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ORIGINAL RESEARCH

Cloning Vectors for Rice

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Abstract We developed various binary vectors that can be used for expressing a foreign gene in rice. Vectors pGA3426, pGA3436, and pGA3626 are intended for overexpression of a gene using the maize *Ubiquitin* promoter, whereas pGA3780 is for rather mild expression of a gene using the rice *Actin1* promoter. Vector pGA3777 is for expressing two genes simultaneously. We also developed binary vectors for expressing a fusion protein with a tag. Four vectors (pGA3427, pGA3428, pGA3429, and pGA3438) are for protein tags with sGFP, HA, His, and Myc, respectively. Vector pGA3383 is for analyzing promoter activity using the *GUS* reporter. In this vector, multiple cloning sites in front of *GUS* can be utilized for accepting a promoter fragment. We also generated transient expression vectors for studying the subcellular localization of a protein. Vectors pGA3452, pGA3651, and pGA3652 are for GFP fusion; pGA3574 for RFP fusion; pGA3697 for Myc tag; and pGA3698 for HA tag. In addition, we generated pGA3506, pGA3516, pGA3592, and pGA3593 for facilitating the subcloning of full-length cDNA clones into our binary vectors.

Keywords Cloning vector · Binary vector · *GUS* · Overexpression · RNAi · Tag · Transformation

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Rice is a major staple food for humans, especially in Asia. Public concern for food security has now escalated in response to climate change [4] and bioenergy production from edible crops [20]. In addition to its economic importance, rice is a model for the study of monocot species because it is a diploid, its entire genome has been sequenced [8, 18], and it is easy to transform [11, 15].

The challenge is great to identify the function of all genes present in the genome. To achieve that goal, insertional mutant populations by T-DNA, *Tos17*, and *Ac/Ds* have been generated, and the locations of those insertion sites have been determined [1, 3, 10, 14, 17]. However, resources are not large enough to cover the entire genome. Moreover, when a mutant in a given gene does not show any clear phenotypes, double mutations must be generated. In these cases, antisense or RNAi suppression approaches could produce valuable data. Overexpression of a gene often also provides a clue to its function.

Binary vectors, based on the Ti plasmid of *Agrobacterium*, have been widely used for plant transformation [2]. Several binary vectors have been developed for rice [12, 15, 16]. In this paper, we introduced several binary vectors for inducing antisense, RNAi, and ectopic expressions. Binary vectors for expression of a target-tag fusion protein also were developed. These vectors are available for subcellular localization, protein interactions using co-immunoprecipitation, and protein purification according to the characteristics of tags. For promoter analysis, we constructed a binary vector that harbors the *GUS* reporter gene. In addition, vectors for transient expression of a target-tag fusion protein (sGFP, eGFP, mRFP, HA, or Myc tags) were constructed for analysis of subcellular localization of target proteins and protein–protein interactions via co-immunoprecipitation.

