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Invited Review

Gateway vectors for plant transformation

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Abstract The availability of complete plant genome sequences has opened a new era of plant molecular research using approaches such as comprehensive expression analysis, ectopic expression and gene knockdown. For such purposes, new tools realizing easy and rapid construction of recombinant plasmids are required. Here, we describe Gateway cloning compatible binary vectors for post-genomic research in plant genetic engineering. The vectors comprise a variety of reporters, epitope tags and selective markers that should make them useful for construction of plasmids for *Agrobacterium*-mediated transformation of plants. We also describe vectors for promoter swapping using Gateway cloning technology.

Key words: Binary vector, epitope tag, Gateway cloning, reporter.

Genome projects of higher plants have provided abundant sequence information for a decade, and now genome-wide studies of gene function and gene regulation are actively carried out. In these areas of research, transgenic analyses using genetically modified plants are essential. For example, promoter-reporter genes are widely used for examining the temporal and spatial regulation of gene expression, fusion genes encoding reporter-fused proteins are powerful tools for analyzing subcellular localization of the gene products, and ectopic expression of cDNA clones and RNAi are the most commonly used strategies for identifying biological functions of each gene product. For gene manipulation in plants, the binary system of *Agrobacterium*-mediated transformation is most widely used. This system consists of two kinds of 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*. However, most of the widely used binary vectors established in the 1990s had been constructed by traditional restriction digestion and ligation, and thus they were large and retained many restriction sites outside their cloning sites. Therefore, it was time consuming and laborious to construct modified genes on the binary vectors using the limited number of available restriction sites. This disadvantage made it difficult to perform high-throughput analysis of plant genes. To overcome this technical difficulty, a new cloning system to realize the efficient and reliable construction of modified genes for plant research was desired; the Gateway cloning system provided by Invitrogen (Carlsbad, CA, USA) is one of these solutions.

Gateway cloning

Gateway cloning technology is an application of the site-specific reversible recombination reactions occurring during λ phage integration into and excision from *E. coli* DNA (Figure 1) (Walhout et al. 2000). In the integration, the *att*P site (242 bp) of λ phage and the *att*B site (25 bp) of *E. coli* recombine and the λ phage genome is integrated into the *E. coli* genome. As a result, λ phage genome is flanked by the *att*L (100 bp) and *att*R (168 bp) sites (the BP reaction). In the reverse reaction, the phage DNA is excised from the *E. coli* genome by recombination between the *att*L and *att*R sites (the LR

Abbreviations: ALS, acetolactate synthase; CFP, cyan fluorescent protein; Cm^r, chloramphenicol resistance; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; G3GFP, G3 green fluorescent protein; GPT, UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase; HPT, hygromycin phosphotransferase; Hyg^r, hygromycin resistance; Km^r, kanamycin resistance; LUC, luciferase; NOS, nopaline synthase; NPTII, neomycin phosphotransferase II; mRFP, monomeric red fluorescent protein; ORF, open reading frame; P_{35S}, cauliflower mosaic virus 35S promoter; P_{NOS}, nopaline synthase promoter; RFP, red fluorescent protein; Spc^r, spectinomycin resistance; sGFP, synthetic green fluorescent protein; TAP, tandem affinity purification; T_{NOS}, nopaline synthase terminator; YFP, yellow fluorescent protein.
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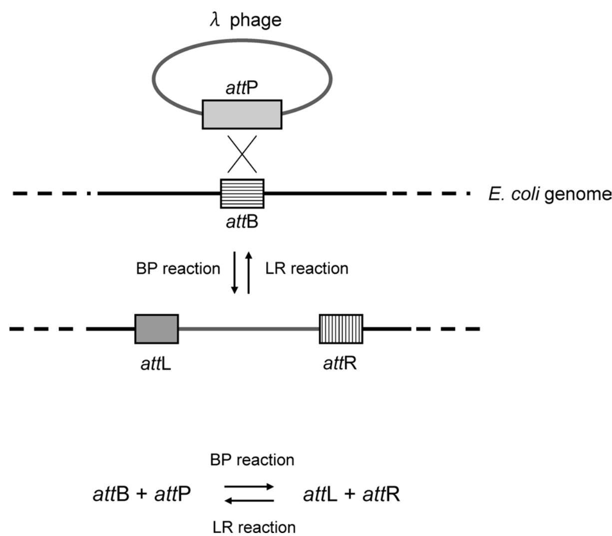

Figure 1 Integration and excision of λ phage into and from the *E. coli* genome. The *att*P site (242 bp) of λ phage recombines with the *att*B site (25 bp) of *E. coli* (BP reaction), resulting in generation of *att*L (100 bp) and *att*R (168 bp) located at each end of the λ phage genome. The BP and LR reactions are reversible reactions.

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 plasmid *in vitro* (Hartley et al. 2000, Walhout et al. 2000).

