2

Gateway Vectors for Plant Genetic Engineering: Overview of Plant Vectors, Application for Bimolecular Fluorescence Complementation (BiFC) and Multigene Construction

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1. Introduction

Transgenic technologies for the genetic engineering of plants are very important for basic plant research and biotechnology. For example, promoter analysis with a reporter such as green fluorescent protein (GFP) is typically used to determine the expression pattern of genes of interest in basic plant research. Moreover, downregulation or controlled expression studies of target genes are used to determine the function of these genes. In plant biotechnology, overexpression of heterologous genes by transgenic methods is widely used to improve industrially important crop plants. Recently, genome projects focusing on various higher plants have provided abundant sequence information, and genome-wide studies of gene function and gene regulation are being carried out. In these areas of research, transgenic analyses using genetically modified plants will become more essential. For example, high-throughput promoter analysis to examine the temporal and spatial regulation of gene expression, the subcellular localization of the gene products based on reporter genes, and ectopic expression of cDNA clones and RNAi will reveal the functions of a variety of genes. For gene manipulation in plants, the binary system of *Agrobacterium*-mediated transformation is most widely used. This system consists of two plasmids derived from Ti plasmids, namely disarmed Ti plasmids and binary vectors (Bevan, 1984). The former contains most genes for T-DNA transfer from *Agrobacterium tumefaciens* to plants, whereas the latter is composed of a functional T-DNA and minimal elements for replication both in *Escherichia coli* and in *A. tumefaciens*. Most of the widely used binary vectors established in the 1990s were constructed by a traditional restriction endonuclease based method. Therefore, it was time consuming and laborious to construct modified genes on

binary vectors using the limited number of available restriction sites because of their large size and the existence of many restriction sites outside their cloning sites. To overcome this disadvantage and perform high-throughput analysis of plant genes, a new cloning system to realize rapid and efficient construction of modified genes on binary vectors was desired. The Gateway cloning system provided by Invitrogen (Carlsbad, CA, USA) is one of these solutions. We have constructed a variety of Gateway compatible Ti binary vectors for plant transgenic research.

2. Basic Ti-binary vector for *Agrobacterium*-mediated transformation and Gateway cloning

Transformation mediated by the soil bacterium *A. tumefaciens* is widely used for gene manipulation of plants. This bacterium has huge Ti-plasmids (larger than 200 kb) and the ability to transfer the T-DNA region of the Ti-plasmid to infect plant chromosomes. The natural Ti-mediated transformation system can be applied to transfer novel genes into a plant genome. To be useful for gene manipulation, binary vectors possessing the T-DNA region were developed. The vectors must possess a plant selection marker gene, a bacterial antibiotic resistance gene, a site for cloning foreign genes, T-DNA border sequences for gene transfer to the plant genome, an origin of replication (*ori*) for a broad host range of the plasmid and an *ori* for *E. coli*. Although binary vectors are much smaller than native Ti–plasmids, they are still large and cause difficulties in gene cloning by traditional methods. Gateway Technology (available from Invitrogen) is based on the site-specific recombination system between phage lambda and *E. coli* DNA. This system was modified to improve its specificity and efficiency to utilize it as a universal cloning system. The advantages of Gateway cloning are as follows: it is free from the need for restriction endonucleases and DNA ligase, has a simple and uniform protocol, and offers highly efficient and reliable cloning and easy manipulation of fusion constructs. Therefore, the development of a variety of Gateway cloning compatible vectors for many purposes will expand the usefulness of this system in plant research.

2.1 Ti-binary vector for *Agrobacterium*-mediated plant transformation

A. tumefaciens harboring a Ti-plasmid can transfer a specific segment of the plasmid, the T-DNA region, which is bounded by a right border (RB) and a left border (LB) sequence, to the genome of an infected plant (Figure 1). Expression of the T-DNA genes causes the overproduction of phytohormones in the infected cells, which causes crown gall tumors. Although T-DNA genes are required for crown gall tumor formation, other genes called the *vir* genes outside of the T-DNA region are essential for transfer of T-DNA into the host plant genome. These *vir* genes work even when they reside on another plasmid in *A. tumefaciens*. Based on these findings, a Ti-binary vector system was developed to overcome the difficulty of manipulating the original Ti plasmids *in vitro* by recombinant DNA methods due to their huge size (Bevan, 1984). A wide range of shuttle vectors for *E. coli* and *A. tumefaciens* was constructed that contain T-DNA border sequences flanking multiple restriction sites for foreign DNA cloning and marker genes for selection in plant cells. Using this vector system, DNA manipulation and vector construction can be done in *E. coli*; the vector is then transferred to *A. tumefaciens* harboring an artificial Ti-plasmid in which the T-DNA has been deleted. The vector is maintained stably in *A. tumefaciens,* and the cloned foreign DNA and

marker gene between RB and LB can be transferred to the host plant genome by the transformation system encoded by *vir* genes on the T-DNA deletion Ti-plasmid. In early studies, several dicot plants were transformed by an *Agrobacterium* method. However, various dicot and monocot plants can now be transformed by co-cultivation of leaf slices or cultured calli with chemicals inducing expression of *vir* genes. Transformed cells are selected by marker gene phenotype such as antibiotic resistance and regenerated to transgenic plants. The most important model plant, *Arabidopsis thaliana*, can be easily transformed by *A. tumefaciens* using a floral dip procedure.

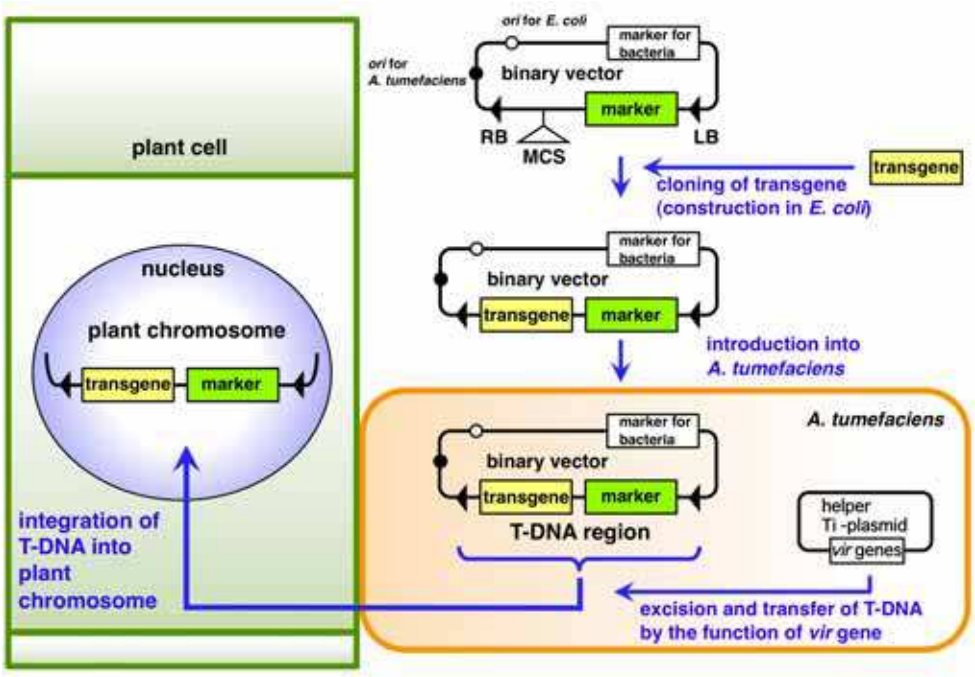

Fig. 1. Ti-binary vector system for *Agrobacterium*-mediated plant transformation. A binary vector, in which a target gene and plant selection marker gene are cloned between the two border sequences (RB and LB), is transformed into *A. tumefaciens* harboring a disarmed Ti-plasmid without the T-DNA region. Plant cells are infected by the transformed *A. tumefaciens* and then the target gene and marker gene are transferred into a plant chromosome by the *vir* genes on Ti-plasmid

2.2 Outline of Gateway cloning

Gateway cloning technology is based on the lambda phage infection system, in which site-specific reversible recombination reactions occur during phage integration into and excision from *E. coli* genome (Figure 2). In this process, the *att*P site (242 bp) of lambda phage and the *att*B site (25 bp) of *E. coli* recombine (in a BP reaction) and the lambda phage genome is integrated into the *E. coli* genome. After the recombination reaction, the lambda phage genome is flanked by the *att*L (100 bp) and *att*R (168 bp) sites. In the reverse reaction, the

phage DNA is excised from the *E. coli* genome by recombination between the *att*L and *att*R sites (in an LR reaction). The BP reaction needs two proteins, the phage integrase (Int) and the *E. coli* integration host factor (IHF). The mixture of these two proteins is called BP clonase in the Gateway system. In the LR reaction, Int, IHF and one more phage protein, excisionase (Xis), are required, and this mixture is called LR clonase. The Gateway cloning method uses these *att* sites and clonases for construction of recombinant DNA *in vitro*.

