GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Modification of vectors for functional genomic analysis in plants

J.T. Li[1]*, G. Yu[1]*, X.H. Sun[1], C.G. Jia[1], Q. Du[2], Q.Y. Li[2] and H.Y. Pan[1]

[1]College of Plant Science, Jilin University, Changchun, China
[2]Jilin Academy of Agricultural Science, Changchun, China

*These authors contributed equally to this study.
Corresponding author: H.Y. Pan
E-mail: panhongyu@jlu.edu.cn

Genet. Mol. Res. 13 (3): 7815-7825 (2014)
Received August 1, 2013
Accepted December 10, 2013
Published September 26, 2014
DOI http://dx.doi.org/10.4238/2014.September.26.20

ABSTRACT. Simple, efficient, and economical recombinant plant binary expression vectors for deciphering large-scale functional genomic research in plants and promoting crop improvement by genetically engineering and biotechnology is in great demand. In this research, using the pCHF3, pCAMBIA1301, pCAMBIA3300, pCAMBIA3301 vectors, we successfully constructed general plant binary expression vectors carrying CaMV35S and *Arabidopsis rd29A* promoters mediating multiple cloning sites (MCS: *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, and *Pst*I). Meanwhile, a series of applicative binary expression vectors that can be utilized for subcellular localization were constructed by fusion of the MCS and eGFP. Subsequently, the recombinant vectors were successfully transferred into *Arabidopsis thaliana* and *Nicotiana benthamiana* for further investigation of functional elements in these plant binary expression vectors. Our results demonstrated that this system was a convenient and versatile vector system for phenotypic, functional, subcellular localization, and promoter activity analysis, and it provided a relatively high-efficiency and reliable platform for researchers in vector construction and may

facilitate large-scale functional genomics analysis in plants.

Key words: Plant binary expression vector; Stable expression; Stress-inducible expression; Subcellular localization

INTRODUCTION

With the development of high-throughput sequencing and complete genome sequencing from many organisms, we have entered to a post-genome era (Wang et al., 2013). Conventionally, two ways to investigate the gain of function and loss of function of target genes are to create transgenic plants via overexpression and knockout or knockdown, respectively (Curtis and Grossniklaus, 2003; Zhang et al., 2006). Several approaches can be employed in gene knockout or knockdown, such as transfer (T)-DNA insertion (Krysan et al., 1999), RNA silencing (Yan et al., 2012), and the emerging technologies transcription activator-like effector nucleases (TALENs) (Li et al., 2011) and zinc-finger nucleases (ZFNs) (Gaj et al., 2013). When it comes to overexpression, the demand for functional identification and characterization of candidate genes, as well as the way in which they interact, is increasing. The construction of an expression vector is usually the first step in obtaining overexpression or ectopic expression. In these circumstances, convenient, efficient, and high-throughput expression vectors are desired; using these vectors, foreign genes are introduced into plant cells and stably integrated into plant genomes, and the transformed cells are regenerated to obtain transgenic plants for further investigation. However, gene manipulation and modification in plants have already experienced the bottleneck problem for large-scale functional genomic research (Wang et al., 2013). Thus, developing an efficient, convenient, timely, and cost-saving approach for gene manipulation has become compulsory for researchers.

Collective efforts of several laboratories in the past two decades resulted in new approaches, such as the Gateway cloning system, the Creator cloning system, the ligation-independent cloning (LIC) system, and TA-based expression system (Curtis and Grossniklaus, 2003; Earley et al., 2006; Chen et al., 2009; Rybel et al., 2011; Wang et al., 2013). High-throughput gene construction based on these technologies is widely used by researchers. However, several limitations of the approaches cannot be ignored. The Gateway cloning system and Creator cloning system, two-step manipulations, continue to be time and labor consuming, and the patented BP and LR enzymes are very expensive, which reduces their application in relatively small laboratories and laboratories without sufficient grants. At the same time, the long primers containing the specific attachment site (*att*) occasionally add weight to the difficulty of gene amplification (Rybel et al., 2011; Wang et al., 2013). Despite the obvious advantages of the LIC cloning as a single-step method to clone in a moderately high-throughput fashion, there are still two obvious drawbacks to the system. The most obvious downside is that every fragment needs to be sequenced when used in different vectors, and another disadvantage is that cloned fragments cannot be recombined as with Gateway cloning (Rybel et al., 2011). On the other hand, the conventional binary expression vectors, such as the pCAMBIA series and pBI121, lack convenient and appropriate restriction enzyme digestion sites, promoter, and terminator, have a relatively high false-positive rate in transformants when the *npt*II gene was utilized as a selective marker in plants, or are not suitable for some transgenic plants because of their antibiotic selective marker (Chen et al., 2002, 2009). Although the pCAMBIA vector series is not the most suitable as the

backbone from the manufacturer because most pCAMBIA vectors are over 12 kb in size, which can add difficulty to the molecular manipulation and ligation efficiency, many researchers are still employing them by remodeling (Chen et al., 2009; Wang et al., 2013).