In the early version of the 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, *att*R1 and *att*R2, and a recombination reaction can occur only in combination of *att*B1 and *att*P1, *att*B2 and *att*P2, *att*L1 and *att*R1, or *att*L2 and *att*R2, since this recombination strictly depends on *att* sequences (Hartley et al. 2000; Walhout et al. 2000). In addition to these *att* sites, *ccd*B whose protein product inhibit 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

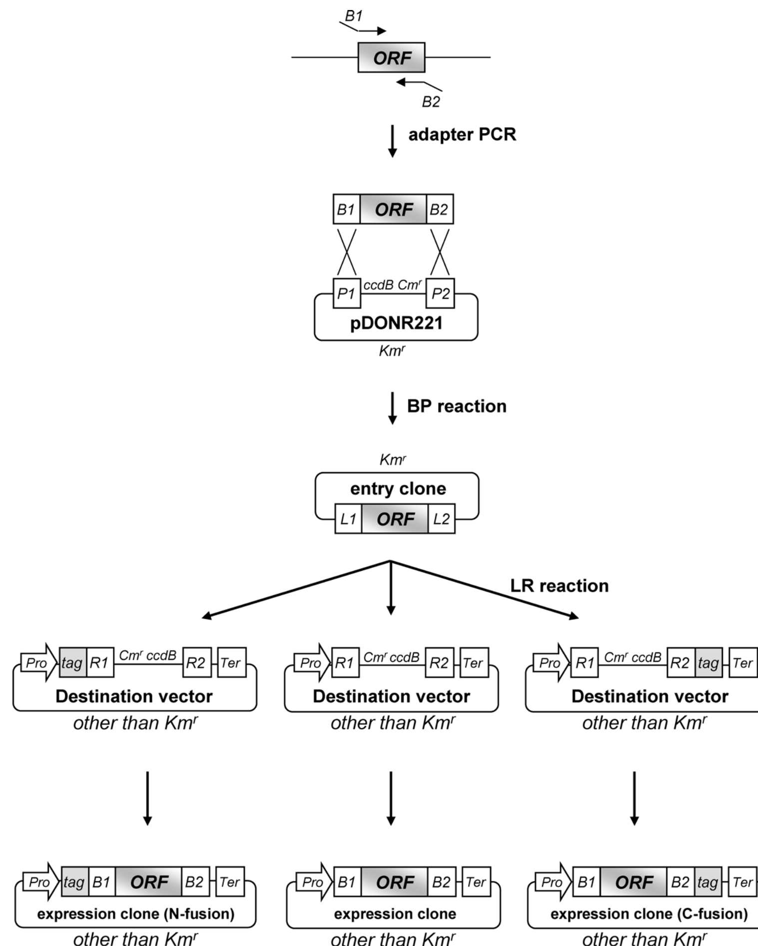

Figure 2 Schematic illustration of Gateway cloning. The ORF region is amplified by adapter PCR and the resulting *att*B1-ORF-*att*B2 fragment is cloned into pDONR221 by a BP reaction to generate an entry clone containing *att*L1-ORF-*att*L2. Subsequently, the ORF is cloned into Destination vectors by an LR reaction to generate expression clones including tag fusion constructs. *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.

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the open reading frame (ORF) and *att*2 is located at the 3′ end. This orientation is maintained in all cloning steps. Figure 2 shows the scheme of Gateway cloning. First, the *att*B1 and *att*B2 sequences are added to the 5′ and 3′ ends of the ORF, respectively, by adapter PCR. The product (*att*B1-ORF-*att*B2) is subjected to a BP reaction with a Donor vector, which possess an *att*P1-*ccd*B-Cm^r-*att*P2 cassette. Because of the existence of the negative selection marker *ccd*B between *att*P1 and *att*P2, only the 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 rapidly 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 resistance marker that is different from that carried by the entry clone, only the recombined Destination vectors carrying *att*B1-ORF-*att*B2 (the expression clone) will be selected. The Gateway cloning is designed so that the smallest *att* site, *att*B (25 bp), appears in the final product (the expression clone) to minimize the length of cloning junctions after the clonase reaction. Many Destination vectors have been developed for different purposes, such as vectors for expression in bacteria, mammals, and plants, vectors for fusion with reporter and epitope tags, and vectors for RNAi. In 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. As described above, Gateway cloning has great advantages: it is free from the need for restriction digestion, has a simple and uniform protocol, and offers high efficiency and reliability of cloning, easy manipulation of fusion constructs, and the existence of a variety of Destination vectors for many purposes. The use of Gateway cloning has expanded in many fields of biological research in recent years.

Binary vectors compatible with Gateway cloning

A large number of Gateway cloning compatible binary vectors (Destination vectors) have been developed in several laboratories and are summarized in the recent review (Karimi et al. 2007a). Among them, the pW (Karimi et al. 2002), pMDC (Curtis and Grossniklaus 2003; Brand et al. 2006) and pEarleyGate (Earley et al. 2006) series contain many types of vector for many purposes. The pW series consists of vectors for overexpression or antisense expression by the cauliflower mosaic virus 35S promoter (P_{35S}), for promoter analysis using luciferase (LUC), GUS, or GFP-GUS, and for construction of gene fusions with GFP, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) or red fluorescent protein (RFP). The pW series also contains a vector to express hairpin RNA for RNAi. The pMDC series consists of vectors for cloning, for overexpression by P_{35S}, for inducible expression by a heat shock promoter or estrogen treatment, for promoter analysis using GFP-6xHis or GUS, and for gene fusions with GFP, GFP-6xHis, or GUS. The pEarleyGate is a Basta resistance series consist 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.

