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A PRIMARY STUDY OF HIGH PERFORMANCE TRANSGENIC RICE THROUGH MAIZE UBI-1 PROMOTER FUSING SELECTIVE MAKER GENE

JINJUAN SHEN[1], PINGZHONG CAI[1,2], FENG QING[1], ZHIYONG ZHANG AND GUIXUE WANG[1]*

[1] *Key Laboratory of Biorheological Science and Technology (Chongqing University), Ministry of Education; Laboratory of Mechano-developmental Biology, Bioengineering College, Chongqing University, Chongqing 400044, China*
[2] *Institute of Biotechnology and Nuclear Technology, Sichuan Academy of Agricultural Science, Chengdu 610066, China*
**Corresponding Author. E-mail: wanggx@cqu.edu.cn. Tel: 86-23-65102508. Fax: 86-23-65102507*

Abstract

Based on the expression vector pBI121, we successfully constructed a plant overexpression vector of Hspa4 gene fusing with selective maker gene (hygromycin-resistance gene) driven by the Ubi-1 promoter (pBI121-Ubi-Hpt-Hspa4, p121UHH). The plant expression vectors p121UHH and pCAMBIA1301-Ubi-Hspa4 (p1301UH) were transformed into the rice callus, mediated by *Agrobacterium tumefaciens*. We screened 17 p121UHH-positive transgenic plants and 15 p1301UH-positive transgenic plants by the hygromycin-resistance gene. The pick-up rate of the resistance callus was 51.7% and 42.5%, respectively, and the rate of regeneration for the resistance callus was 51.2% and 49.1%, respectively. The result of polymerase chain reaction (PCR) identification indicated that the pick-up rate of positive transgenic plants was 51.7% and 42.5% and the total transformation efficiency was 16.5% and 6.2%, and the former was 2.66 times of the later. The results of the experiment indicate that the possibility of the appearance of false positive results in the fusing of a plant over-expression vector with a selective maker gene is much less.

Introduction

Rice is one of the major food resources in the whole world, and our land is the biggest producer of rice, comprising about 35% of the rice yield in the world. With the increasing population and decreased availability of land resources and with rice being considered the main food in China, which must raise single birth and boost continuity and production efficiency of the grain's production. It is very difficult as well as important to produce more food for the population. Since originating in 1972, plant genetic engineering has been drawing scientists' attention during the past 30 years. Based on plant genetic engineering theories, rice gene transfer is becoming available as a method for improving rice yield. Genes for insect resistance (Yoza *et al.*, 2005), fungal resistance (Kim *et al.*, 2009), virus resistance (Zhang *et al.*, 2010), herbicide resistance (Gallon *et al.*, 2008; Wei *et al.,* 2008; Gressel *et al.,* 2009), bacterial resistance (Noor *et al.,* 2006; Xiang *et al.,* 2006) and nematode resistance (Lorieux *et al.,* 2003) have been utilized in genetic engineering for transforming rice yield. There are four main methods for gene transfer that are widely used: PEG-mediated rice transformation (Uchimiya *et al.*, 1986), electroporation-mediated gene transfer (Toriyama *et al.*, 1988; Chaudhury *et al.*, 1995), microprojectile bombardment-mediated gene transfer (Sanford 1988; Christou *et al.*, 1991) and *Agrobacterium*-mediated gene transfer (Raineri *et al.*, 1990; Chan *et al.*, 1992; Bhatti *et al.*, 2009; Khan *et al.*, 2007). Compared with the other three methods, transformation methods based on the use of *Agrobacterium* are more preferred in many instances because of the following properties: (i) easy to handle, (ii) higher efficiency, (iii) more predictable pattern of foreign-DNA integration, and (iv) low copy number of integration. However, with the progress in research, a large number of questions has arisen. The up gradation of transformation condition and increasing of transformation efficiency have deserved further research, given that rice is not a natural host for *Agrobacterium tumefaciens*. The latest advancements in genetic engineering research of rice indicate that the major influence factors of transformation rice were mediated through *Agrobacterium tumefaciens*. Further elucidation of the source of explanation (Li *et al.*, 1990; Hamid *et al.*, 1996), addition of phenolic compounds (Li *et al.*, 1990), categories of nutritive medium, the strain of *Agrobacterium tumefaciens* and vectors (Liu *et al.*, 1987; Yu *et al.*, 1992), selective marker gene, rice gene type, time of co-culture (Yu *et al.*, 1992), kind of promoters, resistance gene and so on are required.