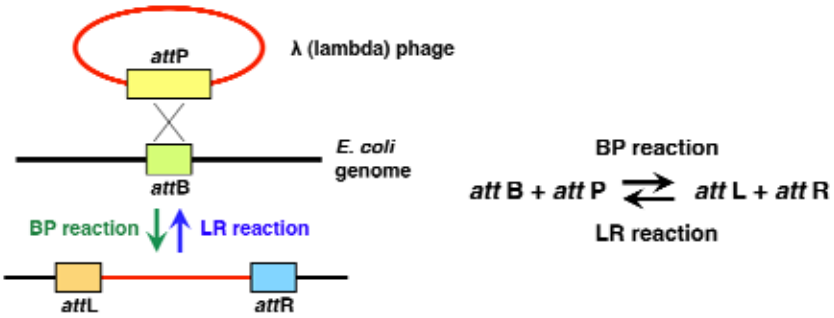

Fig. 2. BP and LR reactions in lambda phage infection of *E. coli*. The site-specific reversible BP and LR recombination reactions occur during lambda phage integration into and excision from the *E. coli* genome

Basic strategies for application of Gateway technology to plasmid construction are shown in Figure 3. For the basic Gateway system, four pairs of modified *att* sites were generated for directional cloning. They are *att*B1 and *att*B2, *att*P1 and *att*P2, *att*L1 and *att*L2, and *att*R1 and *att*R2; a recombination reaction can occur only in the combinations of *att*B1 and *att*P1, *att*B2 and *att*P2, *att*L1 and *att*R1, or *att*L2 and *att*R2, since recombination strictly depends on *att* sequences (Hartley et al., 2000; Walhout et al., 2000). In addition to these *att* sites, the negative selection marker *ccd*B, the protein product of which inhibits DNA gyrase, and a chloramphenicol-resistance (Cm^r) marker are used for selection and maintenance of Gateway vectors. Usually, *att*1 is located at the 5‘ end of the open reading frame (ORF) and *att*2 is located at the 3‘ end. This orientation is maintained in all cloning steps. First, the gene of interest should be cloned in an entry vector by TOPO cloning (pENTR/ D-TOPO), a BP reaction (pDONR221), or restriction endonuclease and ligase (pENTR1A). Each vector is available from Invitrogen. To make an entry clone by a BP reaction, the *att*B1 and *att*B2 sequences are added to the 5‘ and 3‘ ends, respectively, of the ORF by adapter PCR. The product (*att*B1-ORF-*att*B2) is subjected to a BP reaction with a donor vector, pDONR221, which possesses an *att*P1-*ccd*B-Cm^r-*att*P2 cassette. Because of the negative selection marker *ccd*B between *att*P1 and *att*P2, only transformants harboring the recombined vectors carrying *att*L1-ORF-*att*L2 (the entry clone) can grow on the selection plate. Once the entry clone is in hand, the ORF is transferred to a destination vector that possesses an *att*R1-Cm^r-*ccd*B-*att*R2 cassette. Since destination vectors also contain *ccd*B between *att*R1 and *att*R2, and have a selection marker gene that is different from the entry clone, only the recombined destination vectors carrying *att*B1-ORF-*att*B2 will be selected. Gateway cloning is designed so that the smallest *att* sequence, *att*B (25 bp), appears in the final product to minimize the length of cloning junctions after the clonase reaction. In N- or C-terminal fusion constructs, the ORF is linked to a tag with eight or more amino acids encoded by the *att*B1 or *att*B2 sites. Because

the reading frame of *att*B1 and *att*B2 is unified in the Gateway system, any entry clone incorporated into a destination vector is correctly fused to the tag sequence.

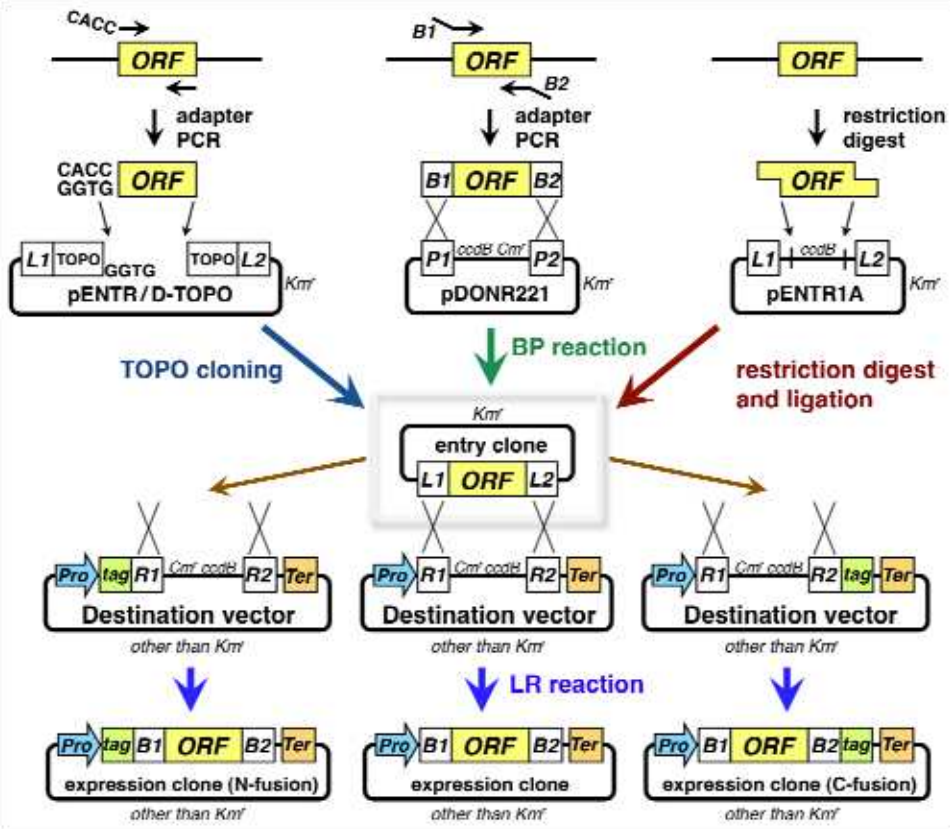

Fig. 3. Schematic illustration of Gateway cloning. An entry clone is constructed by TOPO directional cloning, a BP reaction or restriction digestion and ligation. For construction using the BP reaction, the ORF region is amplified by adapter PCR and the resulting *att*B1-ORF-*att*B2 fragment is cloned into pDONR221 by a BP reaction to generate an entry clone containing *att*L1-ORF-*att*L2. Subsequently, the ORF is cloned into destination vectors by an LR reaction to generate expression clones including tagged fusion constructs. For D-TOPO cloning, CACC is added to the ORF by adapter PCR, and the resulting CACC-ORF fragment is cloned into pENTR/ D-TOPO. *B1*, *att*B1; *B2*, *att*B2; *P1*, *att*P1; *P2*, *att*P2; *L1*, *att*L1; *L2*, *att*L2; *R1*, *att*R1; *R2*, *att*R2; *Pro*, promoter; *Ter*, terminator; *Cm^r*, chloramphenicol resistance marker; *ccd*B, negative selection marker in *E. coli*.; *Km^r*, kanamycin-resistance marker

3. Binary vectors compatible with Gateway cloning

A large number of binary vectors compatible with Gateway cloning, known as destination vectors, have been developed and are summarized in a review (Karimi et al., 2007b). Gateway compatible binary vectors for promoter analysis have the general structure *att*R1-

Cm^r-*ccd*B-*att*R2-tag-terminator, and after an LR reaction with an *att*L1-promoter-*att*L2 entry clone, they yield an *att*B1-promoter-*att*B2-tag-terminator binary construct. Gateway compatible binary vectors for expression of tagged fusion proteins have the general structure promoter-tag-*att*R1-Cm^r-*ccd*B-*att*R2-terminator (for N-terminal fusions) or promoter-*att*R1-Cm^r-*ccd*B-*att*R2-tag-terminator (for C-terminal fusions). After an LR reaction with an *att*L1-ORF-*att*L2 entry clone, they respectively yield promoter-tag-*att*B1-ORF-*att*B2-terminator or promoter-*att*B1-ORF-*att*B2-tag-terminator. The tag added to the N-terminus of the ORF is linked by the peptide encoded by the *att*B1 sequence (XSLYKKAGX), and the tag added to the C-terminus is linked by the peptide encoded by the *att*B2 sequence (XPAFLYKVX). Gateway compatible binary vectors for RNAi analysis (Helliwell & Waterhouse, 2003; Hilson et al., 2004; Karimi et al., 2002; Miki & Shimamoto, 2004) generally have the inverted structure of cassettes: promoter-*att*R1-*ccd*B-*att*R2-linker-*att*R2-*ccd*B-*att*R1-terminator. By an LR reaction with an *att*L1-trigger-*att*L2 entry clone, the trigger sequence is incorporated into both sites in opposite orientations, yielding a promoter-*att*B1-trigger-*att*B2-linker-*att*B2-(complementary trigger)-*att*B1-terminator construct. When the construct is introduced into plants, hairpin RNA is expressed and processed into small interfering RNA that functions in gene silencing.

