

Communication

Gateway Binary Vectors with the Bialaphos Resistance Gene, *bar*, as a Selection Marker for Plant Transformation

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We constructed two series of Gateway binary vectors, pGWBs and R4pGWBs, possessing the bialaphos resistance gene (*bar*) as a selection marker for plant transformation. The reporters and tags employed in this system are sGFP, GUS, LUC, EYFP, ECFP, G3GFP, mRFP, TagRFP, 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7 and TAP. Selection of *Arabidopsis* transformants with BASTA[®] was successfully carried out using both plate-grown and soil-grown seedlings. Transformed rice calli and suspension-cultured tobacco cells were selected on plates containing BASTA[®] or glufosinate-ammonium. These vectors are compatible with existing pGWB and R4pGWB vectors carrying kanamycin and hygromycin B resistance.

Key words: bialaphos resistance gene (*bar*); binary vector; Gateway cloning; glufosinate; plant transformation

Bialaphos and its degraded derivative glufosinate (also known as phosphinothricin) are active ingredients of widely used herbicides (Herbiace[®] and BASTA[®]) that inhibit glutamine synthetase, an essential enzyme for ammonium assimilation in plants. Although these compounds also inhibit the corresponding enzyme of the bialaphos-producing actinobacterium, *Streptomyces hygroscopicus*, it has a bialaphos resistance gene (*bar*) encoding phosphinothricin acetyltransferase, and thus is resistant to them.^{1,2} The *bar* gene has been used to create herbicide-resistant plants for both research and commercial purposes.

We have constructed many Gateway binary vectors containing various reporter proteins and epitope tags.^{3,4}

These vectors have two alternative selection markers for plant transformation, the neomycin phosphotransferase II gene (*NPTII*), conferring kanamycin resistance (Km^r), and the hygromycin phosphotransferase gene (*HPT*), conferring hygromycin B resistance (Hyg^r). Such selectability is especially important for multiple transformations. For example, a second marker gene is necessary for the retransformation of plants that already have a primary transgene with a primary marker gene. However, plants to be used for transformation sometimes have both marker genes already, because they are commonly and widely used markers for plant transformation. Hence, we decided to develop a new series of Gateway binary vectors with the *bar* gene to serve as a third choice for drug resistance. Another advantage of such herbicides in selection of transformants is the absence of any necessity of sterile agar media, which is required for selection with kanamycin or hygromycin B, because spraying herbicides easily kills soil-grown plants that do not have the corresponding resistance gene.

In this study, we developed two series of Gateway binary vectors with the *bar* marker gene: pGWB-type vectors containing *attR1* and *attR2* sites, suitable for promoter analysis and ectopic expression of cDNA,³ and R4pGWB-type vectors with *attR4* and *attR2* sites, suitable for the expression of cDNA under a favorite promoter.⁵ These vectors were denominated with 600-series numbers, the “6” representing the *bar* marker gene, and the last two digits (which are common to all our Gateway binary vectors) representing the kind of reporter and tag. In total, 46 pGWB6xx series and 17 R4pGWB6xx series vectors are available in making fusion proteins with 16 various reporters and tags. The

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Abbreviations: *bar*, bialaphos resistance gene; CaMV, cauliflower mosaic virus; Cm^r, chloramphenicol resistance; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; G3GFP, G3 green fluorescent protein; GUS, β -glucuronidase; HPT, hygromycin phosphotransferase; Hyg^r, hygromycin B resistance; Km^r, kanamycin resistance; LUC, luciferase; mRFP, monomeric red fluorescent protein; NPTII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; RFP, red fluorescent protein; sGFP, synthetic green fluorescent protein with S65T mutation; Spc^r, spectinomycin resistance; TAP, tandem affinity purification; TagRFP, tag red fluorescent protein; Tnos, nopaline synthase terminator

bar marker gene is driven by the nopaline synthase promoter (*Pnos*) and is followed by the nopaline synthase terminator (*Tnos*).