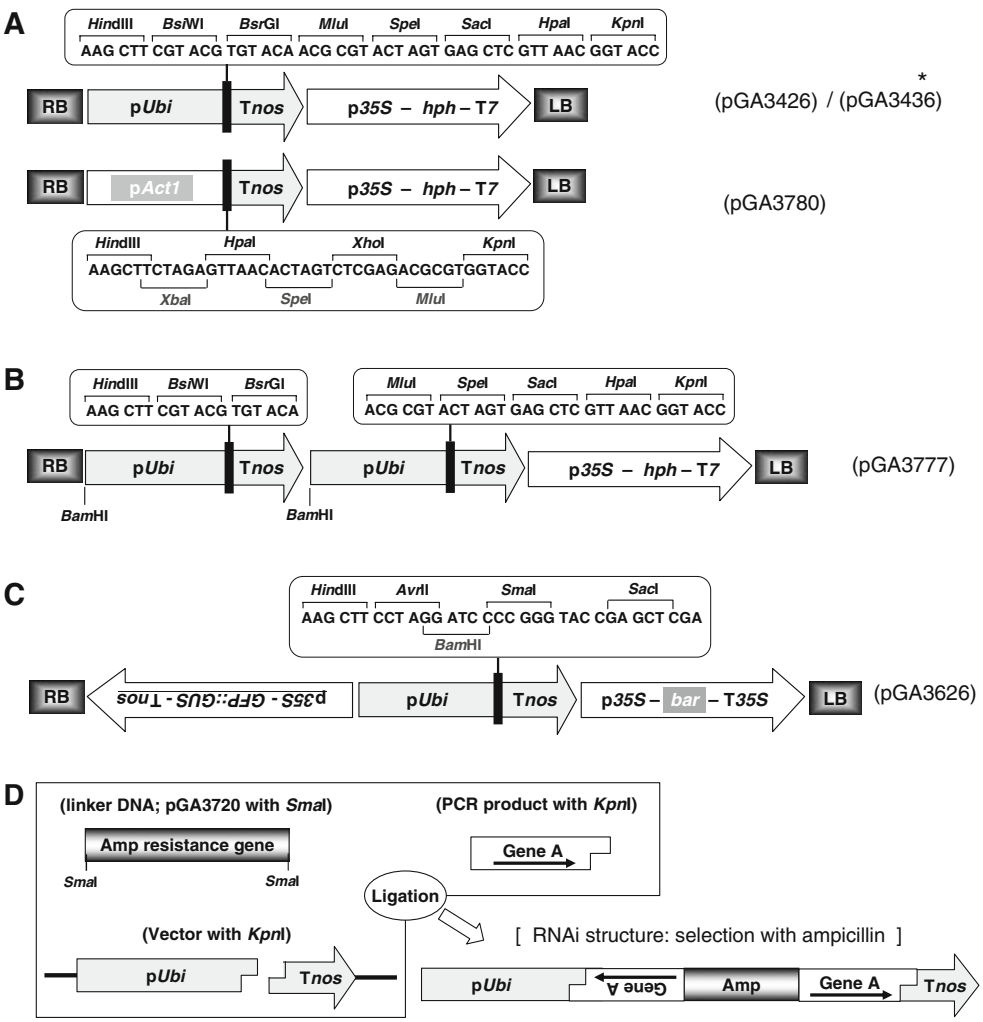

Fig. 1 Binary vectors for rice transformation. Vector maps are depicted in **A**–**C**. *MCS of pGA3436 is in reverse orientation compared with pGA3426. Unique sites for cloning are represented in *big boxes*. **D** Schematic diagram for RNAi construction. *RB* right border of T-DNA; *LB* left border of T-DNA; p*Ubi* maize *Ubiquitin-1*promoter [7] containing the promoter, first exon, and first intron; T*nos nopaline synthase* (*nos*) terminator; p*35S* CaMV *35S* promoter; p*Act1*, rice *Actin1* promoter; *hph hygromycin phosphotransferase* gene; *T7′*, terminator sequence of T-DNA gene 7; *GFP* green fluorescent protein; *bar* bialaphos resistance gene; *T35S* terminator sequence of CaMV *35S*

Materials and Methods

Modification of Multiple Cloning Sites

We used synthetic oligomers (Bionics, Korea) for modification of multiple cloning sites (MCS). Briefly, 100 pmol of complementary single-strand oligomers was denatured by boiling for 5 min in 100 μL of 1× STE buffer then cooling to room temperature (RT) for annealing. Approximately 50 to 100 ng of vector DNA was digested with an appropriate restriction enzyme (Enzynomics, Korea, and NEB, USA), and 0.5 μL of the annealed oligomers was ligated using T4 DNA ligase (Roche, USA) in 2× rapid ligation buffer [60 mM Tris–HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% PEG 8000] for 1 h at RT. Approximately 2 μL of the total 10 μL ligation reaction was then transformed with *Escherichia coli* strain TOP10.

Tissue Bombardment

For their transient expression, we delivered molecules by particle bombardment as previously described [22]. Rice seeds were germinated and grown on Murashige and Skoog media in the dark. Afterward, 7-day-etiolated seedling shoots and onion epidermis were bombarded via a biolistic particle delivery system (Model PDS-1000, Dupont), with parameters of 0.4 mg/shot of gold beads coated with approximately 2 to 4 μg/shot of each vector DNA and 900 psi pressure in a 25-in. Hg vacuum. After 10 to 20 h of incubation at 28°C under darkness, the samples were either assayed for GUS activity by staining or measured for GFP and RFP activities using a Zeiss Axioplan fluorescence microscope.