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 construct. Vectors for expression of tagged fusion proteins have the general structure promoter-*att*R1-Cm^r-*ccd*B-*att*R2-tag-terminator (for C-terminal fusions) or promoter-tag-*att*R1-Cm^r-*ccd*B-*att*R2-terminator (for N-terminal fusions), and after an LR reaction with an *att*L1-ORF-*att*L2 entry clone, they yield promoter-*att*B1-ORF-*att*B2-tag-terminator or promoter-tag-*att*B1-ORF-*att*B2-terminator, respectively, such that the tag added to the N-terminus of the ORF is linked by the *att*B1 peptide (XSLYKKAGX) and the tag added to the C-terminus is linked by the *att*B2 peptide (XPAFLYKVX). Vectors for RNAi (Karimi et al. 2002; Helliwell and Waterhouse 2003; Hilson et al. 2004; Miki and Shimamoto 2004) generally have a Gateway cassette of promoter-*att*R1-*ccd*B-*att*R2-linker-*att*R2-*ccd*B-*att*R1-terminator. By the LR reaction with *att*L1-trigger-*att*L2, 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. Hairpin RNA is expressed from this construct and processed into small interfering RNA for gene silencing.

Although the vectors described above are useful, sometimes it is necessary to use a different series if a series does not have a vector of the required type. In order to carry out most experiments with the same series (with a unified backbone and a unified junction), we planned to make a comprehensive plant Gateway vector system containing many reporters and tags based on the same backbone.

Development of Gateway binary vectors (pGWBs)

We first tried to establish a systematic construction method for Gateway vectors for plant research. For this purpose, platform vectors pUGW2 and pUGW0 (Nakagawa et al. 2007a) were made using pUC119 as the backbone. These vectors include P_{35S} and the nopaline

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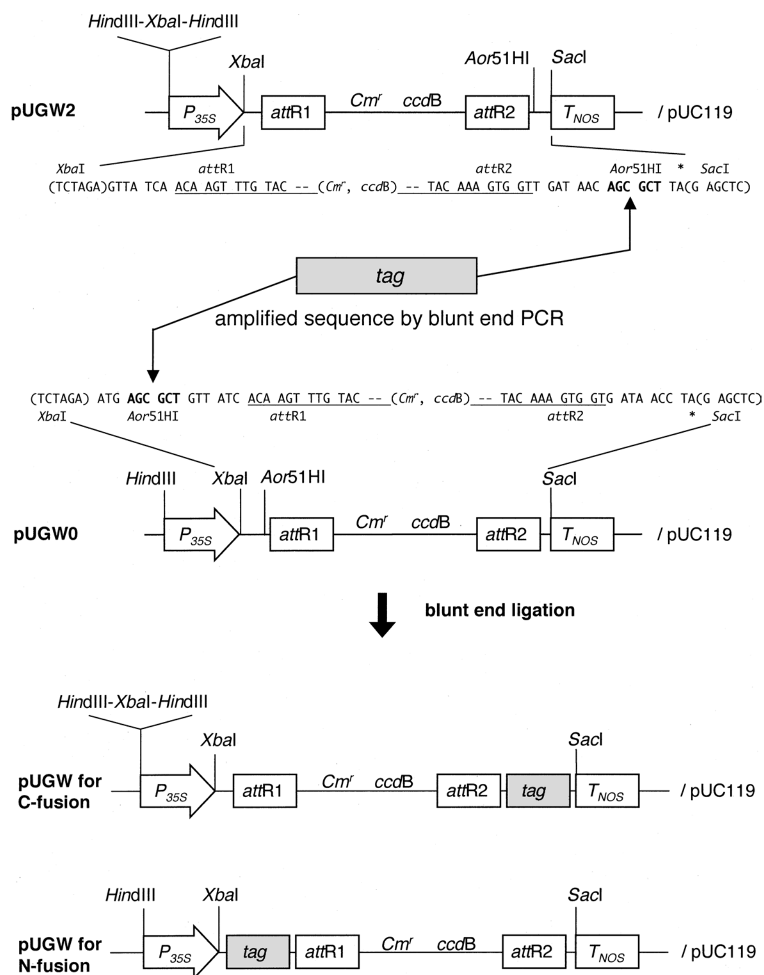

Figure 3 Procedure for construction of pUGWs. pUGW2 and pUGW0 are the starting vectors for construction of new pUGW derivatives. The tag sequence amplified by blunt-end PCR is introduced into the *Aor*51HI site of pUGW2 or pUGW0, then pUGWs for C-fusion or N-fusion are obtained. The region between P_{35S} and T_{NOS} is indicated. The nucleotide sequence corresponding to the region from *att*R1 to *att*R2 is underlined.

synthase terminator (T_{NOS}) as shown in Figure 3. 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-T_{NOS}. A reporter or epitope tag sequence amplified by blunt-end PCR was introduced into the *Aor*51HI site (blunt end) to yield a *Hin*dIII-*Xba*I-*Hin*dIII-P_{35S}-*Xba*I-*att*R1-Cm^r-*ccd*B-*att*R2-tag-*Sac*I-T_{NOS}. In the case of a small epitope tag, a DNA oligonucleotide could be introduced directly. The P_{35S} could be easily removed by digestion with *Xba*I followed by self-ligation for construction of promoterless pUGWs. 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- T_{NOS}. Reporter and epitope tag sequences were introduced by the same method used for pUGW2. Translation is initiated at the ATG located just upstream of the *Aor*51HI site. With these simple procedures, a pUGW series containing a variety of tags was efficiently generated and used as a source for Gateway cassette for construction of a Gateway binary vector (pGWB). Moreover, the pUGWs themselves were Gateway compatible plant vectors useful for transient expression analyses by particle bombardment or protoplast transformation because of their small size and high copy number in *E. coli*.

