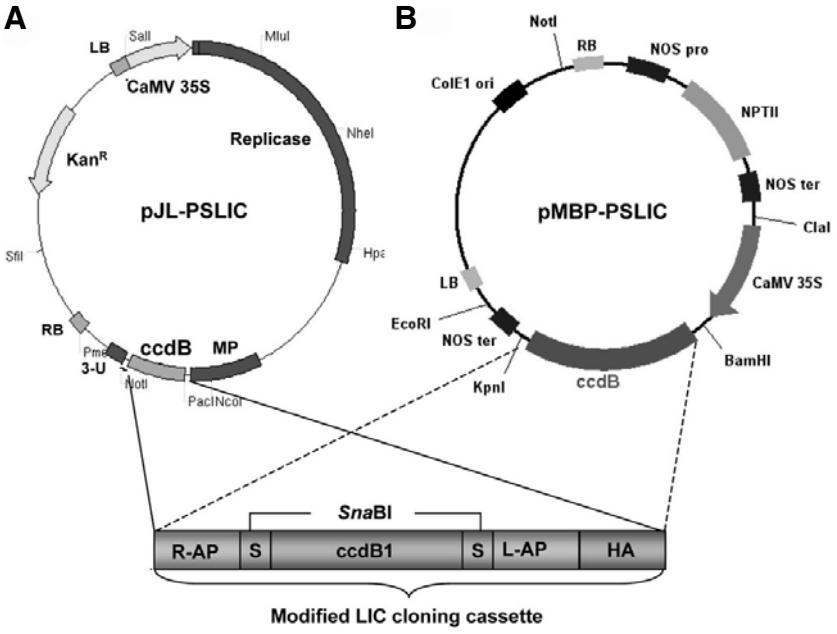

Fig. 2. Construction of a ligation-independent cloning vector: maps of the pJL- and pMBP1-PSLIC vectors used in this study. All pPSLIC plasmids were transformed and maintained in the *E. coli* strain DB3.1 [F- *gyr*A462 *end*A1] (Invitrogen). RB, Right border sequence; LB, Left border sequence; CaMV35S, Cauliflower Mosaic Virus 35S promoter; Replicase, TMV 126K/183K; MP, Movement protein; NPTII, Kanamycin resistance gene; NOS-pro, Nopaline synthase promoter; NOS-ter, Nopaline synthase terminator; HA, Hemagglutinin; S, *Sna*BI enzyme; R-AP, Right region Adapter sequence; L-AP, Left region Adapter sequence; *ccdB*1, controlling cell death B1 gene.

create the pMBP1-PSLIC vector (Fig. 2). The pJL-PSLIC and pGR106-PSLIC vectors were constructed using the same methods as those used for the pMBP1-PSLIC vector. All PSLIC plasmids were transformed into and maintained in *E. coli* strain DB3.1 [F- *gyr*A462 *end*A1 ∆ (sr1-*rec*A) *mcr*B *mrr hsd*S20 (rB-, mB-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 (SmR) *xyl*-5 λ- *leu mtl*1] (Invitrogen, USA).