Results and Discussion

Binary Vectors for Rice Transformation

We have previously reported the development of binary vector pGA1611, which can be used for strong expression of a foreign gene or cDNA in rice [15, 13]. Its T-DNA region harbors a hygromycin resistance gene for selection of transformants, plus multiple cloning sites (*Hin*dIII, *Sac*I,

Table 1 Summary of vectors

Vector	Characteristic and application	Selectable marker	Vector size (bp)
pGA3426	Binary vector, overexpression, RNAi, antisense	tet, hph	13,497
pGA3436	Binary vector, overexpression, RNAi, antisense	tet, hph	13,509
pGA3780	Binary vector, overexpression, RNAi, antisense (rice *Actin1* promoter)	tet, hph	12,870
pGA3777	Binary vector, double expression casstte (p*Ubi*-MCS-T*nos*) x2	tet, hph	15,897
pGA3626	Binary vector, bar selection, overexpression, RNAi, antisense	km, bar	About 14,000
pGA3427	Binary vector, expression of target-sGFP fusion protein	tet, hph	14,211
pGA3428	Binary vector, expression of target-3xHA tag fusion protein	tet, hph	13,623
pGA3438	Binary vector, expression of target-4xMyc tag fusion protein	tet, hph	13,665
pGA3429	Binary vector, expression of target-6xHis tag fusion protein	tet, hph	13,512
pGA3383	Binary vector, promoter analysis using GUS reporter gene	tet, hph	12,981
pGA3452	Transient expression of target-sGFP fusion protein, subcellular localization	amp	6,130
pGA3574	Transient expression of target-mRFP fusion protein, subcellular localization	amp	6,082
pGA3651	Transient expression of target-eGFP fusion protein, subcellular localization	amp	5,695
pGA3652	Transient expression of eGFP-target fusion protein, subcellular localization	amp	5,676
pGA3697	Transient expression of target-6xMyc fusion protein	amp	5,179
pGA3698	Transient expression of target-3xHA fusion protein	amp	5,026
pGA3506	Rice full-length cDNA subcloning vector, blue/white selection	amp	2,910
pGA3516	Rice full-length cDNA subcloning vector, blue/white selection	amp	2,934
pGA3592	Rice full-length cDNA subcloning vector, blue/white selection	km	2,804
pGA3593	Rice full-length cDNA subcloning vector, blue/white selection	km	2,828
pGA3720	Linker DNA (ampicillin resistance gene) for RNAi construction	amp, km	3,822

tet tetracycline, *hph* hygromycin, *km* kanamycin, *amp* ampicillin, *bar* bialaphos

*Hpa*I, and *Kpn*I) between the maize *Ubi* promoter and *nos* terminator. Here, to improve its utility, we made a new vector, pGA3426, by adding four unique restriction enzyme sites (*Bsi*WI, *Bsr*GI, *Mlu*I, and *Spe*I) at the multiple cloning site of pGA1611 (Fig. 1a). We also constructed pGA3436 in which the ordering of the restriction enzyme sites is in a reverse orientation compared with pGA3426 (Fig. 1a). Another expression vector, pGA3780, was constructed from pGA1611 by replacing the maize *Ubi* promoter with the rice *Actin1* promoter. Because the latter is weaker than the former [9], the vector can be used for lower expression of a foreign gene.

