BioVector, a flexible system for gene specific-expression in plants

Xu Wang, Chengming Fan†, Xiaomei Zhang†, Jinlong Zhu† and Yong-Fu Fu*

Abstract

Background: Functional genomic research always needs to assemble different DNA fragments into a binary vector, so as to express genes with different tags from various promoters with different levels. The cloning systems available bear similar disadvantages, such as promoters/tags are fixed on a binary vector, which is generally with low cloning efficiency and limited for cloning sites if a novel promoter/tag is in need. Therefore, it is difficult both to assemble a gene and a promoter together and to modify the vectors in hand. Another disadvantage is that a long spacer from recombination sites, which may be detrimental to the protein function, exists between a gene and a tag. Multiple GATEWAY system only resolves former problem at the expense of very low efficiency and expensive for multiple LR reaction.

Results: To improve efficiency and flexibility for constructing expression vectors, we developed a platform, BioVector, by combining classical restriction enzyme/ligase strategy with modern Gateway DNA recombination system. This system included a series of vectors for gene cloning, promoter cloning, and binary vector construction to meet various needs for plant functional genomic study.

Conclusion: This BioVector platform makes it easy to construct any vectors to express a target gene from a specific promoter with desired intensity, and it is also waiting to be freely modified by researchers themselves for ongoing demands. This idea can also be transferred to the different fields including animal or yeast study.

Keywords: Expression vector, Gene specific-expression, Functional genome study, Gene cloning, Restriction enzyme/ligase strategy, Gateway DNA recombination

Background

Along with the fulfilment of the whole genome sequencing of different organisms including many crops (http://genomesonline.org/cgi-bin/GOLD/index.cgi?page_requested=Statistics), the challenge of how to comprehensively study gene functions on a large scale emerges. Gene cloning, gene expression in different modes (such as constitutive, tissue-/developmental-specific, or inducible ectopic expression), protein localization and interaction, silencing the gene, and promoter function and regulation are always the issues a researcher concerns. Therefore, a series of high efficient cloning and expression vectors are developed in different labs [1-10]. The primary cloning approaches available can be mainly classed into two groups: the traditional restriction enzyme/ligase (REL) strategy including TA cloning technology and the DNA recombinational cloning systems including the Gateway cloning system from Invitrogen and the Creator cloning system from CLONTECH. The former restriction enzyme/ligase strategy, even though cheap, suffers from various problems, mainly no suitable restriction enzyme sites in binary vectors compatible for different genes and promoters. Therefore, it is rather limited for projects on a large scale and multiple functions. TA cloning technology increases the cloning efficiency, and at the same time it increases the cost too, because it is difficult to generate a high-quality TA cloning vector in home-made and a researcher has to purchase different TA cloning kits from the market.

The DNA recombinational cloning system, especially the Gateway Cloning System from Invitrogen, is recently developed and widely used. The first step of this system (BP recombination reaction) is to clone the PCR product...
into a donor vector to produce an entry clone [11]. Once an entry clone is available, the gene of interest is easily transferred into different expression vectors through an LR reaction, which is a high efficient reaction. Due to its many advantages, many cloning systems related were developed [1-4]. However, there are still some limitations for this technology. For example, the primers at the first step need a long extra sequence for recombination reaction, which decreases the efficiency of PCR and BP reaction and increases the cost. Aimed at these problems, higher efficiency vector systems are developed. Invitrogen develops a high efficiency kit (TOPO Gateway Entry vectors), which combines TA cloning and BR reaction to eliminate the BP reaction, and the resulting vector can be directly used to LR reaction (http://www.invitrogen.com/), but it is at the cost of a high price. The ZeBaTA system introduces the TA cloning into plant binary vectors and results in zero background vectors [12]. This ZeBaTA system obviously needs to make a lot of home-made and low efficiency T-binary-vectors for different purposes, such as to label a gene of interest with different tags. TA cloning has another problem waiting to be solved: the gene orientation, which decreases the cloning efficiency. Multiple Gateway Systems are developed for cloning multiple DNA fragments (such as, promoters, genes and terminators) into an expression vector in one step [5-8], but it also suffers from low efficiency because of many recombination sites. The type IIs endonucleases recognize asymmetric DNA sequences, 4–7 bp long, and cleave both strands at specific locations up to 20 bases away from their recognition site [13]. Recently, two excellent papers reported new gene cloning strategies, referred to Golden Gate Cloning (mainly based on the type IIs endonucleases Bsa I and Bbs I) [9] and GoldenBraid (mainly based on the type IIs endonucleases Bsa I and BsmB I) [10], with which it is possible to seamlessly assemble multiple reusable gene modules, including promoters, genes and terminators, together in a binary vector in a single restriction-ligation.