Ligation-independent cloning procedures

Hundreds of pepper (*Capsicum annuum*) and other solanaceous ESTs (http://pdrc.lribb.re.kr) were amplified with primers that included identical 14-nucleotide tails: pCLIC-For, 5′-CCA ATCCCTCTACG-vector (gene)-specific sequence-3′ and pCLIC-Rev, 5′-TATCCTCCTACGAT-vector (gene)-specific se-quence-3′. The amplified PCR fragments were purified with either the DNA clean up or the DNA recovery gel extraction kit (Zymo Research, USA) to eliminate any nonspecific PCR products and unbound primers. A minimum of 50 ng of purified PCR product was treated with T4 DNA polymerase (NEB, USA) in 1X (final concentration) reaction buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9) supplemented with 2.5 mM dCTP (final concentration) at 22°C for 30 min, followed by 20 min of inactivation of T4 polymerase at 75°C, and then cooled at 4°C. At the same time, the pPSLIC vector plasmid (1 ug) was digested with *SnaB*I restriction enzymes (2.5 U) at 37°C for 3 h and purified using the DNA recovery gel extraction kit (Zymo Research, USA) for elimination of the enzyme and any nonspecific reagents. The linearized pPSLIC vector was treated with T4 DNA polymerase under the same conditions except that the reaction mix was supplemented with 2.5 mM dGTP instead of the 2.5 mM dCTP used for the insert. This treatment will produce overhangs in inserts complementary to that of vector overhangs. The DNA concentration was determined using a spectrophotometer (Nano-drop, USA) and adjusted to 25 ng/μl. A total of 25 (or 50 ng) of PCR products and the PSLIC vector were mixed and incubated at 65°C for 1 min and at 22 to 25°C for 20 min. In the final procedure for cloning, 5 μl of the mixture was used to transform *E. coli* DH5α or DH10B competent cells (30 μl; Invitrogen, USA) using heat shock (at 42°C, followed by the addition of 450 μl SOC medium and incubation of the bacterial cells for about 20 min at 37°C) (Sambrook and Russell, 2001). Then, 250 μl of the reaction mixture (cells) was plated onto LB agar media supplemented with 50 mg/L kanamycin, and then incubated at 37°C overnight. The transformants were confirmed by PCR using the following primers: for pMBP1-PSLIC, 5′-CTATCCTTCGCAAGACCCTT C-3′ and 5′-AAGACCGGCAACAGGATTCA-3′; for pJL-PSLIC, 5′-CGATGATGATTCGGAGGCTAC-3′ and 5′-CAATCCGTTA TTTATTATGC-3′; and for pGR106-PSLIC, 5′-AATCAATCAC AGTGTTGGCTTGC-3′ and 5′-AGTTGACCCTATGGGCTGTG TTG-3 or 5′-AAGACCGGCAACAGGATTCA-3′. Plas- mids from PCR-based positive clones were purified and DNA sequencing was performed by NICEM (Seoul National University, Korea) using a DNA Analyzer machine (ABI Prism 3730 XL, Applied Biosystems, USA).

Agrobacterium transformation and *in planta* assay

Three binary pPSLIC-inserted plasmids purified from *E. coli* DH5α or DH10B cultures were transformed into *Agrobacterium tumefaciens* strain GV3101 or GV2260 using the freeze-thaw method (Chen et al., 1994). Transformed *Agrobacteria* were plated on YEP (Yeast ext. 10 g, NaCl 5 g, Pepton 10 g, Agar 15 g, and dH_2O 1 L) plates with 50 mg/L kanamycin and 25 mg/L rifampicin for recombinant selection. For *in planta* transient assays, a single colony of transformed *Agrobacterium* cells was grown at 28°C, 220 rpm for 24 h in YEP broth media including the same antibiotics. Cell cultures were diluted 1:20 in the same media supplemented with 20 μM acetosyringone, and grown under the same conditions to an A600 of 0.8. The cell culture was then centrifuged, resuspended in induction buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 μM acetosyringone), and kept at room temperature for 3 h (Oh et al., 2009). Then, the *Agrobacterium* culture was mixed and infiltrated into the expanded leaves of *N. benthamiana* using a needleless syringe (i.e., mixed with pMBP1-PSLIC-Rpiblb2 and pGR106-PSLIC-Avrblb2 construct). The infiltrated plants were placed in a growth room at 24°C at 60% relative humidity with a 14/10-h light-dark cycle.

