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Ti-Plasmid Vectors Useful for Functional Analysis of Rice Genes

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Abstract

We developed new binary vectors, pPZP2H-lac and pPZP2Ha3, for the transformation of rice plants. These binary vectors contain multiple cloning sites and the hygromycin B phosphotransferase gene as a plant-selectable marker. After *Agrobacterium*-mediated transformation, transgenic plants containing a T-DNA could be tightly selected by hygromycin B. The vectors are powerful tools for the functional analysis of genes.

Agrobacterium-mediated transformation is the most widely used method for introducing foreign genes into rice and other plant species. Hiei *et al.* (1994) established an efficient transformation method in rice by using scutellum calli and a super-binary vector. Toki (1997) improved this method by shortening the period of tissue culture. However, it was sometimes difficult to use vectors to clone genomic regions defined by map-based analysis, because those candidate regions sometimes spanned more than 8 kb, and the number of restriction enzymes used for cloning is limited.

We report here the construction of 2 new Ti-plasmid binary vectors, pPZP2H-lac and pPZP2Ha3, which have a hygromycin B phosphotransferase (*HPT*) gene as a selection marker gene and many unique cloning sites. The cloned genomic DNA and cDNA can be directly introduced into plants by *Agrobacterium*-mediated transformation, and the transformants are selected by hygromycin B.

A 2.5-kb fragment containing the cauliflower mosaic virus (CaMV) 35S promoter, the *HPT* gene, and the terminator of the nopaline synthase (*NOS*) gene was cut from plasmid pLAN-101MHYG by *Sal*I and *Hin*dIII, and was blunt-ended. The fragment was ligated with plasmid pPZP200 (Hajdukiewicz *et al.*, 1994), which had been digested with *Eco*RI and then blunted for the construction of pPZP2H. pPZP2H-lac was constructed by insertion of the 0.7-kb *lac*Z gene fragment from pBluescript II SK(-) (Stratagene, La Jolla, CA, USA) into the filled-in *Ecl*136II (*Sac*I)-*Hin*dIII site of pPZP2H. The plasmid vector has 10 unique restriction enzyme sites within *lac*Z (**Fig. 1A**). A DNA fragment cloned in the vector could be amplified by PCR by using T3 and T7 primers, because the multiple cloning sites (MCS) of *lac*Z are flanked by T3 and T7 bacteriophage promoters (**Fig. 1A**).

pPZP2Ha1 was constructed by insertion of the CaMV 35S promoter, which is a 0.8-kb *Sma*I-*Bam*HI fragment from plasmid pSK-HYG, into the *Ecl*136II-*Bam*HI site of pPZP2H. pPZP2Ha2 was constructed by insertion of the polyadenyl site of the *NOS* gene, which is a 0.1-kb *Bam*HI-*Hin*dIII fragment from pSK-HYG, into the *Bam*HI-*Hin*dIII site of pPZP2Ha1. pPZP2Ha2 had been digested with *Hin*dIII, blunted, and self-ligated for the construction of pPZP2Ha2A. pPZP2Ha3(+) and pPZP2Ha3(-) were constructed by insertion of the MCS into the *Bam*HI site of pPZP2Ha2A (**Fig. 1B**). The MCS were constructed by combining the MCS from *Bam*HI to *Sac*I of LITMUS28 (New England Biolabs, Beverly, MA, USA), the MCS from *Sac*I to *Xho*I of pCITE4a(+) (Novagen, Madison, WI, USA), and the MCS from *Xho*I to *Bgl*II of LITMUS29 (New England Biolabs). Thus, the MCS of pPZP2Ha3 contained 6 unique restriction sites. The 2 *Hin*dIII sites in the MCS can be also used for cloning. As pPZP2Ha3(+) and (-) contain the MCS in opposite directions to each other, a gene can be inserted into the vectors in both orientations. A cloned gene can be expressed under the regulation of the CaMV 35S promoter in higher plants.