Obviously, exploiting conventional and economical restriction enzymes for gene recombination is always the direct, simple, and efficient method and is widely used by researchers, especially in developing countries. Thus, employing multiple cloning sites (MCS) driven by cauliflower mosaic virus 35S promoter (CaMV35S) stands out when performing gene manipulation and overexpression in plants. In some cases, transgenic plants expressing a signal transduction pivotal knot or coordinator in a gene expression network, such as transcription factors, phosphatases, kinases, or some other genes driven by the CaMV35S promoter, can cause pleiotropic effects (Kasuga et al., 1999). For example, transgenic *Arabidopsis* overexpressing the *DREB1A* gene causes growth retardation even under normal growth conditions (Kasuga et al., 2004). Additionally, transgenic tomato overexpressing the *CBF1* gene also showed the dwarf phenotype under unstressed normal growth conditions (Hsieh et al., 2002; Kasuga et al., 2004). To some extent, using a stress-inducible promoter to replace a constitutive promoter such as CaMV35S to drive gene expression in transgenic plants could minimize the negative effects on plant growth (Kasuga et al., 2004). To overcome it, the *Arabidopsis rd29A* promoter has been predominantly used all over the world. Additionally, the *rd29A* promoter is a drought-, cold-, and abscisic acid (ABA)-inducible promoter with a dehydration-responsive element and an ABA-responsive element (Wang et al., 2011).

The green fluorescent protein (GFP) of jellyfish (*Aequorea victoria*) is one of the most popular reporter domains because of its small size (238 amino acid residues), no requirements for substrates and co-factors, activity as a monomer, stability in a broad range of buffers and temperatures, and relative harmlessness when illuminated in living cells (Stanisławska-Sachadyn et al., 2006). The enhanced GFP (eGFP) has increased fluorescence and is more suitable for long-term time-lapse imaging in plant live-cell imaging because it is less sensitive to photobleaching. As another competitive reporter system, the β-glucuronidase reporter gene (GUS) system has become a powerful tool to assess gene and promoter activity and gene expression patterns in plants because there is no detectable GUS activity in higher plants, fungi, and most bacteria (Jefferson, 1987; Jefferson et al., 1987).

To develop an easier system for overexpression in plants, we used pCAMBIA-based vectors (pCAMBIA1301, pCAMBIA3300, and pCAMBIA3301), which contain high copy numbers and individual exogenous marker genes (*Hyg*, *Bar*, or *GUS*), as the backbone to construct three intermediate vectors (pCHF1301, pCHF3300, and pCHF3301) that include the CaMV35S promoter, MCS, and *Arabidopsis* rubisco small subunit (RbcS) terminator between the *Eco*RI and *Hin*dIII sites from the pCHF3 vector (Yin et al., 2002). Furthermore, we adopted overlapping polymerase chain reaction (PCR) to obtain the MCS and eGFP fusion expression cassette under control of the CaMV35S promoter, leading to a simpler fusion expression operation in a subcellular expression vector by inserting the target cDNA into this set of subcellular expression vectors via regular restriction enzyme digestion. Additionally, we derived six binary vectors that were driven by the *Arabidopsis rd29A* promoter and exploited these for stress-inducible gene expression in plant transformation.