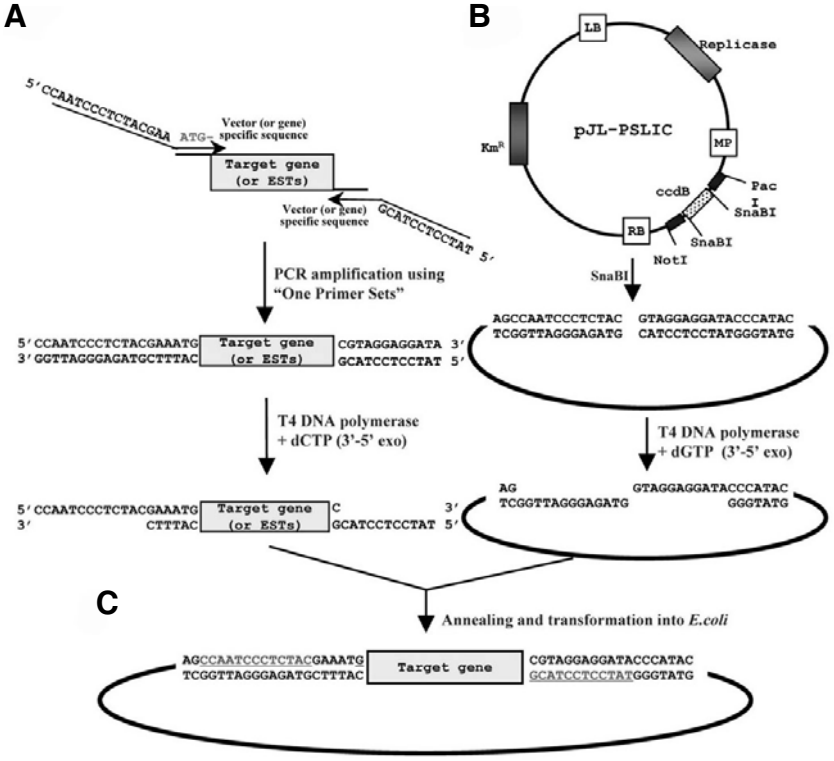

Fig. 3. Ligation-independent cloning (LIC) strategy. (A) Preparation of PCR product for LIC. Target genes were amplified by PCR using gene-specific primers with LIC sequences attached to their 5′ termini. PCR products were purified and incubated with T4 DNA polymerase and a single dCTP to generate an LIC insert with single-stranded 5′ overhangs. (B) Preparation of the LIC plasmid. The pJL-TRBO vector was used to generate the new pJL-PSLIC vector by insertion of a cassette containing adapters and two *Sna*BI sites in two digestion and ligation reactions. The pJL-PSLIC vector was digested with *Sna*BI enzymes. The linearized vector was then purified and incubated with T4 DNA polymerase and a single dGTP to generate single-stranded 5′ termini. (C) Purified vector plasmids and target genes (EST clones) were mixed and annealed without DNA ligase at room temperature. These mixtures were then transformed into *E. coli* DH5α cells.

RESULTS AND DISCUSSION

Alternative to conventional cloning systems

To date, there are over 1 million solanaceous plant ESTs, corresponding to 100,000 unique sequence clones, in the NCBI and pepper EST (http://genepool.kribb.re.kr) databases. Likewise, pathogen-derived "effector" genes, such as the *Phytophthora* RXLR effectors and fungal virulence factors, are also represented by several thousand ESTs in the *Phytophthora* Functional Genomics Database (PFGD) (www.pfgd.org). However, little is known about their biological functions. Accordingly, many groups have developed cloning methods to introduce these ESTs and genomic DNA sequences into plants for functional studies. One currently available method for plant expression is the virus-induced gene silencing vector (VIGS) for reverse genetics approaches to investigate plant genes of interest (Chung et al., 2004; Liu et al., 2002). Together with VIGS approaches, transient- or over-expression methods also offer great opportunity for gain-of-function studies (An, 1987; Lindbo, 2007; Oh et al., 2009). Most expression vectors have been developed for use in bacteria, yeast and animal cells, instead of plant cells. Therefore, we consider that there is a need for improved expression vectors for rapid, efficient and easy-to-use recombinant DNA expression systems for plants.

To overcome several disadvantages of conventional plant expression vectors, we generated the Positive Selection and Ligation Independent Cloning (pPSLIC) plasmids. These plasmids will facilitate large-scale cloning from plant ESTs or fungal ESTs using the universal "One Primer Sets" of vector plasmid sequences (Fig. 3). In addition, they will also enable enzyme- and ligation-free cloning of large genomic DNAs using LIC adaptor primers, including gene specific sequences, to 100% cloning efficiency (Fig. 4 and Table 1). Therefore, we modified several plant expression vectors using a ligation-independent cloning system (Aslanidis and de Jong, 1990) and *ccdB1* as a positive selection marker gene (Bernard and Couturier, 1992).

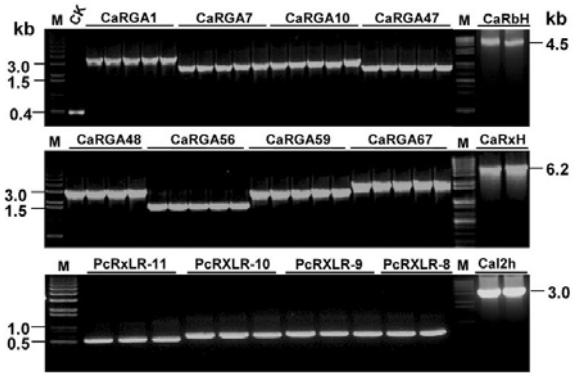

Fig. 4. Cloning efficiency for ligation-independent cloning. The cloning efficiency into pPSLIC-1 was 100% (100% of transformants contained the target DNA insert) as shown by PCR amplification and comparison of fragment sizes. M, DNA size marker; CK, negative control DNA.