To construct the new vectors, plasmids were handled by standard methods.⁶⁾ KOD DNA polymerase (Toyobo, Tokyo, Japan) was used for PCR. All the adapters and primers used in this study are listed in Supplemental Table 1 (see *Biosci. Biotechnol. Biochem.* Web site). The PCR-amplified region and ligation junctions were confirmed by sequence analysis for all vectors. The *Pnos:bar:Tnos* marker was constructed as follows. The Δ *SacI* adapter was introduced into the *SacI* site of pARK5⁷⁾ to destroy the *SacI* site between *bar* and *Tnos*, and the resulting vector was named pARK5 Δ *SacI*. The *bar:Tnos* sequence was prepared by PCR using pARK5 Δ *SacI* as template, with *Pnos-bar-F* and *PshBI-Tnos-R* primers. The *Pnos* sequence was prepared by PCR using pYLTAC⁸⁾ as template, with *PshBI-Pnos-F* and *Pnos-bar-R* as primers. These products were diluted, mixed, and used in a second PCR with *PshBI-Pnos-F* and *PshBI-Tnos-R* primers. The amplified product, in which *Pnos* and *bar:Tnos* were joined (*Pnos:bar:Tnos*), was digested by *PshBI* and used to replace *Pnos:HPT:Tnos* of pGWB500³⁾ to make pGWB600 (*Pnos:bar:Tnos*). LB medium containing 100 mg/l of spectinomycin was used to select *E. coli* DH5 α , which harbors these plasmids. The Gateway cassette and reporter/tag genes were introduced into pGWB600, as described previously,^{3,5,9)} to make pGWB601-661s and R4pGWB601-659s. *E. coli* DB3.1 cultures harboring these vectors were selected on LB medium containing 100 mg/l of spectinomycin and 30 mg/l of chloramphenicol.

Figure 1 shows the structures of the pGWB601-661s and R4pGWB601-659s. All vectors carry the *Pnos:bar:Tnos* marker placed in reverse orientation relative to the direction of the gene cloned by the LR reaction (Fig. 1A). The structures around the Gateway recombination sites are illustrated in Fig. 1B. The *R1-R2* type cassettes in the pGWB601-661s (Fig. 1B, left panel) are the same structures as those in previously constructed vector series pGWB4xx (with the Km^r marker) and pGWB5xx (with the Hyg^r marker),³⁾ and are compatible with *attL1*-(promoter and/or cDNA)-*attL2* entry clones. The *R4-R2* type cassettes in the R4pGWB601-659s (Fig. 1B, right panel) are the same structures as those in R4pGWB4xx and R4pGWB5xx,⁵⁾ and are compatible with a combination of the *attL4*-promoter-*attR1* and *attL1*-cDNA-*attL2* entry clones. Figure 1C summarizes the 16 reporters and tags employed in each vector. The last two digits of the vector names are as with the other pGWB and R4pGWB vectors. The complete nucleotide sequence of the pGWB601-661s and R4pGWB601-659s reported here appear in the GenBank/EMBL/DDBJ database under accession nos. AB543110 to AB543172. The expected linker sequences around the *attB* sites that should be created after the LR reaction are shown in Supplemental Fig. 1 for the pGWB601-661s, and in Supplemental Fig. 2 for the R4pGWB601-659s (see *Biosci. Biotechnol. Biochem.* Web site).

To test the performance of these vectors, we used them in the transformation of *Arabidopsis thaliana* (*Arabidopsis*) plants, *Oryza sativa* (rice) calli, and

suspension-cultured *Nicotiana tabacum* (tobacco) cells, and determined the optimum concentrations of BASTA[®] or glufosinate-ammonium for selection. In *Arabidopsis*, promoter analysis of the F-box family protein 3 gene (*AtFBL3*) was done with pGWB633. *AtFBL3* (AT5G01720) encoded a leucine-rich repeat protein containing an F-box motif in the N-terminal region, and was expressed predominantly in the vascular system (S. Nakamura, unpublished results). The promoter fragment spanning the sequence between -2006 and +3 (the adenosine of the translation initiation codon was designated +1) of *AtFBL3* was prepared from genomic DNA by adapter PCR with primers *AtFBL3-pro-attB1* and *AtFBL3-pro-attB2*. The amplified fragment was cloned into pDONR201 (Invitrogen, Carlsbad, CA) by the BP reaction following the manufacturer's instructions for construction of an *attL1-P_{AtFBL3}-attL2* entry clone. Then the promoter fragment was introduced into pGWB633 by the LR reaction following the manufacturer's instructions, to be fused with a β -glucuronidase (*GUS*) reporter. The *P_{AtFBL3}:GUS* construct obtained was introduced into *Agrobacterium tumefaciens* C58 C1 (pMP90), and was used in the transformation of *Arabidopsis* (Col-0 accession) using a floral dip procedure.¹⁰⁾ In order to determine the appropriate conditions for the selection of transgenic *Arabidopsis* on plates using BASTA[®] as the selection agent, we sowed T1 seed on an agar plate of Murashige and Skoog medium containing 0.0027, 0.0054, or 0.011% BASTA[®] (Bayer CropScience, Monheim am Rhein, Germany). BASTA[®] contained 18.5% glufosinate-ammonium; thus, the final concentrations of glufosinate-ammonium in the selection media were 5, 10, and 20 mg/l, respectively. BASTA[®] at 0.0054% (10 mg/l of glufosinate-ammonium) was the most effective. In a typical result, shown in Fig. 2A, more than 2% of the T1 seed germinated and grew well on a selection medium containing 0.0054% BASTA[®]. Most T1 seedlings surviving on the plate showed strong expression of the *P_{AtFBL3}:GUS* gene, especially in the vascular tissues (Fig. 2B). Alternatively to selection on an agar plate, BASTA[®]-resistant T1 transformants were identified by spraying BASTA[®] solution (0.033% BASTA[®] with 0.01% Silwet L-77) on soil-grown seedlings at 7 and 9 d after germination, and by sowing T1 seed onto a piece of rock wool that was initially soaked with 0.011% BASTA[®] solution (data not shown). Note that a detergent (Silwet L-77) was added to the BASTA[®] solution only when the solution was used for spraying.