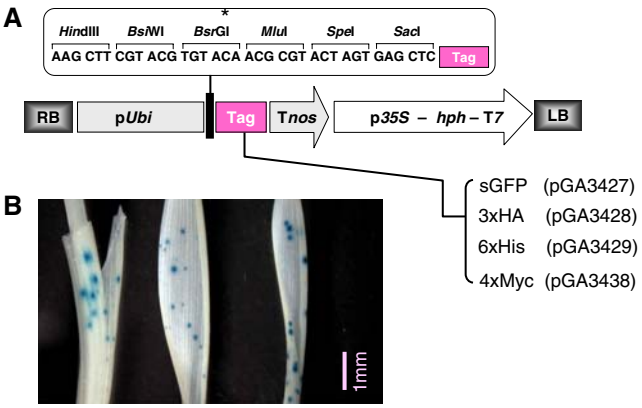

Fig. 2 Binary vectors for generating protein tag. **A** Vector maps. Unique restriction enzyme sites for cloning are denoted in *box*. **Bsr*GI is not unique in pGA3427. **B** Gene expression test using protein tag vector. *GUS* reporter gene was inserted into pGA3427, pGA3428, and pGA3438, and introduced into etiolated leaves via bombardment. GUS activity was measured after overnight transient expression. *RB* right border of T-DNA; *LB* left border of T-DNA; p*Ubi* maize *Ubiquitin-1*promoter containing the promoter, first exon, and first intron; T*nos*, *nos* terminator; p*35S* CaMV *35S* promoter; *hph hygromycin phosphotransferase* gene; *T7′* terminator sequence of T-DNA gene 7; sGFP, synthetic green fluorescent protein [6]; *HA* HA tag; *Myc* Myc tag; *6× His* six histidine residues

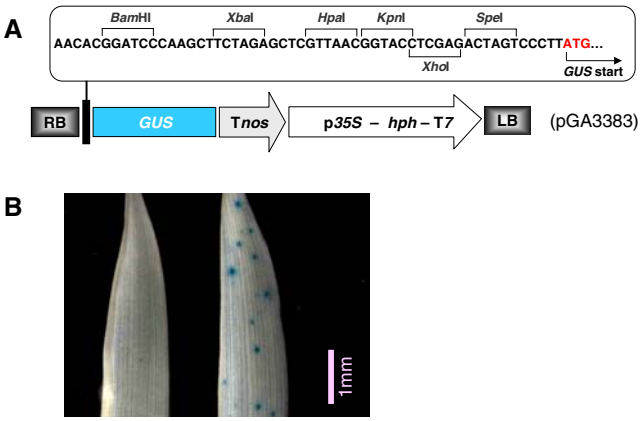

Fig. 3 Binary vector for promoter study using GUS. **A** Vector map of pGA3383. Unique restriction enzyme sites for cloning are denoted in *box*. **B** GUS activity in etiolated leaves caused by bombardment of rice *Actin1* promoter in pGA3383. On the *left* is negative control. *RB* right border of T-DNA; *LB* left border of T-DNA; T*nos*, *nos* terminator; p*35S* CaMV *35S* promoter; *hph hygromycin phosphotransferase* gene; *T7′* terminator sequence of T-DNA gene 7