Even though a lot of cloning systems are now available as discussed above, many common problems still exist in our routine experiment. In modern functional genomic study, one of challenges is to construct a set of vectors for a gene of interest to study gene functions in special spatio-temporal patterns. It includes cloning rapidly and efficiently, labeling proteins with different tags, shuffling randomly promoters and genes, introducing a desired regulatory element for a given promoter or gene, and modifying vectors in hand for ongoing demands. Therefore, the flexibility of vectors appears to be a main challenge. What’s more, GATEWAY technology available now leaves a common question to be answered: after LR reaction, there is a longer spacer existed between genes and tags, which may result in detrimental effect on protein functions [14].

In this study, we developed a highly flexible expression system to cope with on-going demands in plant functional genomic study, based on classical cloning approaches other than to introduce a new method. Firstly, we employed widely-used site-specific recombination (SSR) cloning system (Gateway, Invitrogen) for high cloning efficiency. Secondly, we introduced two pairs of SSRs, one for genes and the other for promoters in two independent entry clones (EC). Thirdly, we adopted traditional restriction enzyme/ligase (REL) strategy in an easy-operating intermediate cloning vector, instead of a difficult-operating binary vector as previous researchers used. This REL is designed for cloning of desired multiple regulatory elements or tags, and it also provide flexible choices for cloning.

**Results and discussion**

Through directly synthetizing sequences of all DNA fragments (Additional file 1) except the backbone of vectors indicated below, we developed our cloning system, BioVector. BioVector is composed of three basic vectors: a gene entry clone (GEC) with one pair of SSRs of attL1/attL2, a promoter entry clone (PEC) with another pair of SSRs of attL3/attL4, and a binary destination vector (BDV) with two pairs of corresponding SSRs of attR1/attR2 and attR3/attR4. Therefore, with a single LR reaction (Invitrogen), a promoter can be fused to a gene and a plant expression vector is constructed.

**Gene entry clone**

The gene entry clone (GEC) (Figure 1, Table 1) was developed from the pUC vector due to its advantage of easy-operating for cloning, and introduced up to 20 restriction enzyme/ligase (REL) sites including T/A cloning sites (Ahd I sites), therefore it is convenient to clone any DNA fragments and to assemble multiple DNA fragments into a GEC. REL sites were inserted inside site-specific recombination (SSR) sites (attL1 and attL2) in GECs, so that it was possible to make a seamless fusion between a gene and a tag in our system, rather than to introduce a SSR spacer (around 20 aa) between them as widely-used GATEWAY Cloning System does, in which the tags are in destination vectors [1,7,15]. Such a spacer has the potential to result in the synthesis of a non-functional or insoluble protein-tag fusion [14]. Another improvement was that a stop code was put at the end of REL sites and inside SSR (attL2). Thus, only the gene without a stop code was needed to be cloned into a GEC, then it can be removed from one GEC to another by a simple REL. In this case, there was no need to clone a gene for the N- or C-terminal fusion, respectively, as wide-used GATEWAY Cloning Systems do [1-4]. Therefore, GECs saved both time and cost for cloning and sequencing of genes.
Therefore, genes, tags, and any regulatory elements of interest from PCR amplification can be directly cloned into GECs after digestion. We constructed a set of alternative GECs for labeling a desired protein with different tags at the N- or C-terminus (Table 1). And all GECs shared identical REL sites, and it was easy to move a gene from one GEC to another with a simple REL.