Initially, pGWB was constructed on the backbone of modified pBI (Mita et al. 1995) containing nopaline synthase promoter (P_{NOS}) driven neomycin phosphotransferase II (NPTII) and P_{35S} driven hygromycin phosphotransferase (HPT) genes as selective markers in plants. The pGWB series consists of 36 vectors, for simple cloning (pGWB1), for overexpression (pGWB2), and for fusion with a variety of tags (pGWB3 through pGWB45) (Table 1). GUS, TAP and LUC are available for C-fusion, and another 10 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. With the pGWBs, promoter activity and subcellular localization of proteins can be effectively

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analyzed (Nakagawa et al. 2007a). Next, we constructed pGWBs carrying the P_{NOS}:*HPT*:T_{NOS} marker instead of P_{35S}:*HPT*:T_{NOS} 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 were pGWB203, 204, 228 and 235, which are listed at the bottom of Table 1. In our previous study, reporter GUS activity was 5-fold higher with pGWB3 than with pGWB203 when the phosphate transporter PHT1 promoter was used for promoter analysis in *Arabidopsis thaliana* (Nakagawa et al. 2007a). Because both types of HPT marker can be expressed in bacteria, all pGWBs listed in Table 1 confer kanamycin and hygromycin resistance to *E. coli* and *A. tumefaciens*.

Although the initial pGWB series described above was useful in transgenic research, their low copy number in *E. coli* results in difficulty in handling of the plasmid, especially in confirmation of sequence. In addition, the existence of both a kanamycin and a hygromycin marker in one plasmid makes it difficult to reintroduce the transgene. To overcome these disadvantages, a new vector series, the improved Gateway binary vectors (ImpGWBs) were constructed (Nakagawa et al. 2007b). As the backbone binary vector, pPZP (Hajdukiewicz et al. 1994) was chosen because of its small size and high copy number in *E. coli*, and it was modified to carry the P_{NOS}:*NPTII*:T_{NOS} or P_{NOS}:*HPT*:T_{NOS} marker. Based on these modified pPZPs, the ImpGWB system, composed of 86 vectors, was generated as summarized in Table 2. A set of 15 tags, sGFP, GUS, LUC, EYFP, ECFP, G3 green fluorescent protein (G3GFP), monomeric red fluorescent protein (mRFP), 6xHis, FLAG, 3xHA,

Table 1. List of pGWBs.

Vector name	Backbone	Bacterial selection[a]	Gateway cassette[b]	Marker for plant	Type
pGWB1	pBI	Km^r, Hyg^r	*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, no tag
pGWB2	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, no tag
pGWB3	pBI	Km^r, Hyg^r	*att*R1-*att*R2-GUS-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-GUS
pGWB4	pBI	Km^r, Hyg^r	*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-sGFP
pGWB5	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-sGFP
pGWB6	pBI	Km^r, Hyg^r	P_{35S}-sGFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-sGFP
pGWB7	pBI	Km^r, Hyg^r	*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-6xHis
pGWB8	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-6xHis
pBWB9	pBI	Km^r, Hyg^r	P_{35S}-6xHis-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-6xHis
pGWB10	pBI	Km^r, Hyg^r	*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-FLAG
pGWB11	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-FLAG
pGWB12	pBI	Km^r, Hyg^r	P_{35S}-FLAG-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-FLAG
pGWB13	pBI	Km^r, Hyg^r	*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-3xHA
pGWB14	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-3xHA
pGWB15	pBI	Km^r, Hyg^r	P_{35S}-3xHA-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-3xHA
pGWB16	pBI	Km^r, Hyg^r	*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-4xMyc
pGWB17	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-4xMyc
pGWB18	pBI	Km^r, Hyg^r	P_{35S}-4xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-4xMyc
pGWB19	pBI	Km^r, Hyg^r	*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-10xMyc
pGWB20	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-10xMyc
pGWB21	pBI	Km^r, Hyg^r	P_{35S}-10xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-10xMyc
pGWB22	pBI	Km^r, Hyg^r	*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-GST
pGWB23	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-GST
pGWB24	pBI	Km^r, Hyg^r	P_{35S}-GST-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-GST
pGWB25	pBI	Km^r, Hyg^r	*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-T7
pGWB26	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-T7
pGWB27	pBI	Km^r, Hyg^r	P_{35S}-T7-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-T7
pGWB28	pBI	Km^r, Hyg^r	*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-TAP
pGWB29	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-TAP
pGWB35	pBI	Km^r, Hyg^r	*att*R1-*att*R2-LUC-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-LUC
pGWB40	pBI	Km^r, Hyg^r	*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-EYFP
pGWB41	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-EYFP
pGWB42	pBI	Km^r, Hyg^r	P_{35S}-EYFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-EYFP
pGWB43	pBI	Km^r, Hyg^r	*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	no pro, C-ECFP
pGWB44	pBI	Km^r, Hyg^r	P_{35S}-*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, C-ECFP
pGWB45	pBI	Km^r, Hyg^r	P_{35S}-ECFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{35S}:HPT (Hyg^r)	35S pro, N-ECFP
pGWB203	pBI	Km^r, Hyg^r	*att*R1-*att*R2-GUS-T_{NOS}	P_{NOS}:NPTII (Kmr), P_{NOS}:HPT (Hyg^r)	no pro, C-GUS
pGWB204	pBI	Km^r, Hyg^r	*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Kmr), P_{NOS}:HPT (Hyg^r)	no pro, C-sGFP
pGWB228	pBI	Km^r, Hyg^r	*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Kmr), P_{NOS}:HPT (Hyg^r)	no pro, C-TAP
pGWB235	pBI	Km^r, Hyg^r	*att*R1-*att*R2-LUC-T_{NOS}	P_{NOS}:NPTII (Km^r), P_{NOS}:HPT (Hyg^r)	no pro, C-LUC

[a] Km^r, kanamycin resistance; Hyg^r, hygromycin resistance.
[b] Chloramphenicol resistance (Cm^r) marker and negative selection marker (*ccd*B) located between *att*R1 and *att*R2 are not shown.