Construction of the "pPSLIC" vector

Recently, two ligation procedures, the Gateway technique (Invitrogen, USA) and LIC, have been used extensively. These techniques provide the opportunity for parallel cloning of multiple genes into different vectors (Hartley et al., 2000). In LIC methods, genes of interest are amplified with 12 to 15 bp end nucleotides and the 5′ → 3′ endonuclease activity of T4 DNA polymerase is then applied (Aslanidis and de Jong, 1990). Most LIC expression systems are restricted to bacteria, yeast, or animals. Therefore, we aimed to generate more improved plant expression cloning vectors that enable easy, rapid, directional,

Table 1. Size-dependent cloning efficiency of the modified ligation-independent cloning vector

Insert (kb)	Vector[a] (ng)	Insert (ng)	Enzyme mix	Number of colonies[b]	% with inserts
0.3-1.0	25	10-25	T4 poly	150-400	99.9
1.1- 3.0	25	25-50	T4 poly	80-250	~
3.1- 6.2	25	50	T4 poly	18-120	100.0

[a]Final concentration of vector plasmid
[b]Number of colonies obtained after transformation of LIC reactions in *E. coli* with the indicated amount of inserted plasmid (minimum to maximum in 250 μl bacterial cell solution).

and cost effective massive cloning of ESTs using combined positive selection markers and ligation-independent cloning methods.

To accomplish this, we created pPSLIC plant expression vectors using the CaMV 35S-based binary vectors pGR106, pJL-TRBO and pMBP1 as their backbone. First, an oligonucleotide cassette was introduced into the pMBP1-PSLIC (or pGR-PSLIC or pJL-PSLIC) plant expression vector via two LIC adaptors located between the *Bam*HI and *Kpn*I sites (Fig. 1). These adaptors consisted of 14 bp of oligo-sequences and an HA-tag ("YPYDVPDYA") in the C-terminal part, and *Sna*BI restriction sites in the central part. Regarding the 14 bp length of the oligo-sequences, we chose a longer length because Aslanidis et al. (1994) reported that an LIC system with 12-nucleotide long single-stranded (SS) tails produced more efficient transformation than an LIC system with 10-nucleotide SS tails. Likewise, Oliner et al. (1993) also demonstrated that the efficiency of LIC system is dependent on the length of the cohesive ends generated. In addition, the cloning and transformation efficiency achieved by our procedure is very stable. Therefore, we did consider that it is suitable for LIC cloning experiments with 14-base longer LIC tails. Second, we inserted the *ccdB* gene (controlling cell death B gene), as a positive selection marker, between the two LIC adapter sites created by *Sna*BI digestion. This created the pPSLIC vectors (the pMBP1-PSLIC, pGR-PSLIC, and pJL-PSLIC vectors). The *ccdB* gene, a new topoisomerase II and DNA gyrase poisoning agent, encodes a potent cell-killing protein that is able to control supercoiling by breaking and rejoining the phosphodiester backbone of DNA strands (Bernard and Couturier, 1992). Bernard et al. (1994) reported that *E. coli* [wild type gyrA+ strain] transformed by *ccdB*-based vectors died. It is known that the ccdB protein acts from within the cell and is able to interfere directly with the viability of transformed bacteria. Therefore, our three PSLIC vector plasmids were derived and maintained in the *E. coli* DB3.1 strain, a ccdB-resistant host with a genetic background [F-gyrA462] that allows propagation of *ccdB*-containing plasmids (Invitrogen, USA). Thus, the *ccdB* gene rapidly and accurately selects putative transformants in similar fashion to the GATEWAY approach. In summary, we constructed three positive-selection, LIC-based plant expression vectors, called pGR-PSLIC, pJL-PSLIC and pMBP1-PSLIC, which rely on the inactivation of the cytotoxic *ccdB1* gene for selection (Fig. 2).

Cloning of target genes into the "pPSLIC" vector and *in planta* expression analysis