A ccdB gene in REL sites (Figure 1) served as a negative selection marker for E. coli DH5α as all GATEWAY entry vectors employ (Invitrogen) [1,7,15]. The chloramphenicol (Cm) was employed as a selection marker in E. coli, which may be compatible to most of binary vectors available.

We designed a primer Entry-F (5′-ACTTGCATTACA GCTTACGAACCGA-3′) for forward sequencing, and the general primer sp6 (5′-ATTTAGGTGACACTATAG-3′) can be used as a reverse sequencing primer.

Plenty of REL sites in GECs provide a flexible platform for ad arbitrium modifying the vector by researcher themselves for their own individual study. This is an important respect of this flexible system, because the plasticity of a system is always demanded in functional genome study, but a shortage of wide-used GATEWAY systems [1-4]. For example, to insert RNA-binding loops (16× BoxB or 6× MS2) [16] inside SSRs in GEC makes it possible to label an RNA. One of our considerations was to develop GECs compatible with the present Gateway destination vectors for plants, E. coli, and yeast, so researchers can keep their vectors in hands when introduce BioVector.

Promoter entry clone

Most of GATEWAY based cloning systems normally put a constitutive promoter, such as CaMV 35S, in the binary vector, in which the promoter is not easy to be replaced by another promoter due to its low cloning efficient. Even though Multiple Gateway System [5-8] clone a promoter inside the specific SSR sites, it bears low efficient in subsequent multiple recombination reactions and it is also difficult to insert a cis-element next to the promoter. The promoter entry clone (PEC) in BioVector (Figure 2) also originated from pUC vector, shared the same backbone as GEC, and embraced up to 27 REL sites inside SSR sites (attL3 and attL4). The plenty of REL sites benefited to freely clone or assemble various promoters or cis-elements together to study their functions. Obviously, as in GECs, PECs were good to be updated by researchers for an ambitious idea.

There were three PECs available with no enhancer, one enhancer, or two enhancers, respectively (Table 2). Thus, a
Table 1: A list of gene entry clones (GEC)

| Vector name | Tag and its position | Restriction enzyme sites at both ends of tags | ABRC stock number* |
|-------------|----------------------|---------------------------------------------|--------------------|
| Fu28        | C-GFP                | Sac I                                       | CD3-1822           |
| Fu30        | N-GFP                | Bgl II                                      | CD3-1823           |
| Fu41        | N-YFP                | Bgl II, Xba I                               | CD3-1824           |
| Fu42        | C-YFP                | EcoR I, Sac I                               | CD3-1825           |
| Fu43        | N-CFP                | Bgl II, Xba I                               | CD3-1826           |
| Fu44        | C-CFP                | EcoR I, Sac I                               | CD3-1827           |
| Fu45        | N-mRFP               | Bgl II, Xba I                               | CD3-1828           |
| Fu46        | C-mRFP               | EcoR I, Sac I                               | CD3-1829           |
| Fu47        | N-3MYC               | Bgl II                                      | CD3-1830           |
| Fu48        | C-3MYC               | Sac I                                       | CD3-1831           |
| Fu49        | N-3FLAG              | Bgl II                                      | CD3-1832           |
| Fu50        | C-3FLAG              | Sac I                                       | CD3-1833           |
| Fu55        | N-3HA                | Bgl II                                      | CD3-1834           |
| Fu56        | C-3HA                | EcoR I                                      | CD3-1835           |
| Fu58        | N-GST                | Bgl II, Xba I                               | CD3-1836           |
| Fu59        | N-StreplI            | Bgl II                                      | CD3-1837           |
| Fu60        | N-NLS                | Bgl II                                      | CD3-1838           |
| Fu61        | C-NES                | Sac I                                       | CD3-1839           |
| Fu62        | C-GUS                | EcoR I, Sac I                               | CD3-1840           |
| Fu63        | C-LUC                | EcoR I, Sac I                               | CD3-1841           |
| Fu64        | N-FLAG; C-YFPc       | Bgl II for FLAGc; EcoR I, Sac I for YFPc     | CD3-1842           |
| Fu65        | N-MYC; C-YFPn        | Bgl II for MYC; EcoR I, Sac I for YFPn       | CD3-1843           |
| Fu66        | N-YFPc; C-HA         | Bgl II, Xba I for YFPc; Sac I for HA        | CD3-1844           |
| Fu67        | N-YFPn; C-MYC        | Bgl II, Xba I for YFPn; Sac I for MYC        | CD3-1845           |
| Fu79        | Tag free             |                                             | CD3-1846           |