In this system, a set of stable gene expression constructs was made by conventional PCR amplification, restriction enzyme digestion, and ligation. Then, some gene constructs for constitutive and stress-inducible expression, protein subcellular localization, and promoter

activity investigation in transgenic plants were successfully generated. With the appropriate restriction sites, PCR products for enzyme digestion and ligation can be manipulated directly and conveniently. What's more, a non-stop codon cDNA fragment can be cloned directly into a construct for eGFP fusion expression. The pCHF3300 and pCHF3301 series vectors containing the *Bar* gene can be directly used in genetically modified plants, specifically in dicotyledons, such as soybean, cotton, alfalfa, and canola. Additionally, the vector system could be used for complementary stable expression for T-DNA insertion mutation lines with different reporter genes. Compared with the Gateway and other expression systems, our results demonstrated that the expression system in this study is a convenient and versatile vector system, and it provides a relative high-efficiency, cost-saving, and reliable platform for gene construction, especially for researchers in small laboratories or developing countries. It may also facilitate large-scale functional genomics analysis in plants.

MATERIAL AND METHODS

Plant and plasmid materials, growth conditions, and plant transformation

The *Nicotiana benthamiana*, *Arabidopsis thaliana* (Columbia ecotype, Col-0), and pCHF3, pMD18T-*eGFP*, pCAMBIA1301, pCAMBIA3300, and pCAMBIA3301 vectors are available from the Integrated Pest Management Laboratory, College of Plant Science, Jilin University. Standard molecular manipulations were performed to make all the constructs. All the intermediate vectors derived from PCR products were characterized by restriction enzyme digestion and sequencing by Invitrogen Corperation. All primers used in this study are shown in Table 1. Wild-type (WT) *A. thaliana* and *N. benthamiana* plants that were used for transformation were grown in the greenhouse under the following controlled environmental conditions: 21 to 23°C, 100 μmol photons·m^{-2}·s^{-1}, 60% relative humidity, and 16 h light/8 h dark (Yang et al., 2012). For GUS staining detection, sterile transgenic *A. thaliana* seeds were germinated on semi-solid Murashige-Skoog (MS) medium supplemented with 0.8% sucrose, pH 5.8, and plants were examined after 2 weeks of growth.

Table 1. Primer sequences for PCR amplification used in this study.

Primer	Sequence
OL-1	CGGAATTCGGTCCCCAGATTAGCCTTTTC (*Eco*RI site underlined)
OL-2	CCTCGCCCTTGCTCACCATACCGCTACCACCGCTACCGTCGACTCTAGAGGATCCC
OL-3	GGGATCCTCTAGAGTCGACGGTAGCGGTGGTAGCGGTATGGTGAGCAAGGGCGAGG
OL-4	GCTGCAGTTACTTGTACAGCTCGTCCATGCCG (*Pst*I site underlined)
pRD29A-1	CGGAATTCCTCGAGGAGCCATAGATGCAATTC(*Eco*RI site underlined)
pRD29A-2	CGAGCTCTTTCCAAAGATTTTTTTCTTTCC (*Sac*I site underlined)
Hyg-F	GATGTTGGCGACCTCGTATT
Hyg-R	TCGTTATGTTTATCGGCACTTT
Bar-F	AAACCCACGTCATGCCAGTTC
Bar-R	CGAGACAAGCACGGTCAACTTC

Construction of pCAMBIA-based plasmids carrying an MCS driven by the CaMV35S or *rd29A* promoter

The intermediate plant expression vectors containing the MCS (*Sac*I, *Kpn*I, *Sma*I,

*Bam*HI, *Xba*I, *Sal*I, and *Pst*I) and rbcS terminator coding sequences controlled by the preferential core CaMV35S promoter were constructed as follows. The foreign fragment of 35S-MCS-rbcS was cleaved from the pCHF3 vector between the *Eco*RI and *Hin*dIII sites and then was ligated into the pCAMBIA1301, pCAMBIA3300, and pCAMBIA3301 vectors that were digested with *Eco*RI and *Hin*dIII. The resulting plasmids were confirmed by enzyme digestion and sequencing, and they were named pCHF1301, pCHF3300, and pCHF3301.

To replace the constitutive CaMV35S promoter with a strong stress-inducible *Arabidopsis rd29A* promoter (Yamaguchi-Shinozaki and Shinozaki, 1994), an *rd29A* promoter fragment was obtained by PCR amplification from *Arabidopsis* DNA with a pair of primers, pRD29A-1 and pRD29A-2 (Table 1), and inserted into the *Eco*RI/*Sac*I-digested plasmids that were constructed above. The resulting plasmids were confirmed by enzyme digestion and sequencing, and they were named pCR-1301, pCR-3300, and pCR-3301.