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Table 2. List of ImpGWBs.

Vector name	Backbone	Bacterial selection[a]	Gateway cassette[b]	Marker for plant	Type
pGWB401	pPZP	Spc^r	*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, no tag
pGWB402	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, no tag
pGWB402 Ω	pPZP	Spc^r	$P_{2x35S\text{-}\Omega}$-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	2×35S-Ω pro, no tag
pGWB404	pPZP	Spc^r	*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-sGFP
pGWB405	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-sGFP
pGWB406	pPZP	Spc^r	P_{35S}-sGFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-sGFP
pGWB407	pPZP	Spc^r	*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-6xHis
pGWB408	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-6xHis
pBWB409	pPZP	Spc^r	P_{35S}-6xHis-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-6xHis
pGWB410	pPZP	Spc^r	*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-FLAG
pGWB411	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-FLAG
pGWB412	pPZP	Spc^r	P_{35S}-FLAG-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-FLAG
pGWB413	pPZP	Spc^r	*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-3xHA
pGWB414	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-3xHA
pGWB415	pPZP	Spc^r	P_{35S}-3xHA-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-3xHA
pGWB416	pPZP	Spc^r	*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-4xMyc
pGWB417	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-4xMyc
pGWB418	pPZP	Spc^r	P_{35S}-4xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-4xMyc
pGWB419	pPZP	Spc^r	*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-10xMyc
pGWB420	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-10xMyc
pGWB421	pPZP	Spc^r	P_{35S}-10xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-10xMyc
pGWB422	pPZP	Spc^r	*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-GST
pGWB423	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-GST
pGWB424	pPZP	Spc^r	P_{35S}-GST-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-GST
pGWB425	pPZP	Spc^r	*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-T7
pGWB426	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-T7
pGWB427	pPZP	Spc^r	P_{35S}-T7-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-T7
pGWB428	pPZP	Spc^r	*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-TAP
pGWB429	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-TAP
pGWB433	pPZP	Spc^r	*att*R1-*att*R2-GUS-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-GUS
pGWB435	pPZP	Spc^r	*att*R1-*att*R2-LUC-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-LUC
pGWB440	pPZP	Spc^r	*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-EYFP
pGWB441	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-EYFP
pGWB442	pPZP	Spc^r	P_{35S}-EYFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-EYFP
pGWB443	pPZP	Spc^r	*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-ECFP
pGWB444	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-ECFP
pGWB445	pPZP	Spc^r	P_{35S}-ECFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-ECFP
pGWB450	pPZP	Spc^r	*att*R1-*att*R2-G3GFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-G3GFP
pGWB451	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-G3GFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-G3GFP
pGWB452	pPZP	Spc^r	P_{35S}-G3GFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-G3GFP
pGWB453	pPZP	Spc^r	*att*R1-*att*R2-mRFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-mRFP
pGWB454	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-mRFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, C-mRFP
pGWB455	pPZP	Spc^r	P_{35S}-mRFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	35S pro, N-mRFP
pGWB501	pPZP	Spc^r	*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, no tag
pGWB502	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, no tag
pGWB502 Ω	pPZP	Spc^r	$P_{2x35S\text{-}\Omega}$-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	2x35S-Ω pro, no tag
pGWB504	pPZP	Spc^r	*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-sGFP
pGWB505	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-sGFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-sGFP
pGWB506	pPZP	Spc^r	P_{35S}-sGFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-sGFP
pGWB507	pPZP	Spc^r	*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-6xHis
pGWB508	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-6xHis-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-6xHis
pBWB509	pPZP	Spc^r	P_{35S}-6xHis-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-6xHis
pGWB510	pPZP	Spc^r	*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-FLAG
pGWB511	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-FLAG-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-FLAG
pGWB512	pPZP	Spc^r	P_{35S}-FLAG-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-FLAG
pGWB513	pPZP	Spc^r	*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-3xHA
pGWB514	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-3xHA-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-3xHA
pGWB515	pPZP	Spc^r	P_{35S}-3xHA-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-3xHA
pGWB516	pPZP	Spc^r	*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-4xMyc
pGWB517	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-4xMyc-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-4xMyc
pGWB518	pPZP	Spc^r	P_{35S}-4xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-4xMyc
pGWB519	pPZP	Spc^r	*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-10xMyc
pGWB520	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-10xMyc-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-10xMyc

[a] Spc^r, spectinomycin resistance.

[b] Chloramphenicol resistance (Cm^r) marker and negative selection marker (*ccd*B) located between *att*R1 and *att*R2 are not shown.

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Table 2. (continued from previous page.)