In experiments with rice, G3 green fluorescent protein (G3GFP) was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The G3GFP sequence was amplified from a cloned *G3GFP* gene¹¹⁾ by adapter PCR with primers *G3GFP-attB1* and *G3GFP-attB2*. The amplified fragment was cloned into pDONR201 by the BP reaction to construct an *attL1-G3GFP-attL2* entry clone. Then the *G3GFP* gene was introduced into pGWB602 by the LR reaction to make *P_{35S}:G3GFP*. This construct was introduced into *A. tumefaciens* EHA101 by electroporation. Transformation of rice (cv. Yukihihikari or Nipponbare) was performed by the method of Hiei *et al.*¹²⁾ Transformed rice calli were selected on N6D selection medium containing 0.025, 0.05, 0.1, or 0.2% BASTA[®] (final

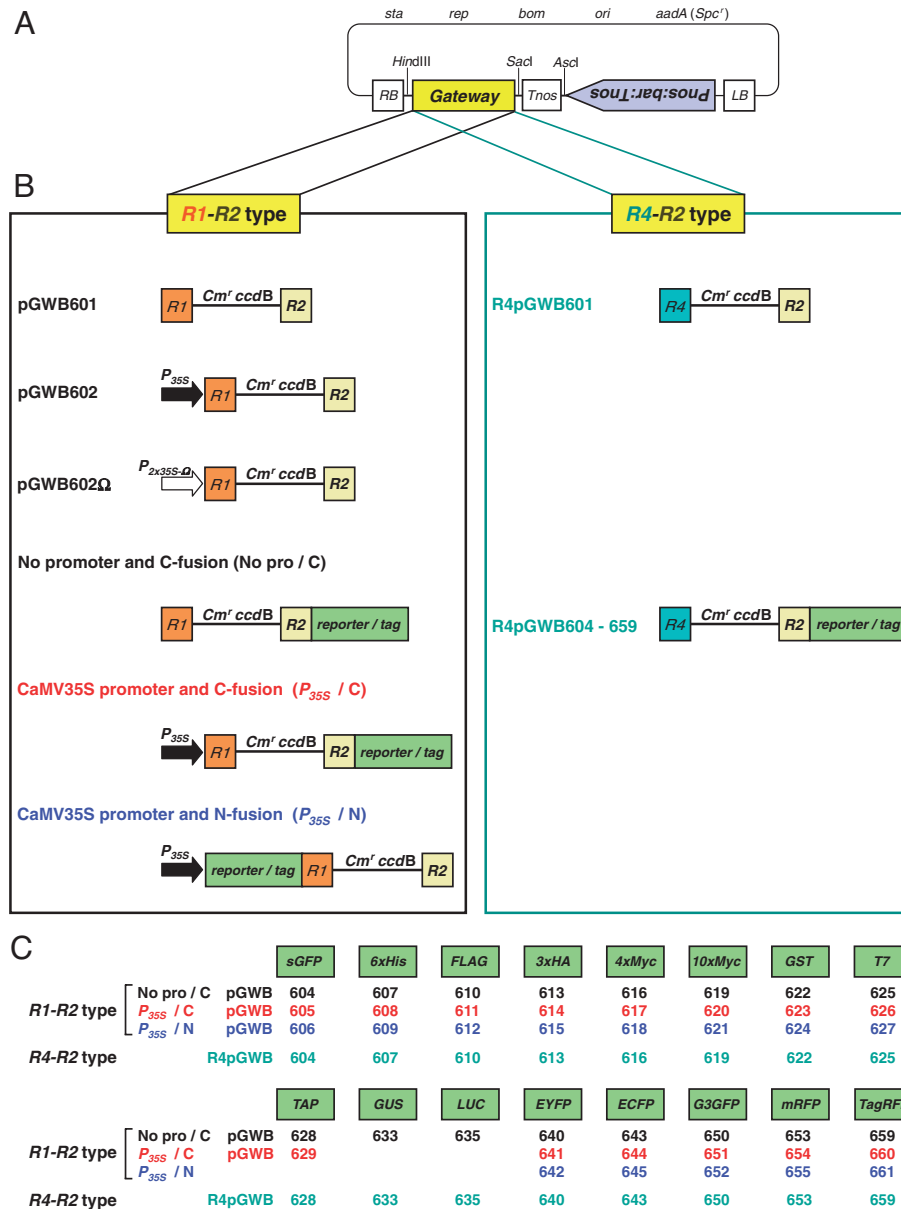


Fig. 1. Schematic Illustration of pGWB601-661 and R4pGWB601-659 Structures.

A, All vectors contain the *Pnos:bar:Tnos* gene, which is a selection marker for bialaphos-resistant plants, in reverse orientation to the Gateway cassette. *RB*, right border; *LB*, left border; *sta*, region conferring stability in *A. tumefaciens*; *rep*, broad host-range replication origin; *bom*, *cis*-acting element for conjugational transfer; *ori*, ColE1 replication origin; *aadA*, gene for spectinomycin resistance (*Spc*^r) used for selection in the bacteria. B, The structure of the region indicated as “Gateway” in (A). The left panel shows the structures of vectors possessing an *attR1-attR2* (*R1-R2*) type cassette (pGWB601-661). The right panel shows the