Construction of 35S-or *rd29A*-based subcellular localization vectors

To investigate the subcellular localization of candidate genes, a set of subcellular localization vectors were obtained. Generally, an *eGFP* gene coding sequence was amplified with two flanking primers (OL-3 and OL-4) from pMD18T-*eGF*P, while CaMV35S was obtained with primers OL-1 and OL-2 from pCHF3. Subsequently, two overlapping primers (OL-1 and OL-4) were used for overlapping PCR, and the resulting segment was named CaMV35S-e*GFP*. After digestion by *Eco*RI/*Pst*I, the PCR fragment CaMV35S-e*GFP* was inserted into the *Eco*RI/*Pst*I-digested intermediate plant expression vectors pCHF1301, pCHF3300, and pCHF3301 to produce C-terminal *eGFP*-fused expression vectors. The resulting plasmids were confirmed by enzyme digestion and sequencing and given the names pCG1301, pCG3300, and pCG3301. A PCR mixture of approximately 25 µL (18.0 µL ddH_2O, 2.5 µL 10X PFU PCR buffer, 1 µL 10 mM dNTP mixture, 1 µL of each PCR primer, 0.5 µL 5 U/µL PFU DNA polymerase, and 0.5 µL *Arabidopsis* DNA and pCHF3 plasmid template) was used for PCR amplification, which followed the following reaction procedure: 10 min at 94°C for pre-denaturation; 5 cycles of 30 s at 94°C for denaturation, 30 s at 45°C for primer annealing, and 3 min at 72°C for extension; 10 min at 94°C; and 25 cycles of 30 s at 94°C for denaturation, 45 s at 57°C for annealing, and 4 min at 72°C for extension.

To substitute the constitutive CaMV35S promoter with the strong stress-inducible *rd29A* promoter, a similar procedure was performed as described before, and the final plasmids were confirmed by enzyme digestion and sequencing, and they were named pCRG1301, pCRG3300, and pCRG3301.

GUS and GFP assays

The destination constructs pCHF1301, pCHF3301, pCR1301, pCR3301, pCG1301, pCG3300, pCG3301, pCRG1301, pCRG3300, and pCRG3301 were introduced into *Agrobacterium tumefaciens* EHA105 based on the protocol established (Weigel et al., 2006). In order to obtain stably transformed *Arabidopsis* plants (T3 homogenous lines), the binary expression vector pCHF1301 and pCHF3301 carrying a *GUS* reporter gene driven by the CaMV35S promoter were transformed into *A. thaliana* (Col-0) by the floral dip method, and

positive transformants were selected on MS medium containing 25 mg/L hygromycin (Hyg) B, and by spraying Basta under controlled environmental conditions as described previously, respectively (Zhang et al., 2006; Peters et al., 2010). All of the *Arabidopsis* transformants were characterized by PCR with primers Hyg-F and Hyg-R, and Bar-F and Bar-R, respectively. To perform transient expression, intact leaves of 4-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain EHA105 harboring all corresponding constructs (Yang et al., 2012).

The GUS assay was performed as previously described with a slight modification (Jefferson, 1989; Liu et al., 2010). Infiltrated *N. benthamiana* leaves were detached 3 days post-agro infiltration and put into GUS staining buffer [50 mM phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 0.1% X-Gluc (w/v)] and then incubated for 12 h at 37°C in the dark with continuous gentle shaking. Leaves were then washed in ddH_2O when blue color appeared and placed into 75% ethanol to fix and remove chlorophyll. The photographs were taken using stereoscopic zoom microscope Nikon SMZ1500 and Canon digital camera EOS 550D. The GUS assay of T3 homogenous transgenic *Arabidopsis* (harboring pCHF1301 and pCHF3301) was performed identically.

To investigate the compartment preference and dynamics of the MCS-*eGFP* fusions in transgenic plants, a variety of *Agrobacterium* strains harboring pCG1301, pCG3300, pCG3301, pCRG1301, pCRG3300, and pCRG3301 are available for transient expression in *N. benthamiana*. The infiltrated 4-week-old *N. benthamiana* leaves were detached 3 days post-agro infiltration, and the localization of GFP in leaves was observed at 488 nm using a confocal laser scanning microscope (Olympus Fluoview FV1000) for transient expression analysis according to a previously reported method (Yang et al., 2012).