Among many Gateway compatible binary vector series, the pW (Karimi et al., 2002), pMDC (Brand et al., 2006; Curtis & Grossniklaus, 2003) and pEarleyGate (Earley et al., 2006) series contain vectors available for many kinds of experiments in plants. The pW series consists of vectors for overexpression or antisense repression by the cauliflower mosaic virus 35S promoter (P_{35S}), for promoter analysis using luciferase (LUC), β-glucuronidase (GUS), or GFP-GUS as reporters, and for construction of gene fusions with GFP, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) or red fluorescent protein (RFP). The pMDC series consists of vectors for cloning, for overexpression by P_{35S}, for inducible expression by heat shock or estrogen treatment, for promoter analysis using GFP-6xHis or GUS as reporter, and for gene fusions with GFP, GFP-6xHis, or GUS. The pEarleyGate is a BASTA®-resistance binary vector series consisting of vectors for overexpression by P_{35S}, for promoter analysis using HA, FLAG, Myc, or AcV5, and for gene fusions with YFP, HA, FLAG, Myc, AcV5, tandem affinity purification (TAP) tags, YFP-HA, or GFP-HA.

The vectors described above are useful tools; however, sometimes it is necessary to use a different series if an existing one does not have a vector of the required type. In order to carry out most experiments within the same series (having a unified backbone and a unified junction sequence), we constructed a comprehensive Gateway compatible binary vector system carrying many reporters and tags based on the same backbone, as mentioned in next section.

4. Development of Gateway binary vector (pGWB) series

To make Gateway compatible binary vectors efficiently, we first tried to establish a systematic method for construction of a vector series. For this purpose, we designed a construction method for introducing a tag sequence by blunt end ligation to save time and labor caused by restriction sites in the tag sequence. Based on this notion, platform vectors pUGW0 and pUGW2 (Nakagawa et al., 2007a) were made using pUC119 as the backbone. As described below, many Gateway binary vector (pGWB) series were constructed from intermediate plasmid pUGWs, which were made with pUGW0 or pUGW2. The

characteristics and accession nos. of each pGWB are summarized in Information of Gaeway Binary Vectors (pGWBs) (http:/ / shimane-u.org/ nakagawa/ gbv.htm).

4.1 Platform vectors pUGW0 and pUGW2 for construction of pGWB series

The platform vectors pUGW0 and pUGW2 include P_{35S} and the nopaline synthase terminator (Tnos), as shown in Figure 4. A pUGW0 was the starting vector for N-terminal fusions, with the structure *Hin*dIII-P_{35S}-*Xba*I-ATG-*Aor*51HI-*att*R1-Cm^r-*ccd*B-*att*R2-*Sac*I-Tnos. A tag (reporter or epitope tag) sequence amplified by blunt-end PCR was introduced into the *Aor*51HI site (blunt end) to yield *Hin*dIII-P_{35S}-*Xba*I-ATG-*tag*-*att*R1-Cm^r-*ccd*B-*att*R2-*Sac*I-Tnos. In the case of a small epitope tag, an oligonucleotide could be introduced directly into the *Aor*51HI site. Translation is initiated at the ATG just upstream of the *Aor*51HI site. pUGW2 was the starting vector for C-terminal fusions, with the structure *Hin*dIII-*Xba*I-*Hin*dIII-P_{35S}-*Xba*I-*att*R1-Cm^r-*ccd*B-*att*R2-*Aor*51HI-*Sac*I-Tnos. Tag sequences were introduced by the same method used for pUGW0. The P_{35S} region could be easily removed by digestion with *Xba*I followed by self-ligation for construction of promoter-less pUGWs. Because there is no need to digest the tag fragment with restriction enzymes to introduce it into the *Aor*51HI site of pUGW0 and pUGW2, any tag fragment can be cloned by the same method. With these simple procedures, a pUGW series containing a variety of tags was efficiently generated. They were sources of Gateway cassettes including tag sequences, and were used for construction of a Gateway binary vector (pGWB). Moreover, the pUGWs are Gateway compatible plant vectors useful for transient expression analysis after particle bombardment or protoplast transformation. Because of their small size and high copy number in *E. coli*, preparation and handling of pUGW plasmids are very easy.

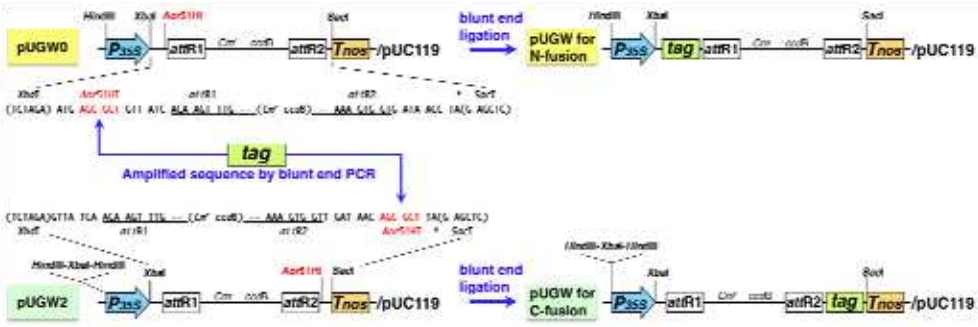

Fig. 4. Procedure for construction of pUGWs. pUGW0 and pUGW2 are the starting vectors for construction of new pUGW derivatives. The tag sequence amplified by blunt-end PCR is introduced into the *Aor*51HI site of pUGW0 or pUGW2, which yields pUGWs for N-fusion or C-fusion. The region between P_{35S} and Tnos is indicated. The nucleotide sequence corresponding to the region from *att*R1 to *att*R2 is underlined. *Cm*^{r}, chloramphenicol resistance marker; *ccd*B, negative selection marker in *E. coli*.; P_{35S}, 35S promoter

4.2 The pGWB series (pGWBxx and pGWB2xx) based on the pBI plasmid

Initially, pGWB was constructed on the backbone of modified pBI carrying a nopaline synthase promoter (Pnos) driven neomycin phosphotransferase II (NPTII) and P_{35S}-driven

hygromycin phosphotransferase (HPT), which confer kanamycin-resistance (Km^r) and hygromycin-resistance (Hyg^r), respectively, to plants (Mita et al., 1995). The initial pGWB series (pGWBxx) consists of 36 vectors designed for simple cloning of genes (pGWB1), for overexpression of ORF clones (pGWB2), and for fusion with a variety of tags (pGWB3 through pGWB45) as shown in the Complete List of pGWB (http:/ / shimane-u.org/ nakagawa/ gbv.htm). GUS, TAP and LUC are available for C-fusion, and 10 other tags, sGFP, 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7, enhanced yellow fluorescent protein (EYFP), and enhanced cyan fluorescent protein (ECFP), are available for both N- and C-fusion. The promoter-less C-fusion vectors can be used for promoter analysis. By an LR reaction with a promoter entry clone, a binary construct of promoter:tag is created. The remaining N- and C-fusion vectors contain P_{35S} for constitutive expression. By an LR reaction with an ORF entry clone, binary constructs expressing tag-ORF or ORF-tag are easily obtained (Figure 5). With the pGWBs, promoter activity, detection of tagged proteins, and subcellular localization of proteins can be analyzed effectively (Nakagawa et al., 2007a).