In this study, constructed a plant over-expression vector of Hspa4 gene fusing with selective maker gene (hygromycin-resistance gene); the plant expression vector was successfully transformed into rice callus mediated by *Agrobacterium tumefaction*. The experiment researched the fusion of resistance gene with Hspa4 gene, under optimal conditions, that was demonstrated to have enhanced transformation efficiency greatly, compared with that of the single resistance gene.

Material and Methods

Bacterial strain and plasmid: *Agrobacterium tumefaciens* EHA105 and expression vector pCAMBIA1301 were preserved by the Institute of Biotechnology and Nuclear Technology of Sichuan Academy of Agricultural Sciences. pmd18T-Hspa4, pmd18T-Ubi and p1301UH [Fig. 1; the domain of T-DNA contains Hspa4 gene and hygromycin (Hpt) screening marker gene; Hspa4 comes from Rattus norvegicus, Hspa4 gene was driven by Ubi-1 promoter and Hpt was driven by CaMV35S promoter] were constructed in our laboratory.

Biomaterial: Rice varieties: Japonica cultivar-group, Zhonghua No.16, Japonica rice H02-117 (Zhejiang University, China) and Zhonghua No.11 were provided by the Institute of Biotechnology and Nuclear Technology of Sichuan Academy of Agricultural Sciences.

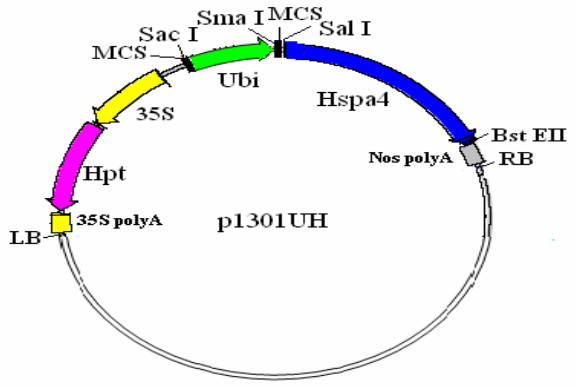

Fig. 1. Plant expression vector p1301UH.

Medium: NB medium composition: N6 medium major element+B5 medium micro element+ B5 medium organism element; Incorporating medium: NB+2 mg/L 2, 4-D + 0.5 mg/L KT + 300 mg/L casein hydrolysate+100 mg/L inose + 500 mg/L proline +300 mg/L Gln+3% sugar +0.7% agar pH5.8; Secondary culture medium: besides 1mg/L 2, 4-D, the rest are the same as including medium; Suspending medium (no added agar): 1/10 NB, pH5.8 ; Co-culture medium: besides pH 5.2, the rest are the same as secondary culture medium; Differential medium: MS+2.0 mg/L KT+0.5 mg/LNAA+0.25 mg/L IAA+ 100mg/L inose + 3% sugar + 0.6% agar pH5.8 ; Rooting medium: MS+0.5mg/L KT+0.5 mg/L NAA+0.5 mg/L IAA+100mg/L inose + 3% sugar + 0.6% agar pH5.8; YEB medium: 10g/L tryptone+1g/L yeast extract + 5g/L beef extract+0.5 g MgS04.7H20+ 15g agar, adjust to pH 7.2.

Primers design: PCR primers were designed, respectively, according to the reported sequences of Hspa4 gene, (accession number: NM-153629) in Rattus norvegicus, and Ubi-1 gene (accession number: DQ141598) in *Zea mays L.* and the sites of enzymes digestion were made out by the sequences.