RESULTS

Constructions of 35S-and *rd29A*-based expression vectors for stable expression, stress-inducible expression, and subcellular localization

Utilizing conventional and economical restriction enzymes, we successfully obtained a set of novel intermediate plant binary expression vectors containing the MCS under the control of the CaMV35S promoter. All of the resulting vectors (pCHF1301, pCHF3300, and pCHF3301) were confirmed by PCR, restriction enzyme digestion, and sequencing, and schematic diagrams of all three constructs are shown (Figure 1). Specifically, the *Kpn*I site is not unique in pCHF3300, and the *Kpn*I and *Sal*I site are not uniquely present in pCHF3301 because identical digestion sites are present in the sequence of the selective markers.

The CaMV35S (930 bp), *eGFP* (765 bp), and 35S-MCS-*eGFP* (1640 bp) PCR products, which contain additional sequence, were amplified by overlapping PCR using the proofreading *Pfu* DNA polymerase (Fermentas Co., China) and inserted into the *Eco*RI/*Pst*I-digested plasmids (pCHF3301, pCHF3301, and pCHF3301) (Figure 2). The resulting vectors (pCG1301, pCG3300, and pCG3301) were shown in Figure 1. Constructs of candidate genes for subcellular localization could be generated by direct amplification of DNA fragments without a stop codon, followed by restriction enzyme digestion at appropriate sites, ligation, and one-step transformation.

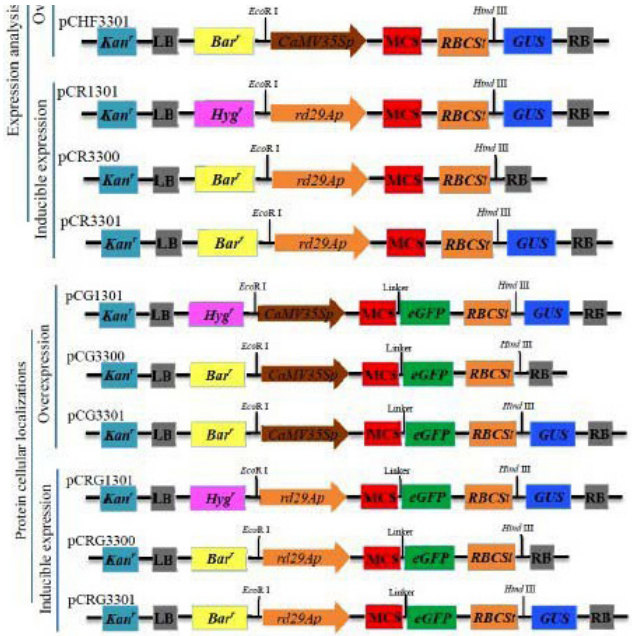

Figure 1. Schematic illustrating the structure of 35S, *rd29A* -based expression vectors for gene overexpression, protein subcellular localization, promoter activity analysis and stress-inducible expression in plant. Schematic structures of constitutive expression vectors with 35S-MCS-rbcSt derived from pCHF3; *rd29A* driven stress-inducible vectors generated by *Eco*RI and *Sac*I sixt-nucleotide recognition sites; Subcellular localization vectors driven by CaMV35S or *rd29A*; others indicated are left and right border (LB and RB), GUS and eGFP reporter gene, and selective marker (Bar or Hyg).

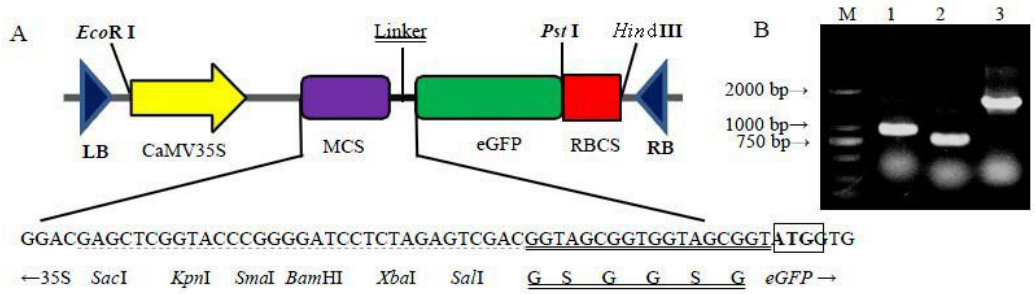

Figure 2. Cloning of 35S-eGFP Fusion by Overlapping PCR. (**A.**) Schematic diagram of CaMV35S-eGFP-RBCS; (**B.**) Agarose gel electrophoresis analysis of PCR products of 35S-eGFP by Overlapping PCR. *Lane M* = DL2000 marker; *lane 1* = Products of PCR of 35S (OL-1, OL-2); *lane 2* = products of PCR of eGFP (OL-3, OL-4); *lane 3* = products of PCR of 35S-eGFP (OL-1, OL-4).

