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Binary cloning vectors for efficient genetic transformation of rice

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The availability of effective vector systems is a prerequisite for genetic manipulation of plants through recombinant DNA technology. We report here construction of a series of binary vectors that have cauliflower mosaic virus 35S promoter-driven genes encoding either resistance to hygromycin or phosphinothricin for selection of the transformants, and high strength constitutive promoters of either *ubiquitin1* or *actin1* genes for efficient expression of the transgenes. The efficacy of the constructs is tested in stably transformed Pusa Basmati 1 rice plants through *β-glucuronidase* reporter gene activity. Availability of vectors with variable promoters and selectable marker genes provides flexibility in stacking two genes. The vectors constructed in this study are suitable for both particle gun and *Agrobacterium*-based transformation protocols.

RICE is the most important food crop. Of late, rice has emerged as a model crop for the analysis of genome and proteome of cereals[1,2]. There is a great deal of progress in stable genetic transformation of rice plants. Compared to conventional breeding, genetic engineering is a relatively faster means for varietal improvement that allows transfer of genes from within as well as outside the primary gene pool[3]. Genetic transformation of rice through use of *Agrobacterium* is a favoured approach as it enables transfer of DNA with defined ends, minimal rearrangement, integration of a small number of copies of the gene and more importantly, the possibility that even large segments of DNA can be efficiently transferred[4–8].

Success achieved in genetic improvement of most of the agronomic characteristics in rice (as well as in other cereals and dicot plants) is limited. Despite the massive support given to rice biotechnology programme in recent years, there is not much success in improving resistance against biotic and abiotic stresses that cause significant damage to rice yield[3,9]. An important reason for this failure is that mostly the stress resistance-related traits are mediated by a number of biochemical reactions/physiological processes, and methods for co-integration of multiple transgenes into the plant genome are not well established.

Earlier studies have shown that *Agrobacterium*-mediated gene pyramiding can possibly be brought about by either sequential transformation or co-transformation with multiple T-DNAs[10–12]. The multiple T-DNAs can be delivered to plant cells either from a mixture of strains (mixture method) or from a single strain which contains more than one T-DNA (single-strain method)[10]. However, the important parameters that must be noted while designing a co-transformation experiment are that (a) there should be different promoters available to drive different transgenes in the same host, so as to avoid homology-based recombination and gene silencing[13], and (b) there should be different selectable markers available to select each transgene separately. We have used two different promoters obtained from *ubiquitin1* and *actin1*

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genes and two different selectable gene markers, namely *hygromycin phosphotransferase* (*hph/hyg*^{r}) which provides resistance to hygromycin and *phosphinothricin acetyltransferase* (*pat/bar*) which confers resistance against phosphinothricin (an active ingredient in the herbicide 'Basta') to develop binary vector systems for genetic transformation of rice. We have tested the efficacy of both the vectors with respect to their promoter activity by subcloning *β-glucuronidase* (*gus*) gene in the multiple cloning site (MCS) followed by stable genetic transformation of an indica rice Pusa Basmati 1.

All *in vitro* DNA manipulations and clonings were carried out using the standard protocols[14]. The plasmids used in this study included pCAMBIA1201, pCAMBIA1300 and pCAMBIA3300 (ref. 15), pActcas (constructed at CSIRO Division of Plant Industry, Canberra, Australia using *Act1*-based vectors)[16] and pLZUbicas[17,18]. The *E. coli* strain XL1BlueMRF' was used for cloning experiments and *Agrobacterium tumefaciens* strain EHA105 was employed to transform rice. The competent XL1BlueMRF' *E. coli* cells were prepared as described by Mandel and Higa[19]. *Agrobacterium* competent cells were prepared and transformation was carried out by the freeze–thaw method[20]. The genetic transformation of rice was carried out according to the protocols of Hiei *et al.*[6] with some modifications[3,18,21]. Transformed calli were selected on hygromycin (30 mg/l) and subsequently subcultured for four weeks. The GUS histochemical staining of callus and leaf segments was performed at 37°C as described by Jefferson[22].