Hygromycin is an effective selection marker because untransformed rice plants barely tolerate it. *HPT*, which confers resistance to hygromycin B in transformed rice plants (Shimamoto *et al.*, 1989), was inserted into the vector as a selection marker gene. The gene lies between the left border and the MCS in the T-DNA region (**Fig. 1A**). Because the

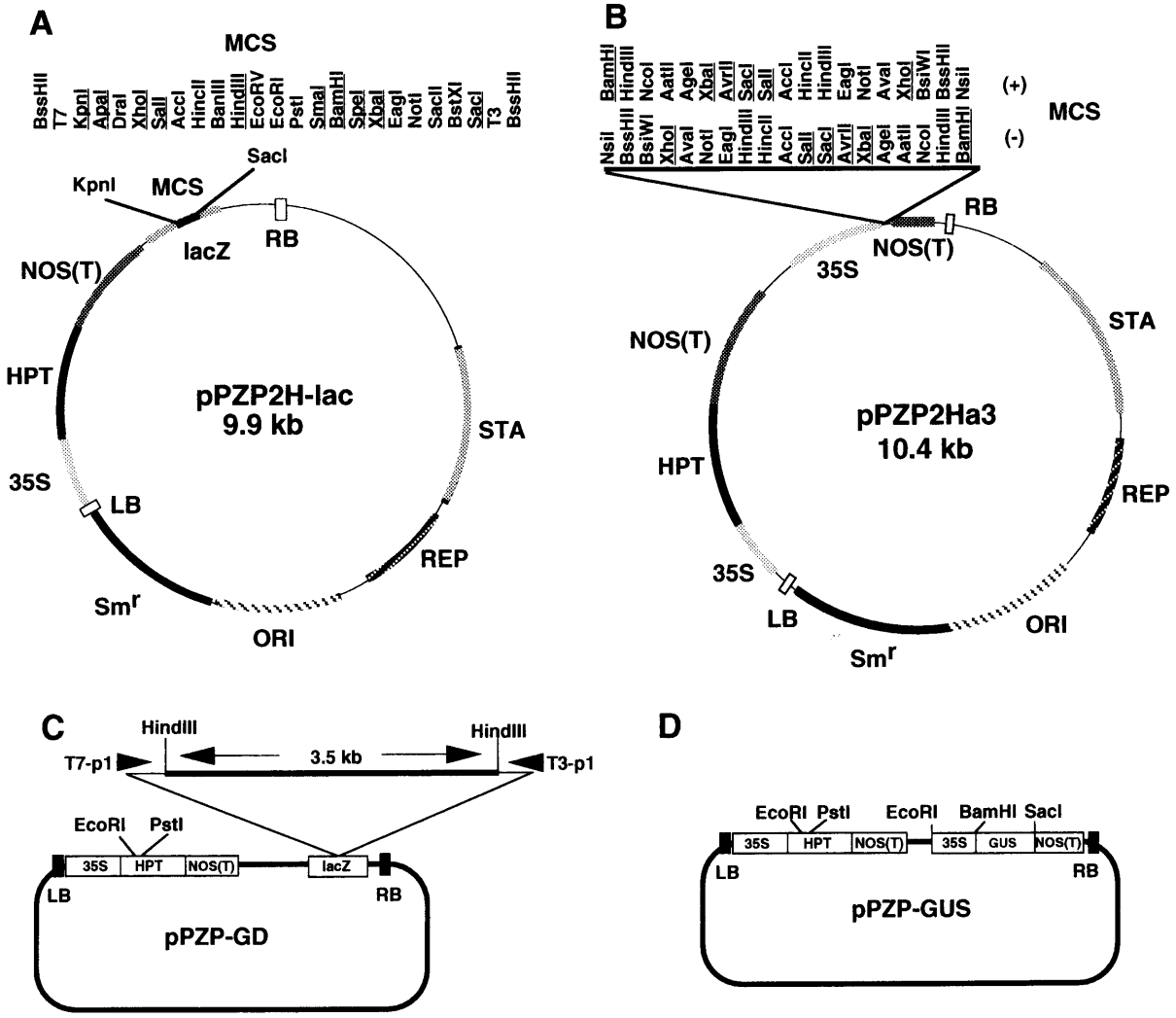

Fig. 1 Structure of pPZP2H-lac (A), pPZP2Ha3 (B), pPZP-GD (C), and pPZP-GUS (D). The unique cloning sites in the multiple cloning sites (MCS) of pPZP2H-lac and pPZP2Ha3 are underlined. 35S, cauliflower mosaic virus 35S promoter; NOS(T), the terminator from the *NOS* gene; LB, left T-DNA border; RB, right T-DNA border; HPT, hygromycin phosphotransferase gene; GUS, β-glucuronidase gene; ORI, the ColE1 replication origin from plasmid pBR322; REP, a replication region of plasmid pVS1; STA, stability region of plasmid pVS1; Sm^r, a bacterial streptomycin-resistance gene; lacZ; the *lacZ* gene from pBluescript II SK(-) (Stratagene); T3, T3 promoter; T7, T7 promoter.

right border is the starting point for T-DNA transfer (Hooykaas and Schilperoort, 1992), the vector system reduces the number of non-transformants in selection with hygromycin.