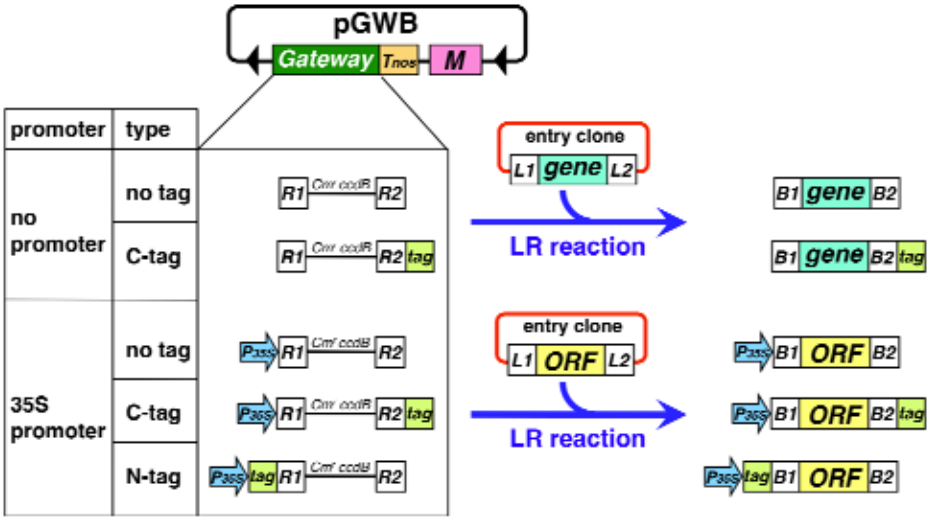

Fig. 5. Cloning into pGWB by LR reaction. The Gateway region in pGWB (top of the figure) represents a variety of acceptor sites (R1-R2) described in the box. The pGWB series includes plasmids with no promoter and no tag, or with no promoter and a C-tag. These are used for expression controlled by a gene's own promoter. The pGWB plasmids also include the following types: a 35S promoter and no tag, a 35S promoter and a C-tag, and a 35S promoter and an N-tag. These are used for constitutive expression using the 35S promoter. After an LR reaction with the entry clone, the expression clones indicated in the right panel are obtained. The tag is fused via the *att*B sequence. *B1*, *att*B1; *B2*, *att*B2; *L1*, *att*L1; *L2*, *att*L2; *R1*, *att*R1; *R2*, *att*R2; *Tnos*, nopaline synthase terminator; *M*, selection marker for plant; *Cm^r*, chloramphenicol-resistance marker; *ccd*B, negative selection marker in *E. coli*.; *P_{35S}*, 35S promoter

We also constructed pGWBs carrying the *Pnos:HPT:Tnos* marker instead of *P_{35S}:HPT:Tnos* (pGWB1-45) to avoid a possible effect of the P_{35S} sequence on the expression pattern and

strength of the cloned gene (Zheng et al., 2007). These vectors are named pGWB203, 204, 228 and 235, and their characters are shown at the bottom of the Complete List of pGWB (http:/ / shimane-u.org/ nakagawa/ gbv.htm). In early experiments, when the phosphate transporter PHT1 promoter was used for promoter analysis in *A. thaliana*, GUS activity in plant extracts was 5-fold higher with pGWB3 than with pGWB203 (Nakagawa et al., 2007a).

4.3 Improved Gateway binary vector (ImpGWB) series (pGWB4xx, pGWB5xx, pGWB6xx and pGWB7xx) based on the pPZP plasmid

We next constructed improved Gateway binary vectors (ImpGWBs) using pPZP as a backbone (Hajdukiewicz et al., 1994). In the ImpGWB system, handling of plasmid is largely improved, transformation efficiency in *E. coli* is drastically increased and much larger amount of plasmid DNA was recovered. The structures and characters of pGWBs (pBI backbone) and ImpGWBs (pPZP backbone) are summarized in Figure 6.

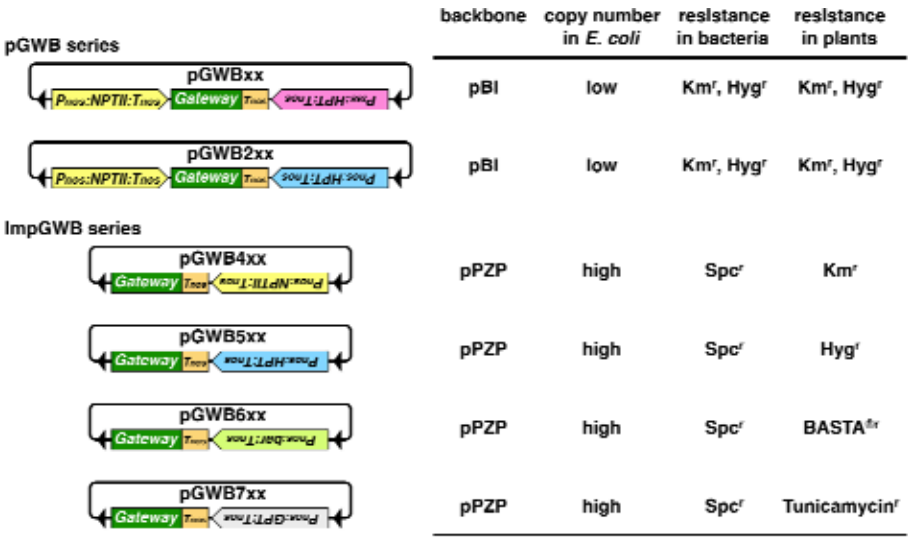

Fig. 6. Characters of pGWBs and ImpGWBs. The Gateway region in vectors represents a variety of acceptor sites as described in the Figure 5. Pnos, nopaline synthase promoter; Tnos, nopaline synthase terminator; P_{35S}, 35S promoter; NPTII, neomycin phosphotransferase II; HPT, hygromycin phophotransferase; *bar*, bialaphos resistance gene; GPT, UDP-*N*-acetylglucosamine: dolichol phosphate *N*-acetylglucosamine-1-P transferase (Koizumi & Iwata, 2008; Koizumi et al., 1999) gene. Km^r, kanamycin-resistance; Hyg^r, hygromycin-resistance; Spc^r, spectinomycin-resistance; BASTA®^{r}, BASTA®-resistance; Tunicamycin^r, tunicamycin-resistance

At present, four kinds of ImpGWB, the Km^r subseries (pGWB4xx) (Nakagawa et al., 2007b), Hyg^r subseries (pGWB5xx) (Nakagawa et al., 2007b), BASTA®-resistance subseries (pGWB6xx) (Nakamura et al., 2010) and tunicamycin-resistance subseries (pGWB7xx) (Tanaka et al., 2011), are available, and they are useful for introducing multiple transgenes into plants by repetitive transformation. Each subseries is composed of 46 vectors as

summarized in the Complete List of ImpGWB (http:/ / shimane-u.org/ nakagawa/ gbv.htm). A set of 16 tags, sGFP, GUS, LUC, EYFP, ECFP, G3 green fluorescent protein (G3GFP), monomeric red fluorescent protein (mRFP), TagRFP, 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7, and TAP, is available in ImpGWB. Because ImpGWB is highly efficient in transformation of *E. coli*, this series was used for development of a new cloning system using multiple LR reactions as described below.

4.4 R4 Gateway binary vector (R4pGWB) series (R4pGWB4xx, R4pGWB5xx, R4pGWB6xx and R4pGWB7xx) for promoter swapping

To assemble multiple DNA fragments in the desired order, an additional four *att* sites (*att*3, *att*4, *att*5 and *att*6) have been developed and applied to MultiSite Gateway cloning (Karimi et al., 2007a; Sasaki et al., 2004). Utilization of these *att* sites (*att*1-6) expanded the availability of cloning technology for more complex gene construction. The cloning system equipped with these *att* sites is useful for swapping of promoters, ORFs and tags, and is also applicable for cloning of multiple transgenes in one vector (Chen et al., 2006). In a typical MultiSite Gateway system, three entry clones containing specialized *att* sites, *att*L4-promoter-*att*R1, *att*L1-ORF-*att*L2, and *att*R2-tag-*att*L3 are simultaneously connected and incorporated into a destination vector carrying *att*R4-Cm^r-*ccd*B-*att*R3 acceptor sites to make an *att*B4-promoter-*att*B1-ORF-*att*B2-tag-*att*B3 construct (Figure 7).