P1: 5 '-atgtcggtggtgggcata-3';
P2: 5 '-gcaggatctggatagggca-3';
P3: 5 '-catgctgctcctttctcca-3';
P4: 5 '-gttggaatcaatcaatgtcca-3';
Ub1: 5 '- CGAGCTCCTGCAGTGCAGCGTGACCC-3' (SacI);
Ub2: 5 '-TCCCCCGGGCTGCAGAAGTAACACCAAACAAC-3'(SmaI).

PCR cloning and sequencing of Hpt gene: Based on the published nucleotide sequences of Hpt gene (accession number: AF234297) in pCAMBIA1301, the primers of Hpt gene were designed. The primers are: Primer 1 (Hpt1), 5 ' -CGAGCTC CTGCAGTGCAGCGTGACCC-3 ' (Nco I); Primer 2 (Hpt2) 5'-TCCCCCGGGCT GCAGAAGTAACAC CAAACAAC-3' (SmaI) . PCR products were named Hpt. The standard reaction system had a total volume of 50μL and the protocol for cyclic amplification was followed as is described in the following: pre-denaturing at 94° for 4 min, denaturing at 94° for 50s, then quenching at 57° for 1 min, thereafter continuing at 72° for 1 min, running the cycle 35 times and then continuing at 72° for 10 min, storing at 4°. Sequencing of PCR products were done by the Takara Co.

Construction of recombinant plasmid p121UHH: The cloning vector pMD18T-Hspa4 was digested with 2 restriction enzymes SmaI and NcoI, cloning vector pMD18T-Hspa4 was digested with 2 restriction enzymes SmaI and NcoI and recombinant plasmid pBI121-Ubi was digested with 2 restriction enzymes SmaI and SacI; then, the Hspa4 fragment of about 2500 bps from pMD18T-Hspa4, the Hpt fragment measuring about 800bps from pBI121-Ubi, big fragment from pBI121-Ubi, finally ligated the 3 target gene fragments at 16°; the reaction system was as follows. The masculine plasmid was screened by kanamycin, detected by PCR and digested with NcoI/SacI or SmaI/NcoI or SmaI/SacI; we named the recombinant plasmid as recombinant plasmid (the procedure for construction has been depicted in Fig.2).

Hspa4 gene fragment	0.1-0.3mmol
Hpt gene fragment	0.1-0.3mmol
pBI121-Ubi big fragment	About 0.03mmol
T4 DNA Ligase	1μl
10×T4 DNA Ligase buffer	2.5μl
ddH2O	Up to 25μl