The aim of this study was to construct binary vectors with different promoters and selectable marker genes for efficient rice transformation. The following clonings were carried out in this study:

1. *Eco*RI–*Hin*dIII-cut 2.3 kb fragment from pLZUbicas containing *ubi1* promoter (along with *ubi1* first exon and first intron), a MCS and *nos* gene terminator was ligated with *Eco*RI–*Hin*dIII-cut pCAMBIA1300, resulting in the construction of a vector with *ubi1* promoter–MCS–*nos* terminator and CaMV35S promoter–*hyg*^{r}–*polyA* terminator. The recombinant clones were checked using *Eco*RI, *Hin*dIII, *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Sal*I and *Xho*I. This vector is referred to as pUH.
2. *Eco*RI–*Hin*dIII-cut 2.3 kb fragment from pLZUbicas containing *ubi1* promoter (along with *ubi1* first exon and first intron), MCS and *nos* gene terminator was ligated with *Eco*RI–*Hin*dIII-cut pCAMBIA3300, resulting in the construction of a vector with *ubi1* promoter–MCS–*nos* terminator and CaMV35S promoter–*bar*–*polyA* terminator. The recombinant clones were checked using *Eco*RI, *Hin*dIII, *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Sal*I and *Pst*I. This vector is referred to as pUB.
3. *Afl*II–*Nco*I-cut 2.1 kb fragment from pCAMBIA1201 was blunted using mung bean nuclease and subsequently ligated with *Sma*I-cut pUH, resulting in the construction of a vector with *ubi1* promoter–*gus*–*nos* terminator and CaMV35S promoter–*hyg*^{r}–*polyA* terminator. The recombinant clones were checked using *Eco*RI, *Hin*dIII, *Kpn*I, *Sac*I, *Sal*I, *Nco*I and *Pst*I. This vector is referred to as pUH/*gus*.
4. *Xho*I–*Cla*I-cut 1.6 kb fragment from pActcas containing *act1* promoter (along with *act1* first exon and first intron), MCS and *nos* gene terminator was treated with T_4 DNA polymerase and subsequently ligated to blunted *Eco*RI–*Hin*dIII-cut pCAMBIA1300, resulting in the construction of vectors with *act1* promoter–MCS–*nos*