*All vectors were delivered to ABRC (http://www.arabidopsis.org/). "N" or "C" indicates that the position of a tag is at the "N" or "C" terminus of a target gene, "n" or "c" for the "n" or "c" half part of YFP fluorescence protein. All vectors contain recombination sites of attL1 and attL2 and have the chloramphenicol selection marker in E. coli. All the sequences are showed in Additional file 1.

A gene can be expressed or over-expressed from a native promoter, and the bona fide functions of a gene could be revealed. Such a strategy was designed to avoid disadvantages of generally over-expressing promoter CaMV 35S, which has overt ectopic effects [17], weak or null functions in some tissues and plants [18-22], and adverse effects on adjacent genes [19,23,24].

Again, the three PECs had identical REL sites, which conferred to easily shift promoters among them. The selection marker and the sequencing primers in PECs were same to that of GECs.

Besides REL strategy, other cloning technology, such as In-Fusion® HD Cloning from Clontech (www.clontech.com), Protocol No. PT5162-1, October 2011), can be an alternative efficient approach for cloning genes or promoters into GECs or PECs, so as to increase the cloning efficiency. If introducing the type IIs endonucleases, such as Bsa I, Bbs I, and BsmB I, GECs and PECs could be compatible to Golden Gate Cloning [9] and GoldenBraid [10].

**Binary destination vector**

All binary destination vectors (BDVs) in this study were developed from pGreen/pCLEAN, which are highly efficient in a wide arrange of plants [25,26]. So, BDVs have smaller size (5~9Kb) than most of gateway-compatible binary vectors (10~18Kb) [1,3,4]. And the small size of binary vectors facilitates to increase the cloning efficiency, plasmid yield and plant transformation [27]. Because the right border of the T-DNA is largely preserved whereas the left border is frequently truncated after integration [17,28,29], a selectable marker in *planta* was placed next to the left border in BDVs to ensure that all transformants with a positive marker always carry the introduced gene.

A set of binary vectors were designed for a genomic gene (BDV1, Figure 3A) or CDS gene expression (BDV2, Figure 3B), gene silencing (BDV3, Figure 3C), and ethanol-inducible expression (BDV4, Figure 3D), respectively. There were more BDVs available with various selection markers in *E. coli* and plants (Table 3), providing multiple choices in different projects. So, BioVector can be used to express a genomic gene spanning the sequence from the promoter to the terminator, to analyze the function of coding sequence from a desired promoter or ethanol-inducible promoter, to monitor proteins with fluorescent and other tags, to study protein-protein interaction, and to silence a gene in specific spatio-temporal mode. The MCS in BDVs facilitates to be modified for extensive demands (Figure 3), such as replacing the preloaded transcription terminator or the selection marker.

Once a gene and a promoter were cloned into GECs or PECs, respectively, the gene and the promoter can be assembled into a binary vector through a single LR reaction (Figure 4), which is a high efficient and directional reaction (Invitrogen). Several systems, such as MultiSite Gateway Cloning [15], provide a possibility to combine promoters and genes at will, but with limited numbers of promoters and tags available. BioVector, however, made it easy to express a gene from different desired promoters with different intensities.

Gene entry clones from wide-used GATEWAY system made by most researchers shared the same SSR sites attL1/2 at both ends with GECs. Thus, Gene entry clones at hand were compatible with our BDVs, rather than discarding them when introduce BioVector.