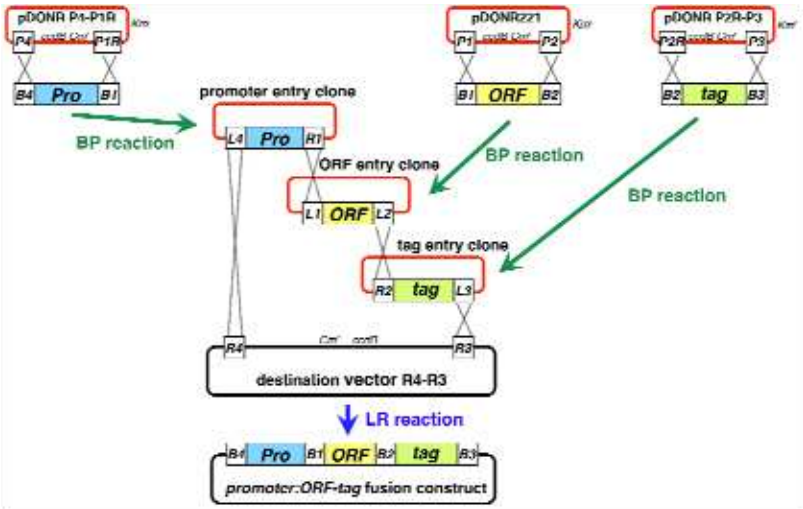

Fig. 7. MultiSite Gateway system. In the MultiSite Gateway system, *att*1, *att*2, *att*3 and *att*4 sequences are used for cloning of multiple DNA fragments into one vector. A promoter entry clone (L4-Pro-R1), ORF entry clone (L1-ORF-L2), tag entry clone (R2-tag-L3) and destination vector R4-R3 are subjected to an LR reaction. The promoter, ORF and tag sequences are linked and incorporated into the destination vector to form a *promoter:ORF-tag* clone. *B1*, *att*B1; *B2*, *att*B2; *B3*, *att*B3; *B4*, *att*B4; *L1*, *att*L1; *L2*, *att*L2; *L3*, *att*L3; *L4*, *att*L4; *R1*, *att*R1; *R2*, *att*R2; *R3*, *att*R3; *R4*, *att*R4; *P1*, *att*P1; *P2*, *att*P2; *P3*, *att*P3; *P4*, *att*P4; *P1R*, *att*P1R; *P2R*, *att*P2R; *Cm^r*, chloramphenicol-resistance marker; *ccd*B, negative selection marker in *E. coli*.; *Pro*, promoter; *Km^r*, kanamycin-resistance marker

Although MultiSite Gateway cloning is an excellent method for building a complicated multigene construct, it is relatively difficult to obtain the desired clone because four recombinations at each *att* site are required for successful cloning. To facilitate multi-fragment cloning, especially for promoter swapping, we developed the R4 Gateway binary vector (R4pGWB) by reducing the number of recombinations needed from four to three (*att*4, *att*1 and *att*2) (Figure 8, left) (Nakagawa et al., 2008). The R4pGWB series was made by replacing the *att*R1 site of ImpGWBs (promoter-less and C-fusion type with four resistance markers) with the *att*R4 site; all tags used in ImpGWB are also available in the R4pGWB system as shown in the Complete List of R4pGWB (http:/ / shimane-u.org/ nakagawa/ gbv.htm). By an LR reaction with a promoter entry clone (*att*L4-promoter-*att*R1), an ORF entry clone (*att*L1-ORF-*att*L2) and R4pGWB equipped with the appropriate tag, construction of chimeric genes among promoters, ORFs, and tags (*att*B4-promoter-*att*B1-ORF-*att*B2-tag) is achieved very easily. The R4pGWB system is a powerful tool to express an ORF by any desired promoter, *e.g.*, a promoter for strong expression, for tissue or cell specific expression, for developmental stage specific expression, or for induction by biotic or abiotic stimuli.

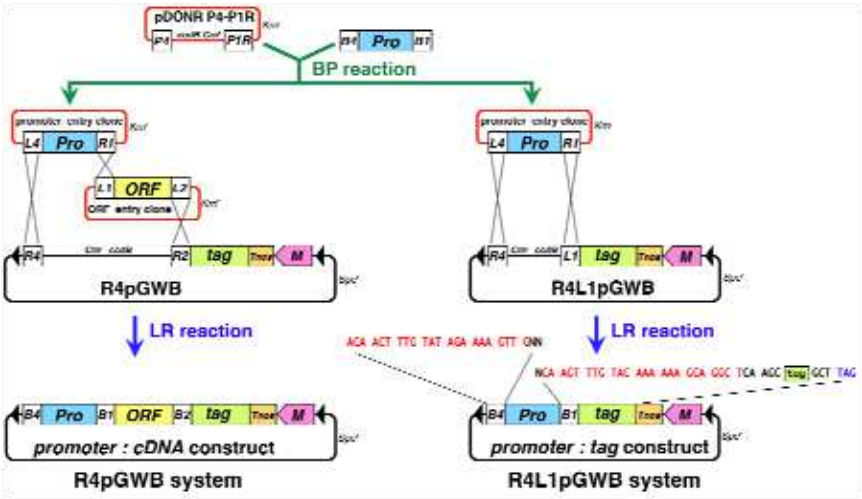

Fig. 8. R4pGWB and R4L1pGWB systems. A promoter entry clone (L4-Pro-R1) is constructed by a BP reaction using pDONR P4-P1R and a B4-Pro-B1 fragment prepared by adapter PCR. Left; in the R4pGWB system, a promoter entry clone (L4-Pro-R1), ORF entry clone (L1-ORF-L2) and R4pGWB are subjected to an LR reaction. The promoter and ORF are linked and incorporated into R4pGWB to form a promoter:ORF-tag clone. Right; in the R4L1pGWB system, only a promoter entry clone (L4-Pro-R1) is used for an LR reaction with an R4L1pGWB. The promoter sequence is incorporated into R4L1pGWB and fused with the tag on the vector. With the R4L1pGWB system using a single LR reaction, a promoter:tag construct is obtained at high efficiency. Nucleotides in red indicate B4 and B1 sequences. *Pro*, promoter; *B1*, *att*B1; *B2*, *att*B2; *B4*, *att*B4; *L1*, *att*L1; *L2*, *att*L2; *L4*, *att*L4; *R1*, *att*R1; *R2*, *att*R2; *R4*, *att*R4; *P4*, *att*P4; *P1R*, *att*P1R; *M*, selection marker for plant; *Cm*^{r}, chloramphenicol-resistance marker; *ccd*B, negative selection marker in *E. coli*.; *Pro*, promoter; *Km*^{r}, kanamycin-resistance marker

4.5 R4L1 Gateway binary vector (R4L1pGWB) series (R4L1pGWB4xx and R4L1pGWB5xx) for promoter analysis

Due to establishment of the R4pGWB system, many kinds of *att*L4-promoter-*att*R1 entry clones were constructed and have been used as a resource for expression of ORFs in plants. We plan to also utilize these resources of *att*L4-promoter-*att*R1 entry clones for efficient promoter:tag experiments, and developed an R4L1 Gateway binary vector (R4L1pGWB) (Nakamura et al., 2009) containing *att*R4-Cm^r-*ccd*B-*att*L1-tag-Tnos. By the simple bipartite LR reaction with *att*L4-promoter-*att*R1 and R4L1pGWB, an *att*B4-promoter-*att*B1-tag-Tnos construct used for promoter assays can be easily obtained in this system (Figure 8, right). The tags in R4L1pGWBs are G3GFP-GUS, GUS, LUC, EYFP, ECFP, G3GFP and TagRFP as shown in the Complete List of R4L1pGWB (http:/ / shimane-u.org/ nakagawa/ gbv.htm).

5. Application of the pGWB system

Because Gateway cloning is efficient, precise, flexible and simple to use, its application will continue to grow in plant research. In this section, we briefly describe two recent advances in our pGWB system, a split reporter for interaction analysis and recycling cloning for multigene constructs.

5.1 Gateway vectors for bimolecular fluorescence complementation (BiFC) assay

BiFC is based on the reconstitution of a fluorescent signal when two interacting proteins or peptides, which are fused to either an N- or C-fragment of a split fluorescent protein, interact. Due to its relative technical simplicity and the ability to use fluorescence microscopes for observation, a growing number of publications describe the use of BiFC to analyze protein-protein interactions. In addition to monitoring protein-protein interactions, this method has expanded to wider application, such as multicolor BiFC to investigate protein complexes (Hu & Kerppola, 2003; Kodama & Wada, 2009; Lee et al., 2008; Waadt et al., 2008), detection *in vivo* (Bracha-Drori et al., 2004; Walter et al., 2004) and combined with bioluminescence resonance energy transfer (BRET; Chen et al., 2008; Gandia et al., 2008; Xu et al., 2007). To date, several BiFC vectors dedicated to plant research have been constructed. Among our efforts in development of Gateway technology, we have generated various destination vectors for BiFC assays. In this section, we introduce our Gateway technology-based BiFC vectors, and describe their application.