Vector name	Backbone	Bacterial selection[a]	Gateway cassette[b]	Marker for plant	Type
pGWB521	pPZP	Spc^r	P_{35S}-10xMyc-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-10xMyc
pGWB522	pPZP	Spc^r	*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-GST
pGWB523	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-GST-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-GST
pGWB524	pPZP	Spc^r	P_{35S}-GST-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-GST
pGWB525	pPZP	Spc^r	*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-T7
pGWB526	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-T7-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-T7
pGWB527	pPZP	Spc^r	P_{35S}-T7-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-T7
pGWB528	pPZP	Spc^r	*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-TAP
pGWB529	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-TAP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-TAP
pGWB533	pPZP	Spc^r	*att*R1-*att*R2-GUS-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-GUS
pGWB535	pPZP	Spc^r	*att*R1-*att*R2-LUC-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-LUC
pGWB540	pPZP	Spc^r	*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-EYFP
pGWB541	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-EYFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-EYFP
pGWB542	pPZP	Spc^r	P_{35S}-EYFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-EYFP
pGWB543	pPZP	Spc^r	*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-ECFP
pGWB544	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-ECFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-ECFP
pGWB545	pPZP	Spc^r	P_{35S}-ECFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-ECFP
pGWB550	pPZP	Spc^r	*att*R1-*att*R2-G3GFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-G3GFP
pGWB551	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-G3GFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-G3GFP
pGWB552	pPZP	Spc^r	P_{35S}-G3GFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-G3GFP
pGWB553	pPZP	Spc^r	*att*R1-*att*R2-mRFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	no pro, C-mRFP
pGWB554	pPZP	Spc^r	P_{35S}-*att*R1-*att*R2-mRFP-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, C-mRFP
pGWB555	pPZP	Spc^r	P_{35S}-mRFP-*att*R1-*att*R2-T_{NOS}	P_{NOS}:HPT (Hyg^r)	35S pro, N-mRFP

[a] Spc^r, spectinomycin resistance.
[b] Chloramphenicol resistance (Cm^r) marker and negative selection marker (*ccd*B) located between *att*R1 and *att*R2 are not shown.

Table 3. List of R4pGWBs.

Vector name	Backbone	Bacterial selection[a]	Gateway cassette[b]	Marker for plant	Type
R4pGWB401	pPZP	Spc^r	*att*R4-*att*R2-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, no tag
R4pGWB404	pPZP	Spc^r	*att*R4-*att*R2-sGFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-sGFP
R4pGWB407	pPZP	Spc^r	*att*R4-*att*R2-6xHis-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-6xHis
R4pGWB410	pPZP	Spc^r	*att*R4-*att*R2-FLAG-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-FLAG
R4pGWB413	pPZP	Spc^r	*att*R4-*att*R2-3xHA-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-3xHA
R4pGWB416	pPZP	Spc^r	*att*R4-*att*R2-4xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-4xMyc
R4pGWB419	pPZP	Spc^r	*att*R4-*att*R2-10xMyc-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-10xMyc
R4pGWB422	pPZP	Spc^r	*att*R4-*att*R2-GST-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-GST
R4pGWB425	pPZP	Spc^r	*att*R4-*att*R2-T7-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-T7
R4pGWB428	pPZP	Spc^r	*att*R4-*att*R2-TAP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-TAP
R4pGWB433	pPZP	Spc^r	*att*R4-*att*R2-GUS-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-GUS
R4pGWB435	pPZP	Spc^r	*att*R4-*att*R2-LUC-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-LUC
R4pGWB440	pPZP	Spc^r	*att*R4-*att*R2-EYFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-EYFP
R4pGWB443	pPZP	Spc^r	*att*R4-*att*R2-ECFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-ECFP
R4pGWB450	pPZP	Spc^r	*att*R4-*att*R2-G3GFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-G3GFP
R4pGWB453	pPZP	Spc^r	*att*R4-*att*R2-mRFP-T_{NOS}	P_{NOS}:NPTII (Km^r)	no pro, C-mRFP
R4pGWB501	pPZP	Spc^r	*att*R4-*att*R2-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, no tag
R4pGWB504	pPZP	Spc^r	*att*R4-*att*R2-sGFP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-sGFP
R4pGWB507	pPZP	Spc^r	*att*R4-*att*R2-6xHis-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-6xHis
R4pGWB510	pPZP	Spc^r	*att*R14-*att*R2-FLAG-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-FLAG
R4pGWB513	pPZP	Spc^r	*att*R4-*att*R2-3xHA-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-3xHA
R4pGWB516	pPZP	Spc^r	*att*R4-*att*R2-4xMyc-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-4xMyc
R4pGWB519	pPZP	Spc^r	*att*R4-*att*R2-10xMyc-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-10xMyc
R4pGWB522	pPZP	Spc^r	*att*R4-*att*R2-GST-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-GST
R4pGWB525	pPZP	Spc^r	*att*R4-*att*R2-T7-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-T7
R4pGWB528	pPZP	Spc^r	*att*R4-*att*R2-TAP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-TAP
R4pGWB533	pPZP	Spc^r	*att*R4-*att*R2-GUS-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-GUS
R4pGWB535	pPZP	Spc^r	*att*R4-*att*R2-LUC-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-LUC
R4pGWB540	pPZP	Spc^r	*att*R4-*att*R2-EYFP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-EYFP
R4pGWB543	pPZP	Spc^r	*att*R4-*att*R2-ECFP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-ECFP
R4pGWB550	pPZP	Spc^r	*att*R4-*att*R2-G3GFP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-G3GFP
R4pGWB553	pPZP	Spc^r	*att*R4-*att*R2-mRFP-T_{NOS}	P_{NOS}:HPTII (Hyg^r)	no pro, C-mRFP

[a] Spc^r, spectinomycin resistance.
[b] Chloramphenicol resistance (Cm^r) marker and negative selection marker (*ccd*B) located between *att*R4 and *att*R2 are not shown.