The *Arabidopsis rd29A* promoter was successfully amplified from *Arabidopsis* DNA by PCR with *Pfu* DNA polymerase and cloned into the pCHF and pCG series binary vectors to replace the CaMV35S promoter at the corresponding *Eco*RI/*Sac*I site. The resulting vectors

were designated as pCRG1301, pCRG3300, pCRG3301, pCR1301, pCR3300, and pCR3301 (Figure 1).

Complete sequences for resulting vectors were then regenerated and deposited in the GenBank database. All the accession numbers are available in Table 2.

Table 2. Information of destination vectors in the GenBank database.

Vector	Size (bp)	GenBank	Vector	Size (bp)	GenBank
pCHF1301	13,336	KF206143	pCR1301	13,429	KF206148
pCHF3300	9915	KF206142	pCR3300	10,008	KF206150
pCHF3301	12,806	KF206146	pCR3301	12,899	KF206152
pCG1301	14,074	KF206144	pCRG1301	14,167	KF206149
pCG3300	10,653	KF206145	pCRG3300	10,746	KF206151
pCG3301	13,540	KF206147	pCRG3301	13,633	KF206153

Measuring eGFP fluorescence by a transient expression system

Series of *Agrobacterium* transformants containing *eGFP* driven by CaMV35S or *rd29A* promoter vectors were infiltrated into *N. benthamiana* epidermal cells for transient expression to investigate eGFP fluorescence, and the eGFP fluorescence was consistently and significantly localized in all subcellular compartments without preference (Li et al., 2012). As shown in Figure 3, the fluorescence driven by the *rd29A* promoter in transient expression in *N. benthamiana* was weaker than the fluorescence driven by the CaMV35S promoter. This might be because *rd29A* is a strong stress-induced promoter, and it can also drive gene expression under normal conditions with slight activity. There was no significant difference in observations of fluorescence in the recombinants with CaMV35S-MCS-*eGFP* (Figure 3).

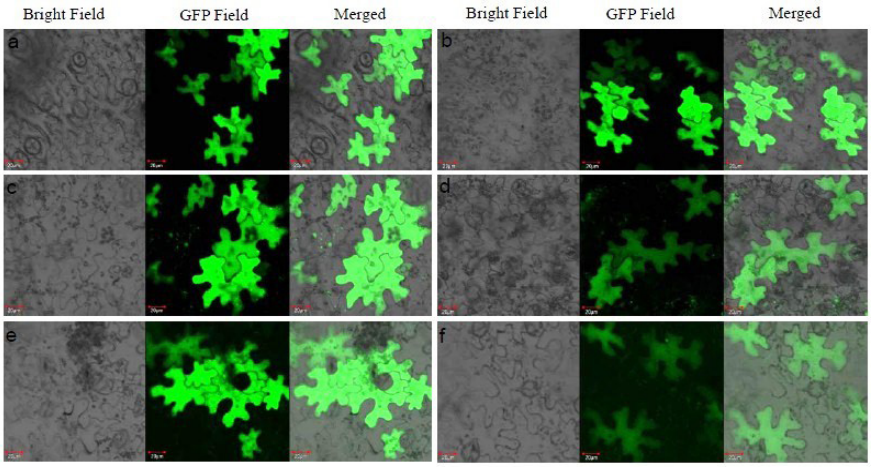

Figure 3. Fluorescence microscopy of transformed *Nicotiana benthamiana* epidermal cells transiently expressing eGFP Micrographs showing cells expressing eGFP from different expression vectors with *eGFP* gene (a = pCG1301; b = pCG3300; c = pCG3301; d = pCRG1301; e = pCRG3300; f = pCRG3301); under Bright Field (left) to examine the epidermal cells, GFP Field (middle) to examine GFP fluorescence, and Merged (right) which is an overlay of bright and fluorescent illumination. Scale bar = 20 µm.