5.1.1 Detection of protein-protein interactions in plant cells by BiFC assay

The investigation of protein-protein interactions provides valuable information in cell biology. In addition to BiFC, several other techniques detect protein-protein interactions, such as co-immunoprecipitation assays (Co-IP), *in vitro* binding assays, the yeast two-hybrid system (Y2H; James et al., 1996), the mating-based split-ubiquitin system (mbSUS; Ludewig et al., 2003; Obrdlik et al., 2004), BRET(Chen et al., 2008; Xu et al., 2007), fluorescence resonance energy transfer (FRET; Day et al., 2001), fluorescence lifetime imaging microscopy (FLIM; Bastiaens & Squire, 1999) and fluorescence correlation spectroscopy (FCS; Hink et al., 2002). The imaging-based approaches such as BiFC and FRET have been utilized in plant research because they enable detection in plant cells, in contrast to Y2H and mbSUS, which

are functional only in yeast cells, and because they do not require specific antibodies or purification of proteins, unlike Co-IP and *in vitro* binding assays.

The BiFC assay is one of the most convenient techniques among the image-based approaches. Although FRET and FLIM are useful and powerful techniques for detection of protein-protein interactions, FRET requires complicated analysis such as of acceptor bleaching and an exclusive device is necessary for FLIM. Although several considerations are required even for BiFC assays, special devices are not required for detection, and complicated analysis is not necessary after obtaining image data. In addition, the BiFC assay provides information on subcellular location of the interacting proteins.

We used our Gateway vector construction system (Hino et al., 2011; Nakagawa et al., 2008; Nakagawa et al., 2007b) to make destination vectors for BiFC assays. Using these vectors, it is easy to make constructs for detection of protein-protein interactions. These Gateway vectors have worked well in plant cells (Goto et al., 2011; Hino et al., 2011; Singh et al., 2009).

5.1.2 Principles of the BiFC assay

In BiFC assays, a fluorescent reporter, such as CFP, GFP, YFP and RFP, is split into two non-fluorescent fragments, N- and C- fragments (Figure 9A,B). Two proteins or peptides, which are to be tested for interaction, are fused at the N- or C-terminus of each fragment. After expression of both fusion genes simultaneously, if an interaction occurs between the two proteins, the non-fluorescent fragments are reconstituted and behave as an unsplit fluorescent protein. Therefore, the detection of fluorescence means the target proteins interact (Figure 9A).

Once the interaction occurs, the reconstituted molecule does not dissociate into non-fluorescent fragments, leading to enhancement of fluorescence due to accumulation of reconstituted fluorescent proteins.

There are eight potential combinations to be tested for protein-protein interactions in a BiFC assay, taking into account which protein of the two partners tested is fused to the N- or C-terminal end of which N- or C- fragment (Figure 9C). However, improper fusion of a split fragment sometimes abolishes protein function and masks information on subcellular targeting. For example, the peroxisome targeting signal 2 (PTS2) must be fused to the N-terminus of the split fluorescent protein (Singh et al., 2009; Figure 10B). In contrast, PTS1 must be fused to the C-terminus of a split fluorescent protein, because its location at the C-terminus is necessary for its function. In these cases, the number of combinations tested is fewer. However, if there is no information on protein function, all combinations should be tested. Viewed in this light, our destination vectors are useful for construction of several fusion genes at the same time.

5.1.3 Destination vectors for the multicolor and in vivo BiFC assays

Various BiFC vectors have been developed and used in plant research (Bracha-Drori et al., 2004; Diaz et al., 2005; Ding et al., 2006; Goto et al., 2011; Hino et al., 2011; Loyter et al., 2005; Maple et al., 2005; Marrocco et al., 2006; Ohad et al., 2007; Singh et al., 2009; Waadt et al., 2008; Walter et al., 2004; Zamyatnin et al., 2006). All the vectors, including ours, use P_{35S} to

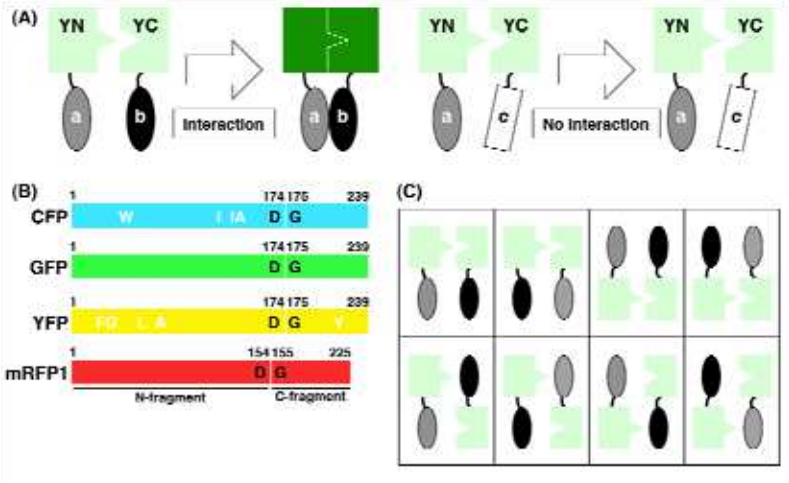

Fig. 9. Principles of the BiFC assay. (A) Nonfluorescent fragments (YN and YC) of a fluorescent protein are brought together through interaction of the tested proteins or peptides (a, b and c) to which they are fused. The interaction of the two proteins causes reconstitution of a fluorescent signal. (B) Diagram of amino acid substitutions among CFP, GFP, YFP and mRFP1, and the positions where they were fragmented. Although there are alternative positions to split a fluorescent protein into two fragments (Hu & Kerppola, 2003; Waadt et al., 2008), the CFP, GFP and YFP in our system were split between residues 174 and 175, and mRFP1, which contains an amino acid substitution of the 66th glutamine to threonine, was split between residues 154 and 155. Amino acids in CFP and YFP that were converted from GFP are depicted in white. In the case of RFP, amino acids that are different from GFP are not represented, since there are many substitutions. (C) Potential combination of two fragments. There are eight possible configurations in the BiFC assay. Each target protein (gray and black) can be fused at its N- or C- terminus to the N- or C-terminal fragment of the fluorescent protein (light green)

express a fusion gene. There are two ways to insert a target gene into the 5' or 3' end of a split fragment of fluorescent protein gene: (1) cloning into a multicloning site using digestion and ligation, and (2) Gateway technology (Hino et al., 2011; Walter et al., 2004). Our BiFC vectors were developed to be compatible with Gateway technology. We generated four kinds of destination vectors for BiFC assays (Figure 10A), enabling the transfer of a gene of interest from the entry clone to the 5' or 3' end of each split fragment. Therefore, researchers are able to easily fuse a gene of interest to the 5' or 3' end of the split fragment, leading to various convenient constructs.

The BiFC vectors were initially generated using YFP (Hu et al., 2002). However, other fluorescent proteins, BFP (Hu & Kerppola, 2003), CFP (Kodama & Wada, 2009; Lee et al., 2008), GFP (Hu et al., 2002; Kodama & Wada, 2009), Venus, (Lee et al., 2008), Cerulean (Lee et al., 2008), DsRed-monomer (Kodama & Wada, 2009), mRFP1 (Jach et al., 2006), mCherry (Fan et al., 2008), and a far-red fluorescent protein, mLumin (Chu et al., 2009), have reportedly been useful for BiFC assay. We adopted CFP, GFP, YFP and mRFP1 to generate vectors (Figure 9B), and verified their usefulness for detection of protein-protein interactions

(Figure 10B-E). PTS2-containing proteins are directed to peroxisomes after binding to a receptor, PEX7, in the cytosol (Hayashi & Nishimura, 2006; Mano & Nishimura, 2005). We were able to observe reconstituted CFP fluorescence as punctate structures, when allowing interaction of *nCFP-PEX7* and *PTS2-cCFP* (Figure 10B), which agrees with a previous report (Singh et al., 2009). Lesion simulating disease 1 (LSD1), a negative regulator of programmed cell death, is a zinc finger protein that forms homodimers. We also tried to detect LSD1 homooligomerization using the combination of LSD1-nYFP and LSD1-cYFP. Reconstituted YFP signals were observed in the cytosol and nucleus (Figure 10C), a result that coincided with previous data (Walter et al., 2004). The localization and interaction of one of the plasma membrane intrinsic proteins, PIP2, which belongs to the aquaporin family, with other PIP members were demonstrated by FRET and FLIM assays in maize cells (Zelazny et al., 2007). An Arabidopsis PIP2 gene, *PIP2;1*, was also fused to split fragments of mRFP1 and used for investigation of homooligomerization (Figure 10D). We were able to detect reconstituted RFP signals at the plasma membrane.