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4xMyc, 10xMyc, GST, T7, and TAP, are available in ImpGWB. In the ImpGWB system, the transformation efficiency in *E. coli* was drastically increased and clear results were obtained in sequence analysis. Because of these advantages in plasmid handling, the ImpGWB system is used in many laboratories worldwide, and construction of new ImpGWBs employing novel tags is ongoing.

R4 Gateway binary vectors (R4pGWBs) for promoter swapping

To assemble multiple DNA fragments in the desired order, other *att* sites (*att*3, *att*4, *att*5 and *att*6) have been generated and applied to MultiSite Gateway cloning (Sasaki et al. 2004; Karimi et al. 2007b). Utilization of these *att* sites is valuable for swapping of promoters and reporters and is also applicable for cloning of multiple transgenes in one vector (Chen et al. 2006). In a typical MultiSite Gateway system, entry clones of specialized *att* sites, *att*L4-promoter-*att*R1, *att*L1-ORF-*att*L2, and *att*R2-tag-*att*L3 were connected and incorporated into a Destination vector carrying *att*R4-Cm^r-*ccd*B-*att*R3 to make an *att*B4-promoter-*att*B1-ORF-*att*B2-tag-*att*B3 construct (Figure 4). Although MultiSite Gateway cloning is an elegant method for building a complicated construct, it is relatively difficult to obtain the clone because four recombinations at each *att* site are required for successful cloning. We developed the R4 Gateway binary vector (R4pGWB) to facilitate multi-fragment cloning, especially for promoter swapping, by reducing the number of recombination to three (*att*4, *att*1 and *att*2) (Figure 5) (Nakagawa et al. 2008). R4pGWBs were made by replacing *att*R1 of ImpGWBs with *att*R4 and all

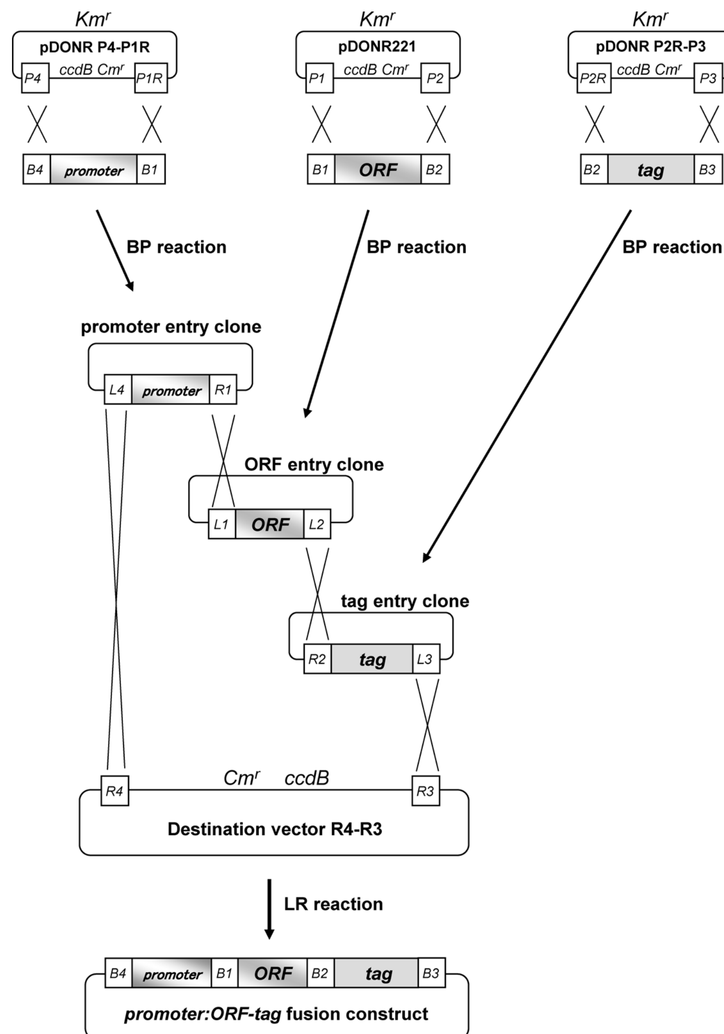

Figure 4 Diagram of construction of a promoter:ORF-tag fusion by MultiSite Gateway cloning. The *att*B4-promoter-*att*B1, *att*B1-ORF-*att*B2 and *att*B2-tag-*att*B3 sequences are amplified by adapter PCR and introduced into pDONR P4-P1R, pDONR221 and pDONR P2R-P3 (Invitrogen), respectively, using a BP reaction. The resulting entry clones containing *att*L4-promoter-*att*R1, *att*L1-ORF-*att*L2 and *att*R2-tag-*att*L3 are connected and cloned into Destination vector R4-R3 by an LR reaction to make the promoter:ORF-tag fusion construct. *P1*, *att*P1; *P1R*, *att*P1R; P2, *att*P2; *P2R*, *att*P2R; *P3*, *att*P3; *P4*, *att*P4; *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.