Measuring the GUS reporter gene by transient and stable expression systems

Considering the activity analysis of some plant promoters by the GUS assay, it was logical to test the efficiency of the GUS reporter gene. For this purpose, we divided our vector expression system into two categories. The first part is for stable transgenic plants, and the rest for transient transformation. Histochemical GUS assays showed that all constructs resulted in GUS expression in T3 homogenous *Arabidopsis* and *N. benthamiana* epidermal cells. Similar results were obtained from three different experiments that were conducted at different times, suggesting that the results were consistent and repeatable (Figure 4).

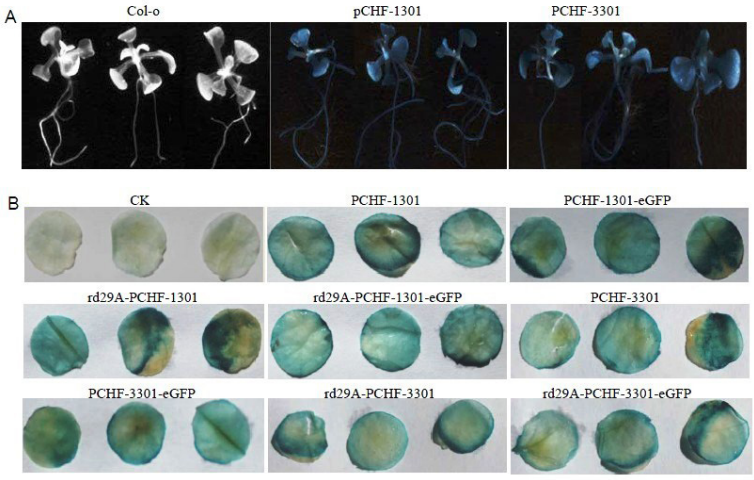

Figure 4. Histochemical staining detection in transient and stable expression transformants (**A.**) Histochemical staining detection in wild-type *Arabidopsis* and the T3 transgenic *Arabidopsis thaliana* of pCHF1301 and pCHF3301 respectively; (**B.**) Histochemical staining detection *Nicotiana benthamiana* for transient expression harboring different vectors. GUS reporter gene was driven by the CaMV35S promoter region.

DISCUSSION

The conventional and economical restriction enzyme digestion method for gene recombination is very simple, highly efficient, and easy to manipulate. In this study, we developed and validated a set of binary vectors for plant functional genomic research. Our results demonstrated that this system is highly efficient for phenotypic analysis, subcellular localization, promoter activity analysis, and stable and stress-inducible expression. In our system, we introduced the CaMV35S-MCS-rbcS fragment into the pCAMBIA-based expression system, which contains replicons for both *Escherichia coli* and *Agrobacterium tumefaciens* with either *Hyg* or *Bar* as the selective marker in plants. Furthermore, we developed vectors for subcellular localization analysis by inserting *eGFP* gene fusions in the C-terminus of the MCS, and there is an essential $(G\text{-}S\text{-}G)_2$ linker between the MCS and eGFP to avoid changing the eGFP conformation (Stanisławska-Sachadyn et al., 2006). To provide strong fluorescence, we employed the *eGFP* reporter marker, which is between 20- and 35-fold more intense and stable than GFP (Cormack et al., 1996). More interesting and useful, the vector system with

different reporter genes could be used to provide compatible vector combinations for generating transgenic plants that co-express two or three candidate genes. In addition, on this basis, we developed more vectors by replacing the CaMV35S promoter with the *Arabidopsis rd29A* promoter for stress-inducible expression of genes of interest, which could cause severe growth retardation and pleiotropic effects under a strong and constitutive CaMV35S promoter. The expression of *eGFP* and GUS reporter genes in the plant binary expression vectors was investigated, and the expression efficiency demonstrated that our vector system is efficient for subcellular localization and promoter analysis.

Although using restriction enzymes to generate constructs is efficient, self-ligation cannot be ruled out because of the potential for incomplete enzyme digestion. The size of these plasmids is also a limiting factor that affects the ligation efficiency. It has been reported that the efficiency of *in vitro* recombination procedures is inversely proportional to the size of the vector (Wang et al., 2013). In particular cases, another limitation of these expression vectors (driven by the CaMV35S promoter) is that they are mainly useful in dicot plants (Cornejo et al., 1993).

To analyze thoroughly this set of vectors, we have developed a simple, rapid, and reliable method to make constructs for stable and stress-inducible expression for phenotypic, functional, subcellular localization, and promoter activity investigation. Thus, our system proves to be promising for large-scale analysis of plant functional genomics.