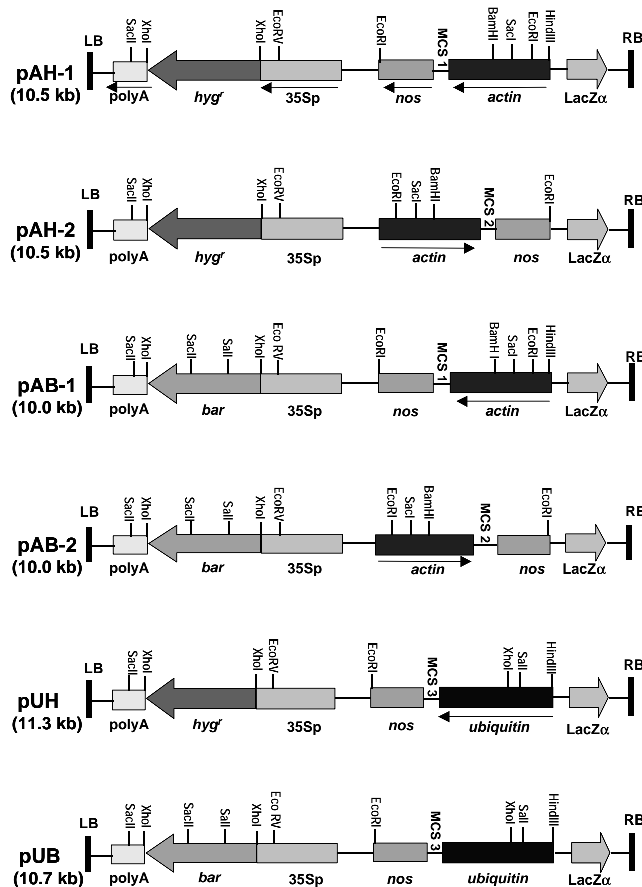

Figure 1. Diagrammatic representation of the T-DNA region of binary vectors constructed. Restriction sites have been indicated and arrow under the DNA region indicates orientation of the promoters used in the constructs. Location of the individual MCS regions is shown. Restriction sites in each MCS are shown in order: MCS1, *Sac*I, ***Kpn*I**, ***Sma*I**, *Bam*HI, ***Xba*I**, ***Sal*I**, ***Pst*I**, *Eco*RI, *Eco*RV and *Hin*dIII; MCS2, *Hin*dIII, *Eco*RV, *Eco*RI, ***Pst*I**, ***Sal*I**, ***Xba*I**, *Bam*HI, ***Sma*I**, ***Kpn*I** and *Sac*I; MCS3, *Pst*I, *Sal*I, *Xba*I, ***Bam*HI**, ***Sma*I**, ***Kpn*I** and ***Sac*I**. Restriction sites indicated in bold represent unique sites; *actin1*, rice *actin1* gene promoter with intron; *bar*, gene for *phosphinothricin acetyltransferase*; *hyg*^{r}, gene for *hygromycin phosphotransferase;* lacZα, lacZα fragment; LB, left border of T-DNA; *nos*, terminator of *nopaline synthase* gene; poly A, poly A signal of 35S cauliflower mosaic virus; RB, right border of T-DNA; 35Sp, 35S promoter of cauliflower mosaic virus; *ubiquitin1*, maize *ubiquitin1* promoter with intron. For complete nucleotide sequence of different DNA segments used in the construction of these vectors, readers may refer to McElroy *et al.*[15] (pCAMBIA vectors), Depicker *et al.*[23] (*nos*), McElroy *et al.*[24] (*actin1* promoter) and Christensen *et al.*[25] (*ubiquitin1* promoter).

terminator and CaMV35S promoter–*hyg*^{r}–*polyA* terminator. The recombinant clones were checked using *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, *Xho*I, *Eco*RV and *Sac*II–*Xma*I. These vectors were referred to as pAH-1 and pAH-2 (depending upon the orientation of the *actin* promoter cassette).

5. *Xho*I–*Cla*I-cut 1.6 kb fragment from pActcas containing *act1* promoter (along with *act1* first exon and first intron), MCS and *nos* gene terminator was treated with T_4 DNA polymerase and subsequently ligated to blunted *Eco*RI–*Hin*dIII-cut pCAMBIA3300, resulting in construction of vectors with *act1* promoter–MCS–*nos* terminator and CaMV35S promoter–*bar*–*polyA* terminator. The recombinant clones were checked using *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, *Xho*I and *Eco*RV. These vectors were referred to as pAB-1 and pAB-2 (depending upon the orientation of the *actin* promoter cassette).

6. *Afl*II–*Nco*I-cut 2.1 kb fragment from pCAMBIA1201 was blunted using T_4 DNA polymerase and subsequently ligated with *Sma*I-cut pAH-1, resulting in the construction of a vector with *act1* promoter–*gus*–*nos* terminator and CaMV35S promoter–*hyg*^{r}–*polyA* terminator. The recombinant clones were checked using *Eco*RV and *Xho*I. This vector is referred to as pAH-1/*gus*.

The restriction maps and other details of the above vectors are provided in Figure 1 (refs 15, 23–25). The pUH, pAH-1 and pAH-2 plasmids have the *hyg*^{r} gene of pCAMBIA1300 for selection, whereas pUB, pAB-1 and pAB-2 have the *bar* gene from pCAMBIA3300 for selection. Both *hyg*^{r} (ref. 26) and *bar*[27] selectable markers are routinely employed for rice transformation. Both these genes are under the control of CaMV35S promoter which has been shown to work reasonably well with the rice system[28]. The maize *ubiquitin1* and the rice *actin1* gene promoters have a high strength of expression and are often used for rice transformation[16,17,29]. Besides the promoter and selectable markers, the other useful feature of these vectors is the availability of several unique restriction sites in the MCS for cloning purpose. The presence of unique *Sma*I site in both the vectors provides flexibility in cloning any gene fragment after blunting through suitable enzymatic reaction. As the foundation vectors used here belong to pCAMBIA series, the cloning vectors designed in this study inherit several useful features. These include kanamycin-based selection in bacteria, high copy number plasmid replication, Kozak sequences for improved expression, minimal extraneous DNA sequences and stability of plasmids under non-selection conditions[15]. Importantly, we have the *act1* promoter–MCS–*nos* terminator and CaMV35S promoter–selection marker–*polyA* terminator configurations in same (pAH-1, pAB-1) and different (pAH-2, pAB-2) directions in our vectors. This should enable cloning of the transgene in either way to avoid the possible influence of one promoter on the activity of the other promoter in the vector cassette.