structures of vectors possessing an *attR4-attR2* (*R4-R2*) type cassette (R4pGWB601-659). *Cm*^r, chloramphenicol resistance; *ccdB*, negative selection marker for bacteria; *R1*, *attR1*; *R2*, *attR2*; *R4*, *attR4*. C, Reporters and tags employed in the Gateway binary vectors illustrated in (B). The first digit, 6, of the vector number represents bialaphos resistance, and the last two digits indicate the reporter/tag and the type of fusion. sGFP, synthetic green fluorescent protein with S65T mutation;^{20,21} 6xHis, hexahistidine tag; FLAG, FLAG-tag;²² 3xHA, triple HA tag;²³ 4xMyc and 10xMyc, four repeats and 10 repeats of the Myc tag, respectively;²³ GST, glutathione *S*-transferase;²⁴ T7, T7-epitope tag;²⁵ TAP, tandem affinity purification tag;²⁶ GUS, β -glucuronidase;²⁷ LUC, modified luciferase, luc+;²⁸ EYFP, enhanced yellow fluorescent protein;²⁹ ECFP, enhanced cyan fluorescent protein;²⁹ G3GFP, G3 green fluorescent protein;¹¹ mRFP, monomeric red fluorescent protein;³⁰ TagRFP, tag red fluorescent protein.³¹

concentrations of glufosinate-ammonium, 46.3, 92.5, 185, and 370 mg/l, respectively). The most efficient selective conditions were 0.05% BASTA[®] (92.5 mg/l of glufosinate-ammonium) for Yukihikari, and 0.1% BASTA[®] (185 mg/l of glufosinate-ammonium) for Nipponbare. Typically, more than 10% of Yukihikari calli grew on the selection medium containing 0.05% BASTA[®] (Fig. 2C). These resistant calli showed GFP fluorescence when observed under a SZX16 fluorescent stereomicroscope (Olympus, Tokyo, Japan) (Fig. 2D and E).