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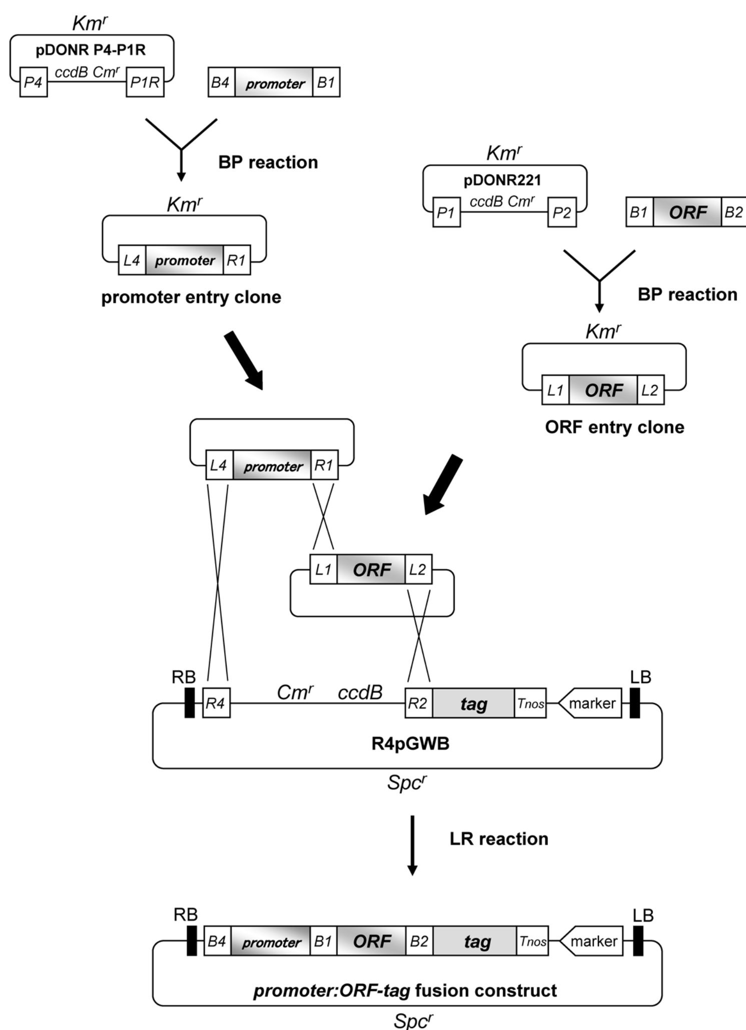

Figure 5 Cloning procedure by the R4pGWB system. The promoter and ORF entry clones constructed as described in Figure 4 are incorporated into R4pGWB (*att*R4-*att*R2-tag) by an LR reaction to make the promoter:ORF-tag construct. *P1*, *att*P1; *P1R*, *att*P1R; *P2*, *att*P2; *P4*, *att*P4; *B1*, *att*B1; *B2*, *att*B2; *B4*, *att*B4; *L1*, *att*L1; *L2*, *att*L2; *L4*, *att*L4; *R1*, *att*R1; *R2*, *att*R2; *R4*, *att*R4; RB, right border; LB, left border.

tags used in ImpGWB are also available in the R4pGWBs (Table 3). With the R4pGWBs, construction of chimeric genes among promoters, ORFs, and tags is achieved very easily. To use the *att*L4-promoter-*att*R1 entry clone for efficient construction of a promoter:tag clone, we also made R4L1pGWB vectors (Nakamura et al. unpublished results) containing *att*R4-Cm^r-*ccd*B-*att*L1-tag-T_{NOS}. By the simple bipartite LR reaction with *att*L4-promoter-*att*R1 and R4L1pGWB, an *att*B4-promoter-*att*B1-tag-T_{NOS} construct can be easily obtained in this system.

Conclusions

Because Gateway cloning is efficient, precise, flexible and simple to use, its application will grow more and more in plant research, and new vectors are expected to be developed. Plant selection markers widely used for Gateway plant binary vectors are kanamycin, hygromycin, and Basta resistance genes. Recently, a mutated acetolactate synthase (ALS) gene conferring bispyribac-sodium resistance (Kawai et al. 2007) was used as a selection marker in plant Gateway vectors (Inplanta Inovations, Yokohama, Japan). In addition to these markers, UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase (GPT) was reported to be applicable for selection by tunicamycin (Koizumi and Iwata 2008). Vectors equipped with new markers such as GPT will be useful for repetitive transformation in order to introduce new transgenes into previously transformed plants.

Many types of fluorescent proteins have been developed and made available for sale. A series of fluorescent proteins with a variety of emission wavelengths is supplied as the Living Colors Fruit Fluorescent Proteins (Clontech, Mountain View, CA, USA) for multicolor labeling of proteins. Photoswitchable fluorescent proteins, such as Kaede

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(MBL, Nagoya, Japan) and Dendra2 (Evrogen, Moscow, Russia) are powerful tools for tracing labeled proteins in the cell. Simultaneous observation of many proteins labeled by different tags such as described above is an effective method to reveal a complex biological process. For this purpose, Gateway plant vectors applicable for cloning of many fusion genes on one plasmid are desired. Because compatibility is a very important property of Gateway cloning, we plan to develop a pGWB system that will realize construction of a multiple fusion gene (e.g., promoter-ORF1-Kaede-terminator+promoter-ORF2-Dendra2-terminator) using conventional *att*L1-ORF-*att*L2 entry clones.

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