To verify the capability of pPZP2H-lac to transfer genes, we constructed pPZP-GD by inserting a 3.5-kb fragment of anonymous rice genomic DNA digested with *Hin*dIII into the *Hin*dIII site of pPZP2H-lac (**Fig. 1C**), and introduced it into *Agrobacterium tumefaciens* EHA101 by freeze-thaw transformation (Holsters *et al.*, 1978). The transformants were selected by plating on AB plates (Chilton *et al.*, 1974) containing 50 mg l^{-1} streptomycin. *Oryza sativa* cv. Nipponbare was transformed by *A. tumefaciens* according to the method of Toki (1997), with some modifications as follows. To induce root formation, the rice plants were cultured in a medium devised by Yoshida *et al.* (1976) instead of MS medium (Murashige and Skoog, 1962). The selected transgenic calli and regenerated plants were cultured on an agar medium containing 20 mg l^{-1} hygromycin B. Plants with roots were transferred to soil in pots and grown in a greenhouse at 25 ℃ . To detect the integrated DNA fragments, we amplified the DNA of 10 regenerated plants by PCR, then fractionated it on 0.7% agarose gel (**Fig. 2**). A band of about 3.6 kb, which included the 3.5-kb fragment of anonymous DNA, was detected in all regenerated plants (**Fig. 2A**). No DNA fragment was amplified in any untransformed control Nipponbare plant. A band of about 0.7 kb derived from the gene for the small subunit of

Table 1. Transmission of the hygromycin resistant gene to progeny.

	Total no. of seeds tested	Number of R_1 seedlings	
		Hygromycin resistant	Hygromycin sensitive
3.5kb genomic DNA			
Nipponbare	20	-	20
GD-3	32	23	9
GD-6	30	21	9
GD-7	31	22	9
GUS			
Nipponbare	20	-	20
GUS-5	30	24	6
GUS-7	30	22	8
GUS-8	20	9	11

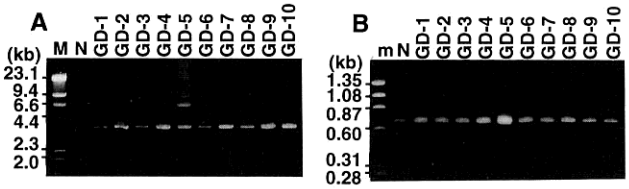

Fig. 2 DNA analysis of transformants containing the 3.5-kb fragment of anonymous DNA. Genomic DNA was prepared from transgenic and wild-type leaves according to the methods of Rogers and Bendich (1988). M, lambda DNA/*Hin*dIII marker; m, ϕX174/*Hae*III marker; N, Nipponbare; GD-1-10, regenerated plants.

(A) The PCR reaction mixture contained 250 ng total DNA from wild-type or transgenic plants, 12.5 μl 2× TaKaRa GC buffer II, 2 units of TaKaRa LA *Taq* polymerase (Takara Shuzou Co. Ltd., Otsu, Japan), and 10 pmol of each primer, in a final volume of 25 μl. The primers used were the T3-promoter-specific primer T3-p1 (5'-GCGCGCAATTAACCCTCACTAAAGGGAACA-3') and the T7-promoter-specific primer T7-p1 (5'-CGTAATACGACTCACTATAGGGCGAATTGG-3') (**Fig. 1C**). The cycle conditions were 20 s at 98 ℃, 7 min at 68 ℃, for 35 cycles. PCR products were separated on 0.7% agarose gel by electrophoresis.

(B) The PCR reaction mixture contained 250 ng total DNA from wild-type or transgenic plants, 2.5 μl 10× TaKaRa *Ex Taq* amplification buffer, 1 unit of TaKaRa *Ex Taq* polymerase (Takara Shuzou), and 10 pmol of each primer, in a final volume of 25 μl. The primers used were specific to the gene for the small subunit of rice ribulose 6-phosphate carboxylase: rbcS-P1 (5'-ACTATCCTTCGCAAGACCCT-3') and rbcS-P2 (5'-GGGTTTCTACAGGACGTAAT-3'). The cycle conditions were 30 s at 98 ℃, 30 s at 62 ℃, 30 s at 72 ℃, for 30 cycles. PCR products were separated on 1.5% agarose gel by electrophoresis.

ribulose 6-phosphate carboxylase was detected in all transgenic and control plants (**Fig. 2B**).