ACKNOWLEDGMENTS

The authors are grateful to Zhao Jun (Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China) for donating the initial pCHF3 vector. Research supported by the National Science and Technology Support Program (#2012BAD19B04) and the project from the Ministry of Agriculture Key Projects of GM Cultivation of New Varieties (#2013ZX08004004).

REFERENCES

Chen PY, Wang CK, Soong SC and To KY (2002). Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. *Mol. Breed.*11: 287-293.

Chen S, Songkumarn P, Liu J and Wang GL (2009). A versatile zero background T-vector system for gene cloning and functional genomics. *Plant. Physiol.* 150: 1111-1121.

Cormack BP, Valdivia RH and Falkow S (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173: 33-38.

Cornejo MJ, Luth D, Blankenship KM, Anderson OD, et al. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol. Biol.* 23: 567-581.

Curtis MD and Grossniklaus U (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133: 462-469.

Earley KW, Haag JR, Pontes O, Opper K, et al. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. *Plan. J.* 45: 616-629.

Gaj T, Gersbach CA and Barbas CF, III (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31: 397-405.

Hsieh TH, Lee JT, Charng YY and Chan MT (2002). Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced resistance to water deficit stress. *Plant Physiol.* 130: 618-626.

Jefferson RA (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5: 387-405.

Jefferson RA (1989). The GUS reporter gene system. *Nature* 342: 837-838.

Jefferson RA, Kavanagh TA and Bevan MW (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene

fusion marker in higher plants. *EMBO J.* 6: 3901-3907.
Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, et al. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* 17: 287-291.
Kasuga M, Miura S, Shinozaki K and Yamaguchi-Shinozaki K (2004). A combination of the *Arabidopsis* DREB1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* 45: 346-350.
Krysan PJ, Young JC and Sussman MR (1999). T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* 11: 2283-2290.
Li T, Huang S, Zhao X, Wright DA, et al. (2011). Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic. Acids. Res.* 39: 6315-6325.
Li XJ, Qian WQ, Zhao YS, Wang CL, et al. (2012). Antisilencing role of the RNA-directed DNA methylation pathway and a histone acetyltransferase in *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 109: 11425-11430.
Liu Q, Wang J, Miki D, Xia R, et al. (2010). DNA replication factor C1 mediates genomic stability and transcriptional gene silencing in *Arabidopsis*. *Plant Cell* 22: 2336-2352.
Peters S, Egert A, Stieger B and Keller F (2010). Functional identification of *Arabidopsis* ATSIP2 (At3g57520) as an alkaline alpha-galactosidase with a substrate specificity for raffinose and an apparent sink-specific expression pattern. *Plant Cell Physiol.* 51: 1815-1819.
Rybel BD, Berg W, Lokerse AS, Liao CY, et al. (2011). A versatile set of ligation-independent cloning vectors for functional studies in plants. *Plant. Physiol.* 156: 1292-1299.
Stanislawska-Sachadyn A, Sachadyn P, Ihle K, Sydorczuk C, et al. (2006). The construction of bifunctional fusion proteins consisting of MutS and GFP. *J. Biotechnol.* 121: 134-143.
Wang CT, Yin XL, Kong XG, Li WS, et al. (2013). A series of TA-based and zero-background vectors for plant functional genomics. *PLoS One* 8: e59576.
Wang CX, Jing RL, Mao XG, Chang XP, et al. (2011). TaABC1, a member of the activity of bc1 complex protein kinase family from common wheat, confers enhanced tolerance to abiotic stresses in *Arabidopsis*. *J. Exp. Bot.* 62: 1299-1311.
Weigel D and Glazebrook J (2006). Transformation of *Agrobacterium* Using the Freeze-thaw Method. Cold Spring Harbor Protocol, Cold Spring Harbor.
Yamaguchi-Shinozaki K and Shinozaki K (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* 6: 251-264.
Yan P, Shen W, Gao X, Li X, et al. (2012). High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. *PLoS One* 7: e38186.
Yang L, Ji W, Gao P, Li Y, et al. (2012). GsAPK, an ABA-activated and calcium-independent SnRK2-type kinase from G. soja, mediates the regulation of plant tolerance to salinity and ABA stress. *PLoS One* 7: e33838.
Yin Y, Wang ZY, Mora-Garcia S, Li J, et al. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109: 181-191.
Zhang X, Henriques R, Lin SS, Niu QW, et al. (2006). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* 1: 641-646.