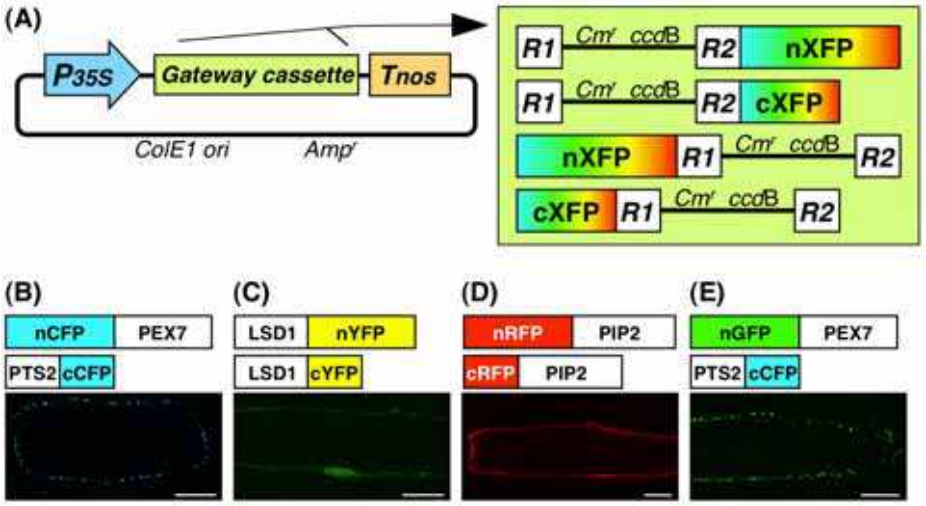

Fig. 10. Schematic representation of the multicolor BiFC vectors and examples of transient expression. (A) Four kinds of destination vectors for transient expression were generated to be compatible with Gateway technology. nXFP and cXFP, the N- or C-fragment, respectively, of CFP, GFP, YFP or RFP; *ColE1 ori*, ColE1 replication origin; *Amp*^{r}, ampicillin-resistance marker used for selection in bacteria; *Cm*^{r}, chloramphenicol-resistance marker; *ccd*B, negative selection marker used in bacteria; P_{35S}, 35S promoter; *Tnos*, nopaline synthase terminator; *R1*, *att*R1; *R2*, *att*R2. (B-E) Fluorescence images of onion epidermal cells expressing the fusion genes indicated above each panel were acquired 18-24 hr after particle bombardment. Bars = 50 μm

Multicolor BiFC assays have been developed to examine protein-protein interactions among various factors, since some combinations of N- and C-fragments of different fluorescent proteins allow reconstitution of signals (Hu & Kerppola, 2003; Kodama & Wada, 2009; Lee et al., 2008; Waadt et al., 2008). We also investigated which combinations among different fragments in our BiFC vectors are practical for reconstitution of signals using nXFP-PEX7

and PTS2-cXFP (XFP means CFP, GFP, YFP or RFP). Combinations among split fragments of CFP, GFP and YFP enabled the reconstitution of fluorescence (Table 1, Figure 10E), although some combinations did not give reproducible results. In contrast, a reconstituted RFP signal was observed only between split fragments from RFP (Table 1).

	nC-PEX7	nG-PEX7	nY-PEX7	nR-PEX7
PTS2-cC	+	+	+	-
PTS2-cG	+	+	+	-
PTS2-cY	±	±	+	-
PTS2-cR	-	-	-	+

Table 1. Summary of the detection of reconstituted signals using various combinations of split fragments from different fluorescent proteins. cC, cG, cY and cR represent the C-fragment of CFP, GFP, YFP and RFP, respectively. nC, nG, nY and nR indicate the N-fragment of CFP, GFP, YFP and RFP, respectively. '+' and '-' denote detection of interaction and inability for interaction, respectively. ''±'' indicates that reproducible results could not be obtained

We adapted our BiFC vectors for transient expression (Figure 10A) to binary vectors for *in vivo* BiFC assays (Figure 11A). Using these binary vectors, researchers are able to easily generate transgenic plants expressing a fusion gene of the N- or C-fragment with a gene of interest. We prepared two kinds of binary vectors, containing either Km^r or Hyg^r markers. Therefore, after crossing transgenic plants expressing either the N- or C-fragment, it will be easier to obtain transgenic plants expressing both N- or C-fragments from screening on medium with both kanamycin and hygromycin.

Agroinfiltration is a powerful technique to express an alien gene *in planta*, and it has been reported that this technique is functional in BiFC assays (Bracha-Drori et al., 2004; Waadt et al., 2008; Walter et al., 2004). We examined whether our binary vectors could also work well in agroinfiltration using *nYFP-Peroxin 6 (PEX6)* and *cYFP-ABERRANT PEROXISOME MORPHOLOGY 9 (APEM9)* (Figure 11B). We already reported the interaction of PEX6 and APEM9 using transient expression of these fusion genes in onion epidermal cells (Goto et al., 2011). We mixed three cultures of *A. tumefaciens* (strain C58C1Rif^R) haboring *nYFP-PEX6*, *cYFP-APEM9* or *CFP-PTS1* as peroxisomal markers, and then co-infiltarted into the leaf cells of *Nicotiana tobaccum*. Reconstituted YFP signal was observed as punctate structures (Figure 11C), and these signals surrounded the CFP-labeled peroxisome matrix (Figure 11C-E), showing that BiFC occurs at the peroxisomal membrane, as reported previously (Goto et al., 2011). These results demonstrated that our binary vectors for BiFC assays work well.

5.1.4 Special considerations for BiFC assays using our vectors

In BiFC assays, fluorescence is derived from reconstituted fluorescence or artificial noise. The same is true for our BiFC vectors. Fluorescence is sometimes observed even in combination with an untagged vector as a negative control. Therefore, it is necessary to test expression using a negative control vector. Conversely, when fluorescence is not observed after expression of two fusion genes, there are two views about the result. One is that the interaction does not occur, although the two fusion genes are expressed properly. The other is that gene expression is inefficient or that the genes were inefficiently introduced into the

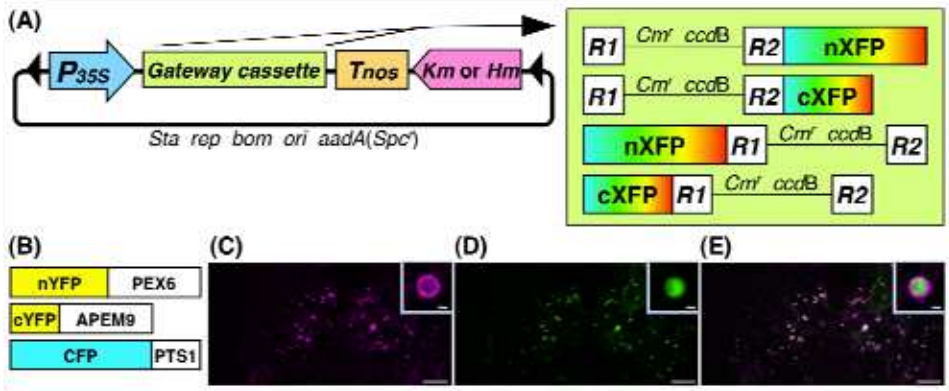

Fig. 11. Schematic representation of the binary vectors for the BiFC assay and examples of an *in vivo* BiFC experiment using an *Agrobacterium*-infiltration technique. (A) Four kinds of destination vectors for an *in vivo* BiFC assay were generated to be compatible with Gateway technology. nXFP and cXFP, the N- or C-fragment, respectively, of CFP, GFP, YFP or RFP; cXFP; *sta*, region conferring stability in *Agrobacterium*; *rep*, broad host-range replication origin; *bom*, *cis*-acting element for conjugational transfer; *ori,* ColE1 replication origin; *Cm*^{r}, chloramphenicol-resistance marker; *ccd*B, negative selection marker used in bacteria; P_{35S}, 35S promoter; *Tnos*, nopaline synthase terminator; *R1*, *att*R1; *R2*, *att*R2; Black arrowheads indicate right border and left border. (B-E) An example of an *in vivo* BiFC experiment. (B) Three fusion genes, *nYFP-PEX6*, c*YFP-APEM9* and *CFP-PTS1* as peroxisome markers were expressed in *Nicotiana tobaccum*. (C-E) Fluorescence images of leaf epidermal cells were acquired 3 days after infiltration. (C) Reconstituted YFP signals. (D) Peroxisomes visualized with CFP. (E) A merged image of (C) with (D). Insets represent magnified images of a peroxisome. Bars = 20 μm and 1 μm for each inset

cells. We always express an additional gene, such as *CFP-PTS1* in Figure 11, to investigate the efficiency of gene expression in transient assays. At the same time, this helps visualize cells and organelles so that it is easier to observe introduced cells that are bombarded or agro-infiltrated. The alternative method is the detection of fusion protein by immunoblotting. Some vectors are developed to add the epitope tag to split fragments so that the detection of accumulation of fusion proteins is carried out by immunoblotting (Bracha-Drori et al., 2004; Waadt et al., 2008; Walter et al., 2004). Of course, if specific antibodies against target protein are possible to obtain, they are useful for verification of protein accumulation.