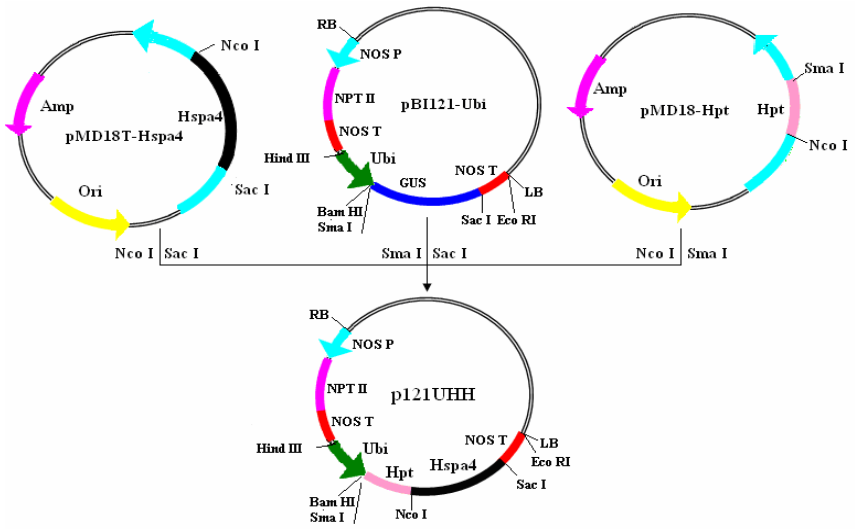

Fig.2. Construction of plant expression vector p121UHH

Genetic transformation of rice: Single colony was taken up from the flat plate and was inoculated into the YEB medium with 50μg/ml rifampicin and 50μg/ml kanamycin at 28° and mixed with shaking (220 rpm) and incubated overnight. When the culture was at log phase or immediately thereafter, the cells were pelleted by centrifugation at 5000rpm for 10 min. The bacterial pellet was suspended in 1/10 NB liquid medium to OD600 measuring about 0.5. Acetosyringone (100 μmol/ L) was also added in the inoculation medium just before the inoculation of callus. The callus was inoculated by submerging it in the inoculation medium for 10-15 min and then dried on sterile filter paper. Co-cultivation was carried out for 3 days in the dark at about 22–24°. After co-cultivation the callus was washed with 250 mg/L cefotaxime (cef) 2–3 times, allowed to stand in the washing solution for about 1 h for the last wash and finally with 500 mg/L cef to kill the bacteria, with the solution allowed to stand 30–50min after which it was dried. When placed on the selection medium with 25 mg/ml of Hpt, it gradually increased the concentration of Hpt to 50 mg/ml. The callus was sub-cultured after every 2 weeks with 300mg/L cef sodium. For the last 14 days of sub-culture the concentration of 2, 4-D used was decreased to 1 mg/ L. After 4–8 weeks of selection, calluses exhibiting strong growth were transferred to the regeneration medium, incubated in the dark for about 1 week and then placed in the growth chamber at 16/8 h light/dark period at 25°. At last the regenerated seedlings were transferred to rooting medium.

Detection of transgenic plants by PCR: The leaf blade genomic DNA was extracted from regenerated seedlings and comparison seedlings using the CTAB method (Wang *et al.*, 1998). If the PCR detection demonstrated positive results, the regenerated seedlings were grown into transgenic plants. The reaction system comprised a total volume of 25μL, and the protocol of cyclic amplification ran as follows:

PCR identification of Hspa4 gene followed by denaturing at 94° for 50s, then quenching at 57° for 1 min, thereafter continuing at 72° for 1 min, and running the cycle 35 times; the primers used were P1and P4.

PCR identification of Hspa4 gene fragment followed by denaturing at 94° for 30s, then quenching at 57° for 30s, thereafter continuing at 72° for 1.5 min, and running the cycle 35 times; the primers used were P3 and P4.

PCR identification of Ubi-1 promoter followed by denaturing at 94° for 50s, then quenching at 57° for 1 min, thereafter continuing at 72° for 2 min, and running the cycle 35 times; the primers used were Ub1 and Ub2.

PCR identification of Hpt gene followed by denaturing at 94° for 50s, then quenching at 57° for 1 min, thereafter continuing at 72° for 1 min, and running the cycle 35 times; the primers used were Hpt1/Hpt2.

Statistics of transformation efficiency

Pick-up rate of resistance callus= the number of resistance callus/ the number of transformed callus×100%;
Regeneration rate of resistance callus= the number of regenerated resistance callus/ the number of resistance callus×100%;
Pick-up rate of regenerated seedlings= the number of positive regenerated seedlings/ the number of regenerated seedlings×100%; overall transformation efficiency= pick-up rate of resistance callus×regeneration rate of resistance callus×pick-up rate of regenerated seedlings×100%.

Results

Isolation and sequence analysis of Hpt gene: Sequence analysis showed that the Hpt gene consists of 1026bps, with one base mutated, which is located at 282bps, with G mutated into T in this base; however, the mutation did not cause a change of the amino acid sequence, therefore its function did not change.

Identification of plant expression vector p121UHH: The Ubi-1 gene fragment was cloned into the vector pBI121 with correct orientation and size, which was identified with PCR amplification and enzyme digestion (Fig. 3). Based on the vector p121U, the plant expression vector p121UHH was constructed. It was difficult to link three large fragments; therefore, the vacant vectors were also detected. The figure showed that negative control had a lighter strap, as the primers were added to the enzyme digestion site, and the site sequences matched with the plant expression vector. PCR amplification had the straps that were the same as that expected (Fig. 4).

