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Full Length Research Paper

A fresh method of DNA transformation to the seeds irradiated by ^{60}Co without the use of antibiotic selection

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To find out a simpler method that can directly transfer the aim gene into plant genomes, the purple medic seeds irradiated by ^{60}Co with 0.375 Gy were transformed by linear DNA containing a β-glucuronidase (GUS) gene (as an aim gene), a betaine aldehyde dehydrogenase (BADH) gene (as a selectable marker) and two pairs of both CaMV35S promoter and Nos terminator. Subsequently, the seeds were planted and grown in perlite media watered with NaCl solution as a kind of selective compound. The results showed that, positive frequency of PCR identification by the GUS gene or the BADH gene was higher than 53.2 and 89.5% in T_0 and T_1 generations, while GUS staining rate was higher than 50%; whereas five T_1 plants assayed by southern hybridization all showed positive reaction. In conclusion, by this method, transgenic plants may be easily obtained with the antibiotic markers for free; moreover, the plant regeneration-system must not be erected by directly transforming the seeds.

Key words: DNA transformation, irradiated seeds, purple medic, salt screening.

INTRODUCTION

There are two main problems of transgenic technology on many plant species. Firstly, the transgenic plants with some selectable markers of antibiotic would face potential risks on the human health and environment (Kai et al., 2002). Secondly, because of plant regeneration system, the technology was complex and limited for some plant species. Now, some methods partly solved the first problem: that is, using co-transformations (Komari et al., 1996) or inserting some transposons in the plant genomes (Hohn et al., 2001; Ebinuma et al., 1997) might be used to obtain some transgenic plants without the selective markers of antibiotics in their offspring. The selectable marker genes might be eliminated by the method of site-specific recombination during their transformation, in which the systems contained Cre/loxP (Gleave et al., 1999), FLP/FRTs (Cregg and Madden, 1989) and R/Rs (Onouchi et al., 1995). Many new selectable markers without the use of antibiotic were used as their transgenic systems, such as the BADH gene (Daniell et al., 2001), pmi gene (Joerbo et al., 1999), xylA gene (Haldrup et al., 1998) and GFP gene which might be partly substituted for the selectable marker of antibiotics (Cubitt et al., 1995). However, these methods were not widely used owing to many reasons, so the aim of this paper was to solve the second problem by finding out a new method.

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MATERIALS AND METHODS

Reagents

The reagents of PCR and southern hybridization were from Sigma or Shanghai Sangon Biological Engineering Technology and Sciences Co., Ltd., while other reagents were from Tianjin Chemical Reagent Co., Ltd.

Origin of the linear DNA transformed into seeds

The linear DNA transformed into seeds were from PCR amplification products of cricoid plasmids (using the prime pairs: P_1 and P_2) (Figure 1).