5.1.5 Perspectives

Our BiFC vectors have wide application to analysis of protein-protein interactions. Future introduction of the R4pGWB system (Nakagawa et al., 2008) to these BiFC vectors will allow regulation of each fusion gene under a specific promoter, leading to examination of the interaction with tissue or developmental stage specificity. Additionally, inducible promoters will be used for transient expression in transgenic plants harboring R4pGWB-based BiFC fragments. Since a great variety of fluorescent proteins with different properties, such as large Stokes' shift, are available, more various combinations for the multicolour BiFC assay

will be generated by adopting our Gateway technology system to new fluorescent proteins, revealing the relationship among several factors in a complex.

5.2 Recycling cloning system for multigene constructs

Multigene transformation of plants is a powerful technology for molecular breeding because it can simultaneously improve multiple enzymes and factors constituting biological pathways (Ha et al., 2010; Nakayama et al., 2000; Naqvi et al., 2009; Ye et al., 2000). For multigene transformation, methods such as re-transformation, co-transformation, and cross-fertilization are available (Dafny-Yelin & Tzfira, 2007), but the most practical method is the utilization of a multigene construct, a vector carrying multiple expression units (Chen et al., 2006). In this section, we introduce a recycling cloning system for cloning multiple expression units by simple repetitive LR reactions.

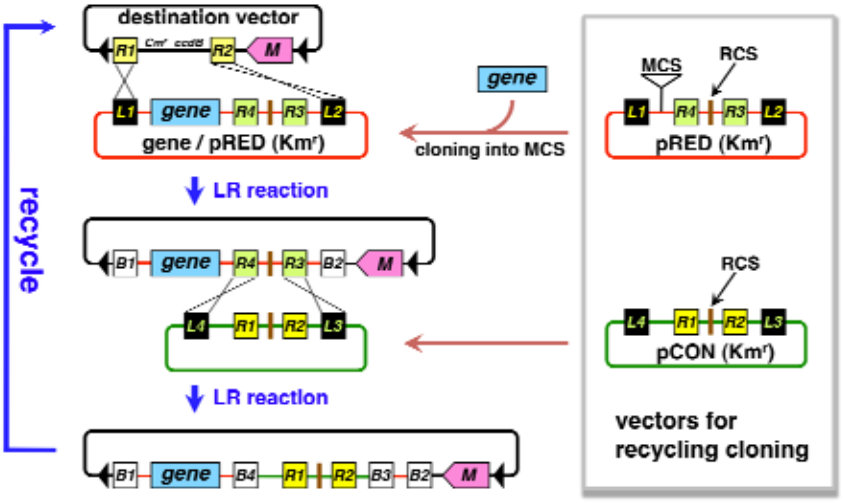

Fig. 12. Schematic illustration of recycling cloning. The pRED vector has the structure L1-MCS-R4-RCS-R3-L2. The gene of interest is cloned into the MCS of pRED419 and subsequently subjected to an LR reaction with a destination vector. In this step, the DNA fragment of gene-R4-RCS-R3 is incorporated into the destination vector and a binary clone carrying B1-gene-R4-RCS-R3-B2 is obtained. Next, the resulting binary clone is subjected to an LR reaction with pCON to introduce the R1-RCS-R2 sequence into the binary clone. The binary clone carrying B1-gene-B4-R1-RCS-R2-B3-B2 is recycled for introduction of another gene by LR reaction with another gene/ pRED in a second cycle. The marker gene (M) is transcribed in the opposite orientation to the cloned gene. *B1*, *att*B1; *B2*, *att*B2; *B3*, *att*B3; *B4*, *att*B4; *L1*, *att*L1; *L2*, *att*L2; *L3*, *att*L3; *LA*, *att*L4; *R1*, *att*R1; *R2*, *att*R2; *R3*, *att*R3; *R4*, *att*R4; *M*, selection marker for plant; *Cmr*, chloramphenicol-resistance marker; *ccd*B, negative selection marker in *E. coli*.; MCS, multiple cloning site; RCS, rare cutter site

As shown in the right panel of Figure 12, two vectors are used for each cloning cycle in this system. The recycle donor vector pRED has four *att* sites, a multiple cloning site (MCS) and a rare cutter site (RCS) in the following order: *att*L1-MCS-*att*R4-RCS-*att*R3-*att*L2. The RCS

has *Asi*I, *Swa*I, *Fse*I, *Pac*I, *Asc*I and *Pme*I sites. The gene of interest is cloned into the MCS of pRED (gene/ pRED) and subjected to an LR reaction with the destination vector containing R1-R2 acceptor sites. In this step, a binary construct carrying gene-*att*R4-RCS-*att*R3 is obtained. Next, conversion vector pCON, containing *att*L4-*att*R1-RCS-*att*R2-*att*L3, is subjected to an LR reaction to introduce the *att*R1-RCS-*att*R2 acceptor site into the resulting binary construct, and the binary construct obtained, which carries *att*R1-RCS-*att*R2, is recycled for the next round of the cloning cycle, together with another gene/ pRED clone (Figure 12). Before the LR reactions, binary constructs are digested by a rare cutter to suppress colonies derived from non-recombinants. With these simple repetitive reactions, genes are introduced sequentially into one vector. Using this recycling cloning system, we made a multigene construct containing four expression units of reporter genes and confirmed expression of all four reporters in transformed tobacco BY-2 cells (Kimura, unpublished results).

6. Conclusions

Gateway cloning is an efficient, reliable, easy and flexible technology, so many types of vectors have been developed and used worldwide. Our pGWBs series consists of many vectors with a variety of tags and four resistance markers. They are constructed on the same vector backbone and provide unified experimental conditions in transgenic research. Because the introduction of a tag sequence into pUGW is very easy (Figure 4), the number of vectors for fusion with new tags is growing in our Gateway vector system. Among them, vectors for fusion with split fluorescent proteins are very important tools for BiFC assays. Our Gateway technology-based BiFC vectors are useful when several fusion genes must be generated for detection of protein-protein interactions among several factors in transient or *in vivo* assays. Introduction of the R4pGWB system (Nakagawa et al., 2008) to these BiFC vectors will lead to wider applications. Recycling cloning has the potential to introduce many expression units in high efficiency and will open a new way for genetic engineering of plants.

6.1 Distribution and information updates

All vectors described in this chapter are available for non-commercial research purposes, although the permission of original developers is required for some tags. The e-mail addresses for requesting the vectors are mano@nibb.ac.jp (for distribution of BiFC vectors) and tnakagaw@life.shimane-u.ac.jp (for distribution of other pGWBs).

The list of pGWBs is updated on our website (http:/ / shimane-u.org/ nakagawa/ gbv.htm).

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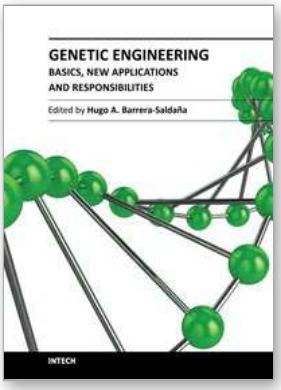

Genetic Engineering - Basics, New Applications and Responsibilities

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Leading scientists from different countries around the world contributed valuable essays on the basic applications and safety, as well as the ethical and moral considerations, of the powerful genetic engineering tools now available for modifying the molecules, pathways, and phenotypes of species of agricultural, industrial and even medical importance. After three decades of perfecting such tools, we now see a refined technology, surprisingly unexpected applications, and matured guidelines to avoid unintentional damage to our and other species, as well as the environment, while trying to contribute to solve the biological, medical and technical challenges of society and industry. Chapters on thermo-stabilization of luciferase, engineering of the phenylpropanoid pathway in a species of high demand for the paper industry, more efficient regeneration of transgenic soybean, viral resistant plants, and a novel approach for rapidly screening properties of newly discovered animal growth hormones, illustrate the state-of-the-art science and technology of genetic engineering, but also serve to raise public awareness of the pros and cons that this young scientific discipline has to offer to mankind.

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