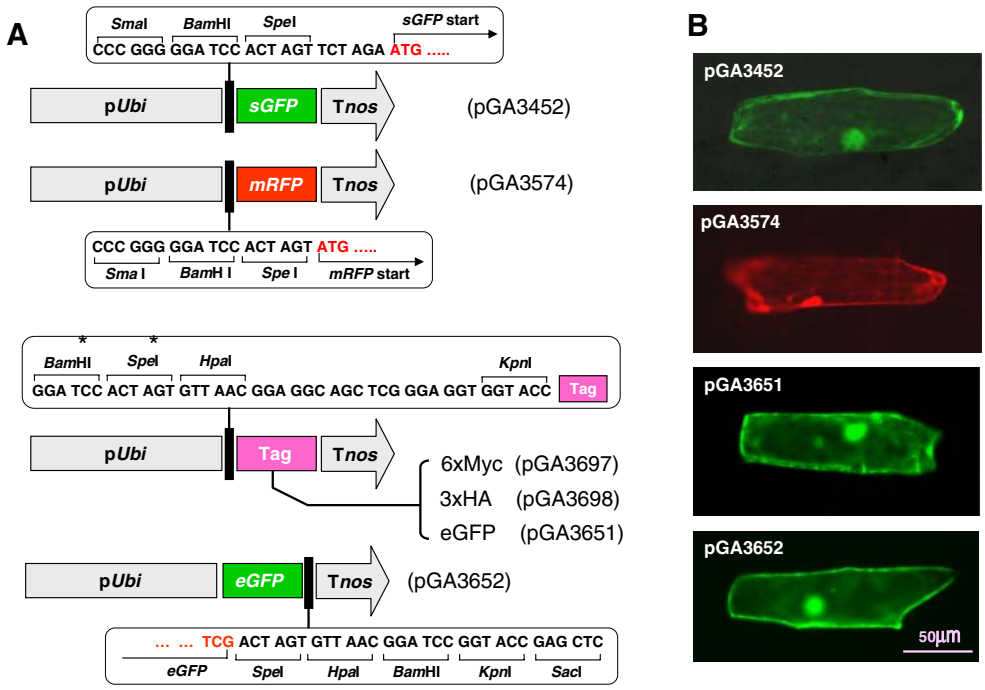

Fig. 4 Vectors for transient expression. **A** Vector maps. Unique sites for cloning are depicted in *boxes*. **Bam*HI and *Spe*I sites are not unique in pGA3697. **B** Detection of GFP and RFP after bombardment of pGA3452, pGA3574, pGA3651, and pGA3652 vector DNAs into onion epidermis. p*Ubi* maize *Ubiquitin-1*promoter containing the promoter, first exon, and first intron; T*nos nos* terminator; sGFP synthetic green fluorescent protein; *eGFP* enhanced green fluorescent protein (Clontech, USA); *mRFP* monomeric red fluorescent protein [5]; *HA* HA tag; *Myc* Myc tag

Binary vector pGA3777 was constructed for expressing two genes simultaneously. It has two multiple cloning sites under independent control by the *Ubi* promoter (Fig. 1b).

The pGA3626 vector was derived from binary vector pC35SB-gfp by replacing the *35S* promoter with the *Ubi* promoter and by adding multiple cloning sites (*Hin*dIII, *Avr*II, *Bam*HI, *Sma*I, and *Sac*I) between the *Ubi* promoter and *nos* terminator. Because this vector has the *bar* gene as a selectable marker, it is useful when one wants to express a gene in a plant that already contains a hygromycin resistance gene. It also carries p*35S-GFP*::*GUS*-T*nos*, which is valuable for detecting early-stage transformants and transgenic gametophytes. Unlike pGA1161 and its derivatives that have a tetracycline resistance gene for bacterial selection, pGA3626 carries a kanamycin resistance gene on the vector backbone as a bacterial selectable marker (Fig. 1c and Table 1).

The gateway recombination system and multistep ligation methods are generally used for RNAi vector construction [21, 16, 23]. Here, we introduced an alternative RNAi construction technique that involved a one-step ligation method (Fig. 1d). Target DNA was amplified with a primer containing GG*GGTACC* at one end. This amplified DNA was cut with *Kpn*I and inserted into the *Kpn*I-digested pGA3426 along with a linker DNA. For easy identification of the RNAi construct, we used the ampicillin resistance gene as a linker, which was obtained from pGA3720 by *Sma*I. The pGA3720 vector was constructed by inserting the ampicillin resistance gene, containing *Sma*I sites at both ends, into a subcloning vector, pGA3591.

Binary Vectors for Protein Tags

For analysis of the subcellular localization of target proteins, protein interactions via co-immunoprecipitation, and protein purification from transformants, we developed four binary vectors (pGA3427, pGA3428, pGA3438, and pGA3429) from pGA3426. These vectors generated a fusion protein between the target protein and tag: sGFP, HA, Myc, or His (Fig. 2a). We verified the molecules by

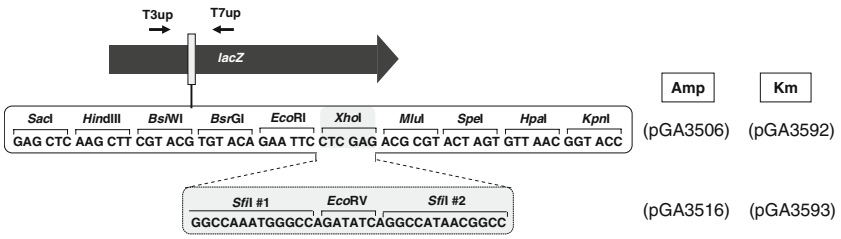

Fig. 5 Vectors for subcloning of rice full-length cDNA. Recombinant clones were selected by blue/white screening. Restriction enzyme sites in *boxes* are unique in each vector. T3up (5-ACAGGAAACAGC TATGACCA-3) and T7up (5-TCCCAGTCACGACGTTGTA-3) were used for sequencing primers

inserting a *GUS* gene in the vectors. Transient expression of the reporter gene was detected in etiolated leaves (Fig. 2b).