To further check the efficiency of the vectors designed, we subcloned *gus* gene in the MCS of both pUH and pAH-1 to obtain pUH/*gus* and pAH-1/*gus* (Figure 2 *a*). These constructs were employed to transform mature

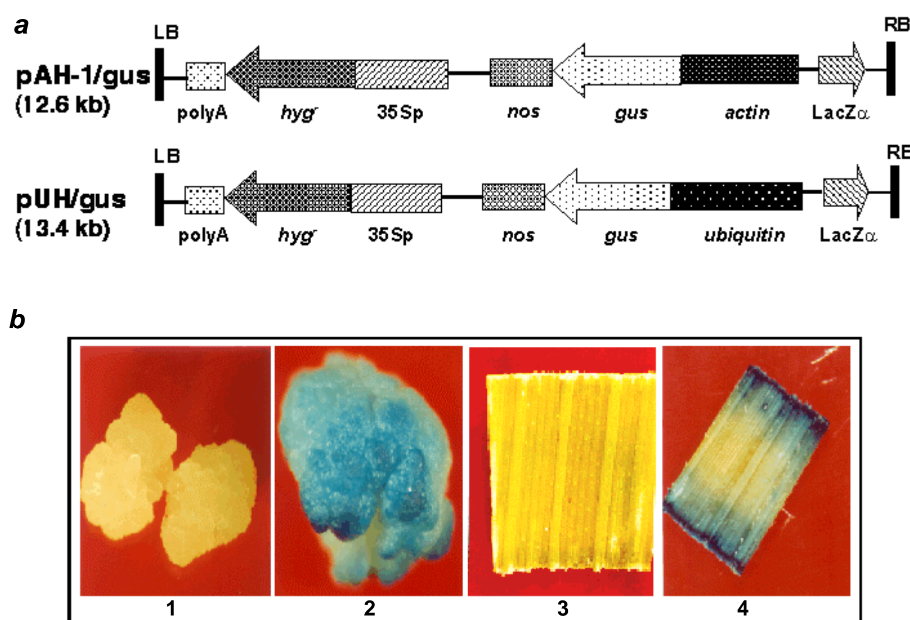

Figure 2. *a*, Diagrammatic representation of binary vectors containing *gus* as a reporter gene used for rice transformation. *gus*, gene for *β-glucuronidase* with a catalase intron. *b*, Histochemical staining for GUS expression in (1), control callus; (2), callus transformed with pUH/*gus*; (3), leaf segment of control plant; (4), leaf segment of transgenic plant transformed with pUH/*gus*.

seed-derived calli of Pusa Basmati 1 rice cultivar. Further, subculturing of the transformed calli on selection medium resulted in healthy, vigorously proliferating white daughter calli, indicating that they possess cells with hygromycin resistance gene. These hygromycin-selected calli showed positive reaction for *gus* expression when histochemically stained, while untransformed calli did not show the reaction (Figure 2 *b*). The calli transformed with pUH/*gus* showed blue colour within a minute, while pAH-1/*gus* showed GUS reaction after nearly 2 h, indicating that the *ubiquitin1* promoter more strongly regulates the expression than the *actin1* promoter. Similar observations have also been reported earlier[17]. Moreover when leaf segments from transgenic rice plants (pUH/*gus*) were tested for GUS expression, intense blue colour was noted indicating stable integration of the transgene (Figure 2 *b*). Subsequent to this work, we have subcloned several agronomically-useful genes in pUH and have noted the expression of the transgenes in stably-transformed rice lines[30].

The advantage of having vectors with two different selectable markers and two different promoters in different orientations as achieved in this study provides flexibility in gene-stacking experiments. We will further aim at bringing in additional promoters and selectable markers so that more than two genes can also be pyramided. While the present study is limited to rice, it is to be noted that *ubiquitin1* and *actin1* promoters are shown to work in several other monocot crops[31]. Likewise, there are several reports indicating that the resistance to hygromycin and phosphinothricin is effective in selection of transformed tissues, generally in monocots[32–36]. We believe that the vectors constructed in this study would have general applicability in genetic transformation of monocots.

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