In transformation experiments with suspension-cultured tobacco cells, red fluorescent protein (RFP) fused with peroxisome targeting signal 1 (PTS1) was expressed and observed to determine intracellular localization. A DNA fragment encoding the RFP-PTS1 fusion protein¹³ was cloned into pENTR2B (Invitrogen) to make an *attL1-RFP-PTS1-attL2* entry clone (pENTRHPTS1). Then the *RFP-PTS1* fragment was introduced into pGWB602 by the LR reaction to construct *P_{35S}:RFP-PTS1* (pRHPTS1-Bs). This construct was introduced into *A. tumefaciens* EHA101 by electro-

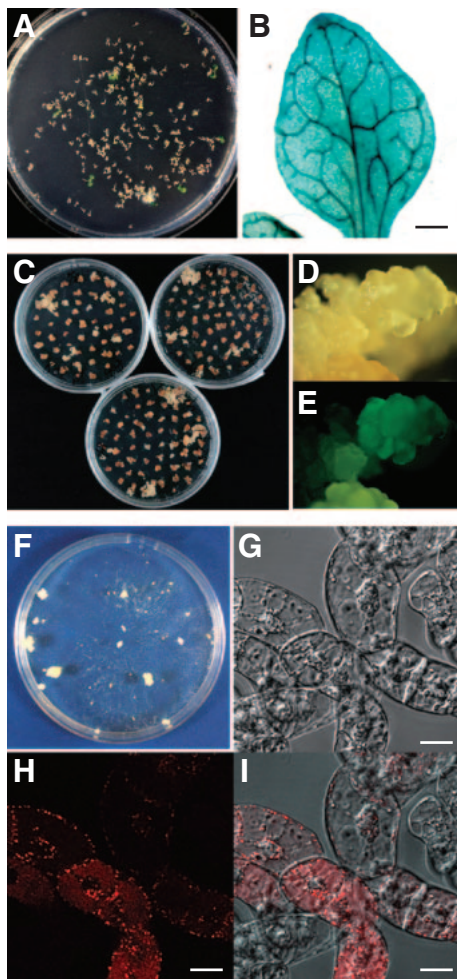


Fig. 2. Selection and Analysis of Transformants.

A, Selection of transgenic Arabidopsis on an agar plate containing 0.0054% BASTA®. The plate was incubated for 9 d at 22 °C under continuous illumination. B, Histochemical analysis of GUS expression in a leaf of a T1 Arabidopsis plant possessing the *P_{AFL3}:GUS* gene. Bar = 1 mm. C, Selection of transgenic rice calli (Yukihikari) on a selection medium containing 0.05% BASTA®. The plates were incubated for 4 weeks at 29.5 °C under continuous illumination. D, E, Microscopic images of transformed rice calli possessing the *P_{35S}:G3GFP* gene. Bright field image (D) and image of GFP fluorescence (E). F, Selection of transgenic BY-2 on a plate containing 2 mg/l of glufosinate-ammonium. The plate was incubated for 3 weeks at 26.5 °C under continuous darkness. G, H, I, Microscopic images of transgenic BY-2 cells possessing the *P_{35S}:RFP-PTSI* gene. Differential interference contrast microscopy (G), RFP fluorescence (H), and merge of G and H (I). Bars = 20 µm.

poration. Transformation of suspension-cultured tobacco Bright Yellow 2 (BY-2) cells was done by the method of Matsuoka and Nakamura.¹⁴⁾ Transformants were selected on a medium containing 1, 2, 5, or 10 mg/l glufosinate-ammonium (Sigma-Aldrich, St. Louis, MO). The most effective selection was with 2 mg/l of glufosinate-ammonium (Fig. 2F). Resistant cells grown on the plate were analyzed with a LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany), as described previously.¹⁵⁾ A typical peroxisomal localization pattern of RFP fluorescence was observed in the transgenic BY-2 cells (Fig. 2G–I).

In conclusion, we developed a new series of Gateway binary vectors, pGWB601-661s and R4pGWB601-659s, possessing the *Pnos:bar:Tnos* marker, conferring bialaphos resistance. Although other Gateway compatible

binary vectors containing the *bar* marker have been made in several laboratories,^{16–19)} our bialaphos-resistant vectors have great advantages in the availability of many reporters and tags to express fusion proteins and compatibility with existing *Km^r* and *Hyg^r* vectors, which have the same structures with the exception of the *bar* marker gene.^{3–5)} By using these Gateway binary vectors, one can make a variety of fusion constructs with the desired selection marker and carry out many transgenic experiments under unified conditions. All the vectors described in this report will be made available for noncommercial research purposes, although the permission of the original developers is required for some reporters. The e-mail address to request the vectors is tnakagaw@life.shimane-u.ac.jp.

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