Obviously, GECs and PCEs can serve as an intermediate vector for cloning due to plenty of MCSs available (Figure 1...
and 2). In addition, GECs, PECs and BDVs can be shared worldwide as a library. We have delivered all vectors in this study to ABRC centre (Table 1, 2 and 3).

Verification of BioVector
With BioVector we succeeded in stable or transient expression of GFP with or without nuclear localization signal (NLS) or nuclear export signal (NES) in Arabidopsis protoplasts (Figure 5A), a GUS gene from the companion cell-specific SUCROSE H+ SYMPORTER2 (SUC2) promoter [30] with or without enhancers in Arabidopsis (Figure 5B), a Myc-tagged gene in Nicotiana benthamiana (Figure 5C), and a luciferase gene under ethanol-inducible pattern N. benthamiana (Figure 5D). The results supported that BioVector was efficient expression vector for plants.

Conclusion
Perfect combination of conventional REL strategy and modern SSR technology confers obvious advantages to BioVector. (1) Exchangeable and Efficient. A gene and a promoter can be easily assembled together to fulfill expressing a gene from a temporal-spatio promoter with different intensity, especially overexpressing genes under the control of native promoters; (2) Flexible. GECs, PECs, and BDVs can be ad arbitrum modified with ongoing demands; (3) Practical and Versatile. BioVector can be applied to almost all fields in functional genome research of various plants; (4) Universal and Time-/Labour-Saving. GECs can be efficiently applied to any plant, yeast, and E. coli destination vectors sharing corresponding SSR sites, and it is possible to construct a worldwide library as shared community resource for GECs, PECs and BDVs; (5) Seamless fusion. It is possible to make seamless fusion between a protein and a tag, rather than to introduce a detrimental SSR spacer as the widely-used Gateway recombination system does; (6) Broad application and interest. The idea of BioVector can also be applied to similar study in animals and yeast.

Methods
Vectors constructing
All basic vectors including gene entry clones, promoter entry clones, and binary destination vectors, were directly synthetized according to the sequences on Additional file 1. Then the PCR amplified products of different tags, fluorescent markers, selection markers, and other elements were inserted into corresponding positions by restriction enzyme/ligase (REL) strategy to produce a set of vectors.

Table 2 A list of promoter entry clones (PEC)

| Vector name | Number of enhancers | Restriction enzyme sites at both ends of enhancers | ABRC stock number* |
|-------------|---------------------|-----------------------------------------------------|-------------------|
| Fu76        | Enhancer free       |                                                     | CD3-1847          |
| Fu77        | 1 x Enhancer        | Nde I                                               | CD3-1848          |
| Fu78        | 2 x Enhancer        | Nde I                                               | CD3-1849          |

* All vectors were delivered to ABRC (http://www.arabidopsis.org/). All vectors contain recombination sites of attL3 and attL4 and have the chloramphenicol selection marker in E. coli. All the sequences are showed in Additional file 1.
Gene and promoter cloning

The original SUC2 promoter is kindly presented by Dr. George Coupland. For making an entry clone of SUC2, the sequence of SUC2 was cloned into Fu76, Fu77, and Fu78 between Sal I and Bgl II sites, respectively, to produce Fu76-SUC2, Fu77-SUC2, and Fu78-SUC2.

The Ubiquitin (Ub) promoter was inserted between Stu I and Fsp I sites in Fu76 to generate Fu76-Ub. 2x35S promoter were subcloned from pLeela (from Dr. George Coupland) by PCR and inserted into Fu76 with Sal I and Pst I restriction sites to make Fu76-2x35S. Fu62 was digested with Bgl II and Bam HI to remove excess MCS and self-ligated to produce Fu62-GUS. Fu63 was digested with Bgl II and Bam HI and self-ligated to make Fu63-LUC. The GFP gene was cloned into Fu26, Fu48, Fu60, and Fu61, respectively, to generate expression vectors of...
Fu26-GFP, Fu48-GFP:3xMyc, Fu60-NLS:GFP, and Fu61-GFP:NES. All genes and promoters were confirmed by sequencing with primers of Entry-F (5′-ACTTGCATTACAGCTTACGAACCGA-3′) and sp6 (5′-ATTTAGGTGACACTATAG-3′).