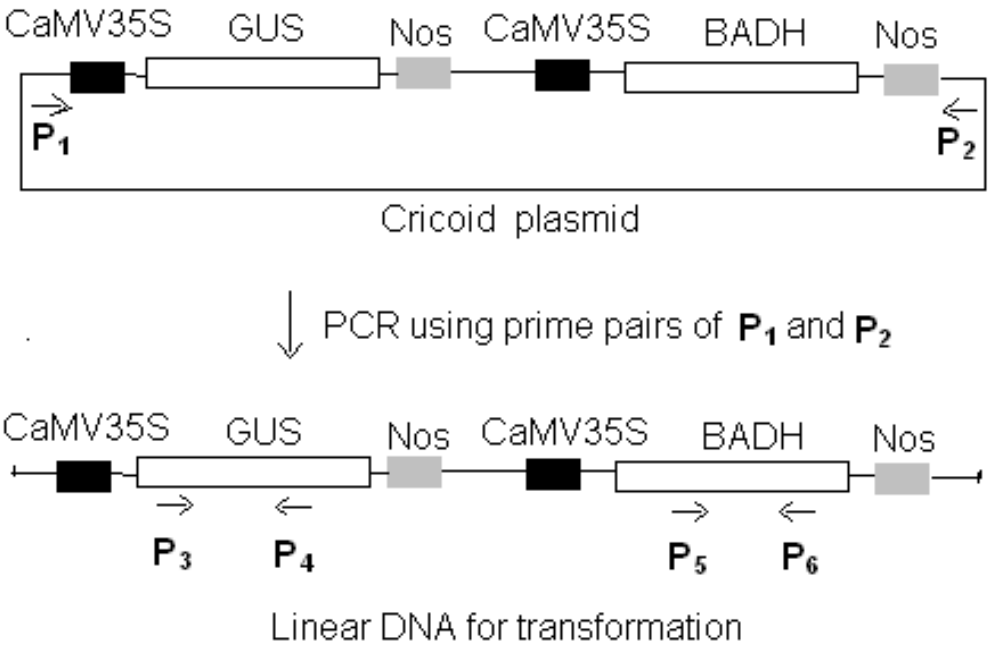

Figure 1. Origin of the linear DNA transformed into seeds. The linear DNA were from the PCR amplification products, in which the templates were the cricoid plasmids, and the primers were the up-stream primer P_1 (its sequence: 5'-GTT GGC GGT AAC AAG AAA GGG AT-3') and down-stream primer P_2 (its sequence: 5'-TTT TCG CGA TCC AGA CTG AA-3'), respectively. There was a pair of CaMV35S promoter (5' up-stream) and Nos terminator (3' down-stream) in a GUS or BADH gene (from GenBank accession No.AY156694), respectively.
The primer sequences of the PCR assay on both GUS and BADH genes are shown as follows. The up-stream primer (P_3) of the GUS gene was 5'-CTC ATT ACG GCA AAG TGT GGG TC-3' and its down-stream primer (P_4) was 5'-ACC ACG ATG CCA TGT TCA TCT GC-3'; whereas the BADH gene's up-stream primer (P_5) was 5'-GTT GAC AAG ATT GCC TTT AC-3' and its down-stream primer (P_6) was 5'-CTT AAC AAA AAC AAC ACC GT-3'.

Preparation of the selective solutions of NaCl

The selective solutions containing 2, 3, 4, 5, 6, 7 and 8 gl^{-1} NaCl were prepared by adding the corresponding NaCl into ½ strength MS media (Murashige and Skoog, 1962), respectively.

Radiating seeds by ^{60}Co

Purple medic seeds were irradiated by ^{60}Co at 0.125 Gy h^{-1} for 3 h, and the dose was 0.375 Gy.

DNA transformation to the seeds

All the experiments were performed three times. About 600 irradiated or non-irradiated seeds were dipped in the solutions containing the DNA fragments (1 μg μl^{-1}) from PCR amplification (Figure 1) for 12 h at about 25°C respectively. Subsequently, the seeds were transplanted into some porous pots with the perlite (granule diameters of about 3 mm). Lastly, they were watered in turn with the selectable solution of 3, 4, 5 and 6 gl^{-1} NaCl every one week.

PCR assay of both GUS and BADH genes

The genome DNA of the plants was extracted using about 1 g of leaves (Smith and Pring, 1987) for PCR. The primer design was based on the GUS and BADH genes' sequences (Figure 1). The positive CK were from PCR amplification products of the cricoid plasmids, which expressed the normal PCR work and the electrophoresis sites of the PCR products on the GUS and BADH genes, respectively (Figure 1), while the negative CK were from PCR amplification products of the genome DNA of the non-transformed plants, which showed the absence of the GUS or BADH genes.

Southern hybridization and GUS staining

The Southern hybridization method was followed as a molecular cloning in a laboratory manual by Joseph Sambrook and David Russell (the third edition). The positive CK was from PCR amplification products using the primer pairs of P_3-P_4 (GUS) or P_5-P_6 (BADH), while the negative CK was from the plants genomes of non-irradiated seeds. However, the PCR identification of all the five T_1 plants was positive by the GUS or BADH genes. Thus, the GUS chemical staining method followed that of Jefferson (1987).

Statistical analysis

Statistical analyses were performed using the two-tailed Mann Whitney nonparametric test, and a p-value of $p < 0.05$ was considered as statistically significant. All statistical analyses were conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL).

Table 1. The germination percentage of the seeds in the perlite media with the selective solutions of NaCl (%).