Following the selection of positive colonies, identification of plant expression vector p121UHH was undertaken by enzyme digestion; 14000 bps and 2000 bps fragments had been obtained by SmaI and SacI enzyme digestion of p121U; 14000 bps and 3500 bps fragments had been obtained by SmaI and SacI enzyme digestion of p121UHH; 14000 bps and 2500 bps had been obtained by SmaI and NcoI enzyme digestion of p121UHH; and 14000 bps and 1000 bps fragments had been derived by Nco I and SacI enzyme digestion of p121UHH. All enzyme digestion had results as anticipated, which confirmed the successful construction of the plant expression vector p121UHH (Fig. 5).

Selection of transformed callus: Selection of transformed callus is a very crucial factor for transformation. This constitutes the basic screening of transformed cells from non-transformed ones. Therefore, by scattering the colour of light yellow and eugonic callus could be used to transform. The eugonic callus was cut to the size of 2–3mm, which was used to co-culture with Hpt added to the screening medium. The transformed callus took on particle diameter and eugenic (Fig. 6).

Gain of transgenic plants: The experiment shown, the regeneration was better when the total culture duration was shorter. The colour of the callus changed to brown, indicating the loss of ability for regeneration. The long duration of the callus phase leads to significant reduction in morphogenetic potential. Thus, we grew about 69 transgenic plants, 40 of which were transgenic p1301UH seedlings and 29 of which were transgenic p121UHH seedlings. These plants were strong and were similar in appearance (Fig. 7).

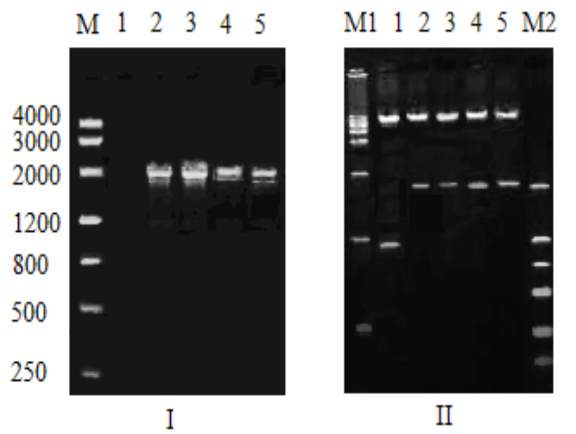

Fig. 3. Identification of p121U plasmids by PCR and striction enzyme digestion
M: Marker 3; M1: DL15, 000 DNA Markers; M2 DL2, 000 DNA Markers
I 1: PCR amplification of pBI121; 2-5: PCR amplification of transplanted plasmids;
II 1: Digestion with *Bam* HI and *Hind* III of pCAMBIA1301; 2-5: Digestion with *Bam* HI and *Hind* III of transplanted plasmids

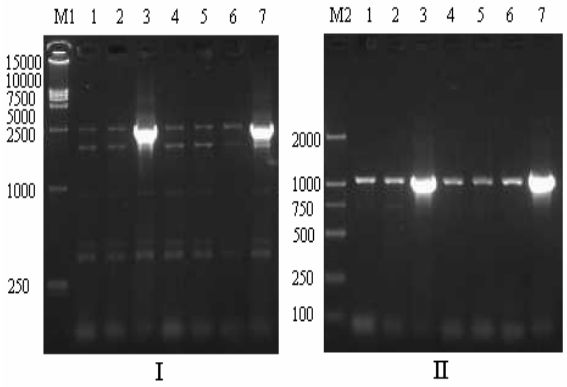

Fig.. 4. Identification of plant expression vector p121UHH by PCR
M1, DL 15000 DNA Marker; M2, DL 2000 DNA Marker
I. Detection of plasmids by PCR with primers P1 and P4
1, Negative control: p121U; 2-7, p121UHH;
II. Detection of plasmids by PCR with primers Hpt1 and Hpt2
1, Negative control: p121U; 2-7; p121UHH.