The progeny of 3 transformants selected from the 10 regenerated plants were examined for resistance to hygromycin (**Table 1**). The R_1 progeny of plants GD-3, GD-6, and GD-7 showed a segregation pattern of 3:1 for hygromycin resistance. The 3.5-kb fragment was detected in all hygromycin-resistant seedlings (data not shown). These results indicate that the T-DNA of the vector can be stably integrated into the rice genome by *Agrobacterium*-mediated transformation.

The pPZP2H-lac vector has been used for the functional complementation of candidate genomic regions that were defined by a map-based strategy (Yano *et al.*, 2000; Y. Takahashi and M. Yano, unpublished data). In both cases, relatively large genomic fragments-7.1 and 8.8 kb-were stably transferred and inherited as single Mendelian factors. The pPZP2H-lac vector could facilitate the isolation of genes from rice by map-based cloning.

To examine whether the pPZP2Ha3 plasmid vectors could be used as expression vectors in rice, we constructed pPZP-GUS by inserting a 1.9-kb *Bam*HI-*Sac*I fragment containing the β-glucuronidase (*GUS*) gene derived from plasmid pBI221 (Clontech, Palo Alto, CA, USA), into the *Bam*HI-*Sac*I site of pPZP2Ha3(+) (**Fig. 1D**). The vector was introduced into *A. tumefaciens* and the rice was transformed as described above. Twenty regenerated plants were analyzed for GUS activity by the 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc) histochemical staining method (Xu *et al.*, 1996). GUS activity was detected in 15 regenerated plants (data not shown). The progeny of 3 transformants selected from the 15 plants were examined for resistance to hygromycin (**Table 1**). The R_1 progeny of plants GUS-5 and GUS-7 showed a segregation pattern of 3:1 for hygromycin re-

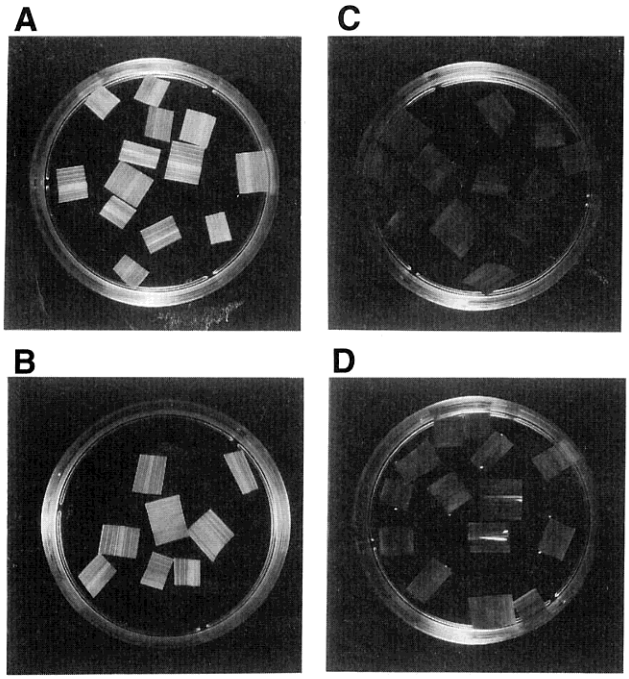

Fig. 3. Histochemical GUS staining of leaves of transgenic plants. (A) Wild-type plants. (B) pPZP2Ha3 R_0 plants. (C) The GUS-7 R_0 plant. (D) GUS-7 R_1 plants. The leaves were incubated for 40 h at 37 ℃.

sistance. GUS activity was detected in all hygromycin-resistant plants (data not shown). GUS expression in plant GUS-7 was examined by X-gluc histochemical staining (**Fig. 3**). It was found in leaves of both the R_0 and R_1 plants of GUS-7 (**Fig. 3C, D**). Leaf sections from untransformed control plants and pPZP2Ha3 R_0 plants displayed no staining (**Fig. 3A, B**). These results indicate that a foreign gene in the vector can be expressed in and be stably inherited by progeny. In our laboratory we have used the pPZP2Ha3 vectors to analyze functions of the *Hd1* gene. The pPZP2Ha3 vectors will be available for analyzing gene functions.

Acknowledgments

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