NaCl content (gl^{-1})	NINTS	NITS	INTS	ITS
0	93.8±6.4^{a}	\	95.4±4.0^{a}	\
2	94.1±5.4^{a}	\	96.6±2.8^{a}	\
3	2.3±0.1^{e}	4.0±0.3^{e}	65.3±5.2^{b}	\
4	0^{e}	0^{e}	22.6±7.3^{cd}	52.9±9.5^{bc}
5	0^{e}	0^{e}	1.6±0.3^{e}	27.0±3.6^{c}
6	0^{e}	0^{e}	0^{e}	14.5±2.9^{d}
7	0^{e}	0^{e}	0^{e}	0^{e}
8	0^{e}	0^{e}	0^{e}	0^{e}

All the experiments were performed thrice, and about 600 seeds were planted in the perlite media every time. The mean ± SD of their germination percentage was counted in a week. There was a significant difference between two different alphabets (a, b, c, d and e; $p < 0.05$). NINTS, non-irradiated and non-transformed seeds; NITS, non-irradiated and transformed seeds; INTS, irradiated and non-transformed seeds; ITS, irradiated and transformed seeds.

Table 2. The survival percentage of the transplant seedlings in the perlite media with the selective solutions of NaCl (%).

NaCl content (gl^{-1})	Seedlings origins at five-leaf phase	
	Non-irradiated seeds	Irradiated seeds
0	98.2±3.6^{a}	96.3±4.7^{a}
2	96.4±1.8^{a}	98.6±2.0^{a}
3	53.3±12.3^{c}	78.3±9.5^{b}
4	6.7±1.1^{e}	48.1±8.0^{c}
5	0^{e}	13.2±4.2^{de}
6	0^{e}	0.3±0.0^{e}
7	0^{e}	0^{e}
8	0^{e}	0^{e}

All the experiments were performed three times, and 50 seedlings were planted in the perlite media at five-leaf phase every time. The mean ± SD of their survival percentages was counted in three weeks. There was a significant difference between two different alphabets (a, b, c, d and e; $p<0.05$).

RESULTS

The seed germination's sensitivity to the selective solutions of NaCl

Four kinds of seeds (non-irradiated and non-transformed seeds, non-irradiated and transformed seeds, irradiated and non-transformed seeds and irradiated and transformed seeds) for the test design were planted in the perlite media with the selective solutions, respectively. Their germination percentages showed that the other seeds could not burgeon in the perlite media with above 3 gl^{-1} NaCl except for the irradiated and transformed seeds (Table 1).

The seedlings' sensitivity to the selective solutions of NaCl

The survival percentages of all the transplanted seedlings in the perlite media with the selective solutions are shown in Table 2. The results indicated that the transplanted seedlings from non-irradiated seeds could not survive in the perlite media containing above 4 gl^{-1} NaCl. Likewise, the seedlings from irradiated seeds could not survive in the perlite media with the selective solution containing above 5 gl^{-1} NaCl. Therefore, using above 5 gl^{-1} NaCl as the selective solution, the transgenic seedlings might be screened of purple medic.

PCR identification of T_0 and T_1 plants

PCR amplification results of T_0 and T_1 generation plants are shown in Table 3. It indicated that the positive frequency of PCR identification by the GUS or BADH gene was higher than 53.2 and 89.5% in T_0 and T_1, respectively.

Southern hybridization of T_1 plants

Five T_1 generation plants were determined by southern

Table 3. The PCR positive frequency of both BADH and GUS genes (%)‡.

Gene	NaCl content (gl^{-1})†	NINTS	NITS	INTS	ITS	
					T_0	T_1§
BADH	3	0 (0/10)	0 (0/10)	0 (0/10)	\	\
	4	\	\	0 (0/10)	\	\
	5	\	\	0 (0/3)	59.6 (28/47)	\
	6	\	\	\	70.8 (17/24)	90.8 (69/76)
GUS	3	0 (0/10)	0 (0/10)	0 (0/10)	\	\
	4	\	\	0 (0/10)	\	\
	5	\	\	0 (0/3)	55.3 (26/47)	\
	6	\	\	\	79.2 (19/24)	89.5 (68/76)
BADH +GUS#	3	0 (0/10)	0 (0/10)	0 (0/10)	\	\
	4	\	\	0 (0/10)	\	\
	5	\	\	0 (0/3)	53.2 (25/47)	\
	6	\	\	\	66.7 (16/24)	89.5 (68/76)

‡ The specific values in brackets were the rate of the positive plant numbers to all plant numbers checked; †, the plants checked from the perlite media watered with the selectable solutions of NaCl; §, the T_1 from some self-cross offspring of T_0 plants in which the PCR identification was positive by both GUS and BADH genes; #, the numbers of the positive plants by PCR identification on both GUS and BADH genes. NINTS, non-irradiated and non-transformed seeds; NITS, non-irradiated and transformed seeds; INTS, irradiated and non-transformed seeds; ITS, irradiated and transformed seeds.