**LR reaction protocol**

Fu26-GFP, Fu48-GFP:3xMyc, Fu60-NLS:GFP, and Fu61-GFP:NES, respectively, with Fu76-2x35S recombined with Fu39-2 through LR reaction (Invitrogen) to make binary vectors of Fu39-2x35S::GFP, Fu39-2X35S::GFP:3xMyc,
Fu39-2x3SS::NLS:GFP, Fu39-2x3SS::GFP:NES. And Fu76-SUC2, Fu77-SUC2, and Fu78-SUC2, respectively, with Fu61-GUS was subjected to LR reaction (Invitrogen) with Fu39-2 to make promoter::GUS expression vectors, Fu39-2-SUC2::GUS, Fu39-2-SUC2::Enh::GUS, Fu39-2-SUC2:2xEnh::GUS. Both Fu63-LUC and Fu76-Lib were subjected to LR reaction with Fu39-8 to get ethanol-induced LUC expression vector Fu39-8-Lib::LUC. 5 μL system reaction was used for multiple-components LR, including Fu39-2 (20 ~ 50 ng), two entry clones (120 ~ 150ng, respectively) and 1 μL LR Clonase II Enzyme mix (Invitrogen). Incubate the mixture at 25°C for overnight and transform the LR reaction product to E. coli strain DH5α for selecting the positive clones through PCR.

**Transient expression in Arabidopsis protoplasts**

*Arabidopsis* (Col) mesophyll protoplasts isolation and transformation were performed according to Sheen's protocol (http://genetics.mgh.harvard.edu/sheenweb/faq.html). Briefly, well-expanded leaves of 3- to 4-week-old *Arabidopsis* plants grown on soil were cut into small strips with a razor blade and incubated in 10 mL of enzyme solution (0.4% Macerozyme R-10, 1.5% Cellulase R-10, 400 mM mannitol, 10 mM CaCl2, and 20 mM MES pH5.6, 20 mM KCl) at 23°C for 3 ~ 4 hours. After incubation, the protoplast suspension was filtered through 100 μm mesh and protoplasts were collected by centrifugation at 100xg for 2 min at 4°C. Wash the pelleted protoplasts once in cold W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES pH5.7) and resuspend protoplasts in the same solution. Keep the protoplasts on ice for 30 min. Spin down protoplasts and resuspend in MMg solution (400 mM mannitol, 15 mM MgCl2, and 4 mM MES pH5.6) at a density of 1-2 × 10^5 protoplasts/mL before PEG transfection. Warm the cold W5 solution to 23°C. To transform DNA into protoplasts, 20 μL plasmid DNA (about 20 μg) was added to 100 μL of protoplast suspension, mixed gently, then added 120 μL of PEG solution (200 mM mannitol, 100 mM CaCl2, and 40% PEG4000). The mixture was mixed gently and incubated for 30 min at room temperature. After incubation, the mixture was diluted with 500 μL W5 solution and spun at 100× g for 3 min at 23°C. The recovered protoplasts were resuspended in 1 mL W5 solution and incubated at 23°C in the dark for 12 ~ 14 hours.

**Transformation of arabidopsis (flower dipping)**

After LR reaction, the resulted BDV was introduced into *Agrobacterium tumefaciens* strain GV3101 with pSoup by electroporation. Prepare the *A. tumefaciens* strain GV3101 carrying the wanted BDV by inoculating a single colony into 5 mL liquid LB medium containing the appropriate antibiotics for binary vector selection. Incubate culture at 28°C for 1 day. Use this feeder culture to inoculate a
100 mL liquid LB with the appropriate antibiotics and grow the culture at 28°C for overnight until the cells grown to OD_{600} = 1.5 ~ 2.0. Collected Agrobacterium cells by centrifugation at 4,000xg for 10 min at room temperature, and gently resuspended the pellet in 100 mL freshly prepared Dipping Buffer (5% sucrose, 10 mM MgCl_2, 0.02% Silwet L-77). The inflorescences of Arabidopsis plants were submerged in the Agrobacterium cell suspension for 20 seconds and then bagged with plastic for 24 hours. After this incubation, plastic bags were removed. The transformed Arabidopsis plants were grown under LD in the greenhouse until seeds could be collected.