LIC is a fast and accurate cloning system, but it has a strict requirement for correct T4 DNA polymerase treatment of both insert and vector (Aslanidis et al., 1994). To improve this LIC system, we introduced the *ccdB* gene, which encodes a potent cell-killing protein, between the two LIC adapter sites in our pPSLIC vectors, for the efficient selection of recombinant clones (Fig. 2). To test the cloning efficiency of pPSLIC vectors, we selected 104 NBS-LRR gene families from pepper ESTs and Solanaceae genomic DNAs (http://genepool.kribb.re.kr) for large-scale functional analysis. Likewise, to demonstrate the efficiency of the pPSLIC vector for high-throughput screening, we also cloned 290 *Phytophthora capsici* or *P. infestans* genomic DNAs or ESTs into these vectors. Briefly, T4-treated PCR products and pPSLIC vectors were mixed and incubated as described in the "Materials and Methods". The T4 DNA polymerase treatment generates overhangs in the PCR fragment that allows subsequent insertion into a complementarily T4-treated vector using a simple annealing procedure (Fig. 3). Because there is no need for restriction enzyme or ligase treatment, the pPSLIC vector is a highly efficient and cost-effective method for mass cloning (Dong et al., 2007). Transformation into *E. coli* cells (DH5α) was efficient; 99.9% of the colonies screened were found to be positive for pPSLIC. Then, cloning efficiency was confirmed by PCR amplification and sequencing analysis to verify the presence of inserts in the vector (Fig. 4). As shown in Fig. 4, the targeted inserts were present in 100% of transformants. From these results, we were convinced that the pPSLIC plasmid containing the ccdB protein is able to act from within the cell to directly interfere with the viability of transformed *E. coli* strain DH5α or DH10B cells (Fig. 4 and Table 1) (Bernard and Couturier, 1992).

To validate *in planta* expression of the cloned gene in pPSLIC vector, we transformed to *Agrobacterium tumefaciens*

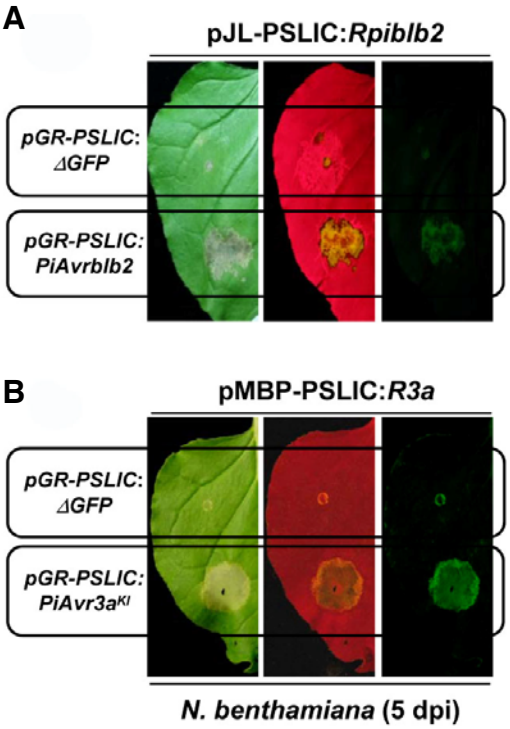

Fig. 5. Activation of *Phytophthora* effector-induced cell death in the Potato R gene of *Nicotiana benthamiana*. *Agrobacterium* cultures containing the *Phytophthora* effector and R constructs were infiltrated at OD 600 nm = 0.3 [(A) pJL-PSLIC-Rpiblb2 and pGR106-PSLIC-Avrblb2 constructs, (B) pMBP1-PSLIC-R3a and pGR106-PSLIC-Avr3a^{KI} constructs]. Photographs were taken five days after agro-infiltration.

strain GV3101 and then co-expressed both pathogen-derived effectors and plant NBS-LRR genes in *Nicotiana benthamiana*. To do this, we infiltrated four-leaf stage *N. benthamiana* leaves with a 1:1 ratio mixture of pGR-PSLIC-Avrblb2 and pJL-PSLIC-Rpiblb2 (Fig. 5A), or pGR-PSLIC-Avr3a and pMBP-PSLIC-R3a genes (Fig. 5B) (Bos et al., 2006; Oh et al., 2009). As shown in Fig. 5, with multiple cases, we observed hypersensitive cell death only in co-expressed combination, but not in single gene expression.

In this study, we present new expression vectors that facilitate high-throughput cloning and transient overexpression in plants. The pPSLIC vector system allows rapid, directional, efficient (100% of transformants were confirmed to contain the target DNA) cloning, and has the added advantage of cost effectiveness. The cloning procedures are simple and do not require restriction enzymes, ligase, or recombinase. The *ccdB*1 gene, which acts as a potent bacterial cell-killing protein, provides a built-in positive-selection feature that helps to ensure low-background cloning. Gene-specific primer sets are not required for massive cloning of ESTs; instead, large-scale cloning of ESTs can be accomplished using the universal "One Fixed Primers" from vector plasmid sequences. Therefore, our improved vector systems provide new and useful tools for high-throughput *in planta* expression strategies for plant functional genomics studies.

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