Binary Vector for Promoter Study with GUS

Analysis of spatial and temporal gene expression is essential for understanding gene functioning. We constructed binary vector pGA3383, which carries a promoterless *GUS* reporter gene. The *GUS* coding sequence together with the *nos* terminator region was amplified with a forward primer containing restriction enzyme sites, and the polymerase chain reaction product was cloned into pBluescript SK(−) (GenBank Accession No. X52324). Finally, the cloned fragment was transferred to binary vector pGA1605 [15]. This resulted in multiple cloning sites containing *Bam*HI, *Xba*I, *Hpa*I, *Kpn*I, *Xho*I, and *Spe*I that were located immediately upstream of the ATG start codon (Fig. 3a). Therefore, insertion of a promoter fragment at a multiple cloning site would activate expression of the reporter gene. We examined the vector by inserting a promoter fragment of *OsActin1*. The molecule was delivered into mesophyll cells of etiolated seedlings, and transient expression of the reporter gene was measured by GUS assay. Figure 3b shows that the introduced molecule rendered *GUS* expression, demonstrating that the vector was working properly.

Vectors for Transient Expression

For analyses of subcellular localization of target proteins, we constructed several vectors that can generate a fusion molecule between a target protein and sGFP (pGA3452), eGFP (pGA3651 and pGA3652), or mRFP (pGA3574; Fig. 4a). These vectors are high-copy plasmids that could be delivered to cells through bombardment or to mesophyll cells by PEG. Here, the reporter proteins were localized in the cytosol and nucleus when they were delivered to onion epidermal cells (Fig. 4b). The pGA3697 and pGA3698 vectors could be used for generating a fusion protein between a target and Myc or HA, respectively, for immunoprecipation experiments.

pGA3651, pGA3697, and pGA3698 harboring C-terminal tag fusions and pGA3652 having an N-terminal eGFP fusion were constructed by inserting the DNA fragment carrying p*Ubi*-MCS-tag-T*nos* into the pUC18 plasmid. Vectors pGA3452 and pGA3574 were constructed from the 326-GFP vector by replacing p*35S*-MCS-mGFP with p*Ubi*-MCS-sGFP or p*Ubi*-MCS-mRFP, respectively.

Vectors for Subcloning of Rice Full-Length cDNA

The Rice Full-Length cDNA Project has generated approximately 30,000 cDNA clones [19]. These are available through the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/index.html.en). The full-length clones are located in three plasmid vectors: pME18SFL-3 (Clone IDs: 001- and 006-), pCMV-SPORT6 (Clone ID: 002-), and Lambda-FLC (Clone ID: J0-). Full-length cDNA can be isolated by *Xho*I from pME18SFL-3 and pCMV-SPORT, and by *Sfi*I from Lambda-FLC. To facilitate the cloning of cDNA into binary expression vectors, we developed subcloning vectors by inserting a synthetic DNA fragment carrying *Xho*I (pGA3506), or *Sfi*I and *Eco*RV (pGA3616) into pBluescript SK(−) (Fig. 5). After subcloning the full-length cDNA into these vectors, the restriction enzyme site located in the MCS could be used for cloning the cDNA into binary expression vectors, e.g., pGA3426, pGA3436, and pGA3626.

The pGA3592 and pGA3593 vectors contained the same MCS as pGA3506 and pGA3516, respectively, but carried a kanamycin resistance gene as a selectable marker. These vectors are useful for direct cloning of cDNA without purification of an insert. Because the full-length cDNA is present in the vectors that carried the ampicillin resistant gene, direct cloning was difficult with pGA3506 and pGA3516. Vectors pGA3592 and pGA3593 were constructed by replacing the ampicillin resistance gene in pGA3506 and pGA3516, respectively, with the kanamycin resistance gene by *Bsp*HI.

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