Table 4. Results of southern hybridization.

Material		Gene	
		GUS	BADH
The positive CK		+	+
The negative CK		-	-
Number of T_1 plants	1	+	+
	2	+	+
	3	+	+
	4	+	+
	5	+	+

'+' indicate a positive reaction (with one or more hybridization blots), whereas '–' indicate a negative reaction (without any hybridization blot).

hybridization on both the GUS and BADH genes. The results are shown in Table 4 and it indicated that all the T_1 generation plants transformed reacted positively.

Chemical staining of GUS

The results of GUS chemical staining of T_0 and T_1 plants are shown in Table 5. Based on Tables 4 and 5, it was suggested that the GUS gene had been inserted into the purple medic genomes and expressed in an activated enzyme GUS in the cells that were transformed.

DISCUSSION

Now, considering the main transformation methods to plants, there are other problems besides the use of some antibiotic genes as markers. For agrobacterium-mediated (Kemal et al., 2003) and biolistic bombardment methods (Men et al., 2003; Bohorova et al., 1999), first, only the plants which erected a regeneration system could be transformed. Secondly, there were very significant differences on the transformation ratio between different plant species and between different explants. Thirdly, the transformation and regeneration ratios were always incompatible at the same explants. Kenneth and Marks (1987) reported that the germinating seeds of *Arabis gunnisoniana* could be directly transformed by the agrobacterium-mediated method, but the method still needed to make use of an antibiotic marker gene even though it was little for other plants.

Table 5. The positive frequency of GUS chemical staining and southern hybridization of both GUS and BADH genes (%).

NaCl content (gl^{-1})	GUS chemical staining					Southern hybridization	
	T_0				T_1§	T_1‡	
	NINTS	NITS	INTS	ITS		GUS gene	BADH gene
3	0 (0/10)	0 (0/10)	0 (0/10)	\	\	\	\
4	\	\	0 (0/10)	\	\	\	\
5	\	\	0 (0/3)	53.2 (25/47)	\	\	\
6	\	\	\	66.7 (16/24)	82.9 (63/76)	100 (5/5)	100 (5/5)

§, The self-cross offspring from T_0 that expressed positive reaction by PCR assay of both GUS and BADH genes; ‡, five plants of T_1§ that expressed positive reaction by GUS chemical staining; the plant numbers of T_0 and T_1§ are same as shown in Table 3. NINTS, non-irradiated and non-transformed seeds; NITS, non-irradiated and transformed seeds; INTS, irradiated and non-transformed seeds; ITS, irradiated and transformed seeds.

In the preparation methods of antibiotic free marker transformation, some research, such as *cre/lox* system (Dipankar et al., 2008; Gleave et al., 1999), had been carried out. The first step was to erect two kinds of binary expression vectors with the antibiotic marker gene (such as *nptII* gene), while the second step was to transform the explants in order to obtain two kinds of plants (containing *cre* or *lox* gene) using the vectors, respectively. The third step was to cross between the plants containing *cre* and *lox*, while the fourth step was to screen the transgenic plants without antibiotic gene in the hybridization generations. Apparently, when comparing the study's method of transforming seeds, the systems had very complicated steps. Daniell et al. (2001) reported that, the BADH gene acted as a selectable marker without the use of antibiotic, and the betaine aldehyde was used as a positive selective compound. The study's method indicated that, the irradiated seeds acted as a kind of acceptor, while the linear DNA with the aim gene (such as the GUS gene) and the selective marker gene of BADH acted as a kind of donor. Thus, 5 or 6 gl^{-1} NaCl selective solutions were used as a selective condition. Moreover, this transgenic system was a characteristic of both the higher transformation ratio and a regeneration-free system of the plants.

With this method, the researchers of this paper had gotten 6 plants species transformed by 3 kinds of genes (not published), in which the aim gene was mainly inserted into the plant genome with a single copy and there was little gene silence in the purple medic. So, to most plant species without the BADH gene in their genomes, this method might rapidly help in achieving the transgenic plants, but before their work, the researchers needed to assay the sensitivity of the seeds and seedlings to the selective solutions of NaCl, by which all labs might easily finish the assay.

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