**Infiltration of nicotiana benthamiana leaf**

A. tumefaciens strain EHA105 harboring the Fu39-8-Lb::LUC vector was grown at 28°C in LB medium supplemented with appropriate antibiotics to stationary phase. Bacteria were pelleted by centrifugation at 4000xg for 10 min at room temperature and wash the pellet once in phosphate buffer pH 7.0, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100] for 100 mL liquid LB with the appropriate antibiotics and grow the culture at 28°C for overnight until the cells grown to OD_{600} = 1.5 ~ 2.0. Collected Agrobacterium cells by centrifugation at 4,000xg for 10 min at room temperature, and gently resuspended the pellet in 100 mL freshly prepared Dipping Buffer (5% sucrose, 10 mM MgCl_2, 0.02% Silwet L-77). The inflorescences of Arabidopsis plants were submerged in the Agrobacterium cell suspension for 20 seconds and then bagged with plastic for 24 hours. After this incubation, plastic bags were removed. The transformed Arabidopsis plants were grown under LD in the greenhouse until seeds could be collected.

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**Protein extraction and immunoblot analysis**

After incubation for 12 ~ 14 h, the transformed Arabidopsis protoplasts were collected by centrifugation at 100xg for 2 min at room temperature. For extraction the total protein, the pelleted cells were incubated in 2x SDS loading buffer (100 mM Tris-HCl pH6.8, 4% SDS, 200 mM DTT, 0.2% bromophenol blue and 20% glycerol) at 95°C for 5 min, and then spun the mixture at 12,000xg for 10 min at room temperature. The extracts were loaded on 12% SDS-PAGE for separation. After electrophoresis, proteins were transferred onto ECL nitrocellulose membrane (GE Healthcare, no. RPN303D) by wet electroblotting. For detection of GFP, a mouse monoclonal GFP antibody (Roche, no. 1181446001) and a goat anti-mouse antibody conjugated to peroxidase (Pierce, no. 31430) were used at 1: 3000 and 1: 5000 dilutions, respectively. Blots were developed using the ECL kit (Pierce, no. 34079) and chemiluminescence emitted from the filter was visualized by a transmission detector. The images were processed with Leica LAS AF and MacBiophotonics ImageJ softwares (http://rsbweb.nih.gov/ij/plugins/mbf/index.html).

**Ethanol induction and luciferase imaging**

For testing the ethanol-inducible luciferase gene expression, the infiltrated N. benthamiana leaves were cut into pieces and imbedded in 1/2 MS liquid medium supplemented with (or without) 2% (v/v) ethanol for indicated hours. After incubation, the luciferin and Triton X-100 were added into the medium to a final concentration at 100 μM and 0.1%, respectively, the bioluminescence signals were imaged by a Princeton Instruments Digital CCD camera.

**Additional file**

Additional file 1: The sequences of vectors, tags/markers, enhancers, resistant genes, and recombination sites.

**Abbreviations**

BDV: Binary destination vector; EC: Entry clones; GEC: Gene entry clone; NES: Nuclear export signal; NLS: Nuclear localization signal; PEC: Promoter entry clone; REL: Restriction enzyme/ligase; SSR: Site-specific recombination; SUC2: SUCCOHE H+ SYMPORTER2.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

X W performed main experiments, analyzed the data, and wrote Methods; C F, X Z and J Z participated in some work; Y-F F conceived and supervised BioVector strategies, analyzed the data, and wrote the main manuscript. All authors read and approved the final manuscript.

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