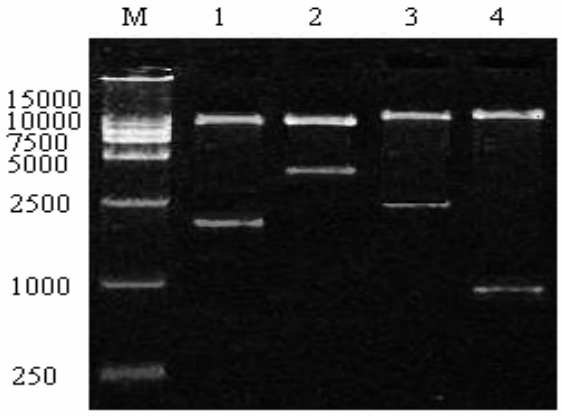

Fig. 5. Identification of plant expression vector p121UHH
M, DL 15 000 DNA Marker
1, Negative control: p121U digested by *Sma* I and *Sac* I.; 2, p121UHH digested by *Sma* I and *Sac* I; 3, p121UHH digested by *Sma* I and *Nco* I; 4, p121UHH digested by *Nco* I and *Sac* I

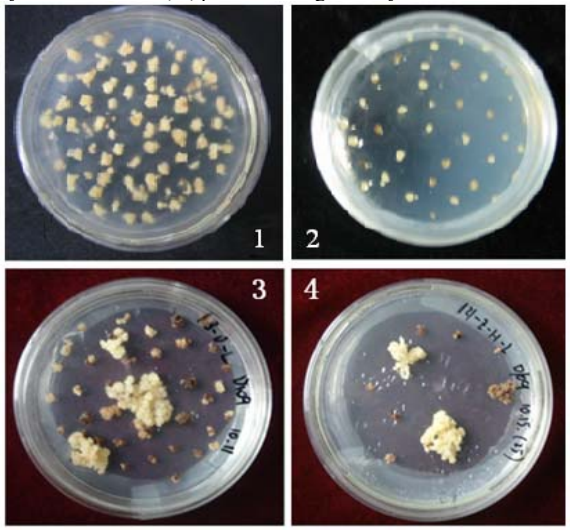

Fig. 6. The picture of rice callus
1, Rice callus induction
2, Preincubate rice callus
3, Resistance callus of transgenic p1301UH
4, Resistance callus of transgenic p121UHH

Fig. 7. Transgenic seedlings
1,Positive transgenic p1301UH seedling; 2(CK),Negative transgenic seedling; 3, Positive transgenic p121UHH seedling.

PCR amplification of transgenic plants: 69 plants had been detected by PCR (Fig. 8, Fig. 9). Every transgenic plant genomic DNA had been introduced Hspa4, Ubi-1, Hpt gene, primers P1 and P2, or primers P3 and P4, or primers Ub1 and Ub2, or primers Hpt1 and Hpt1might be used to detect.

Among these plants, there were 15 transgenic p121UHH plants and 17 transgenic p1301UH plants.

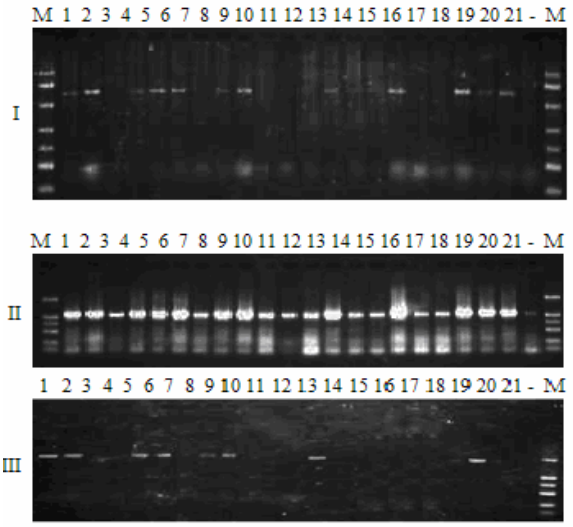

Fig. 8. Detection of transgenic p121UHH plants genomic DNA by PCR

-, Negative control; I.Detection of transgenic plants by PCR with primers P1and P4; II.Detection of transgenic plants by PCR with primers Hpt1 and Hpt1; III.Detection of transgenic plants by PCR with primers Ub1 and Ub1.

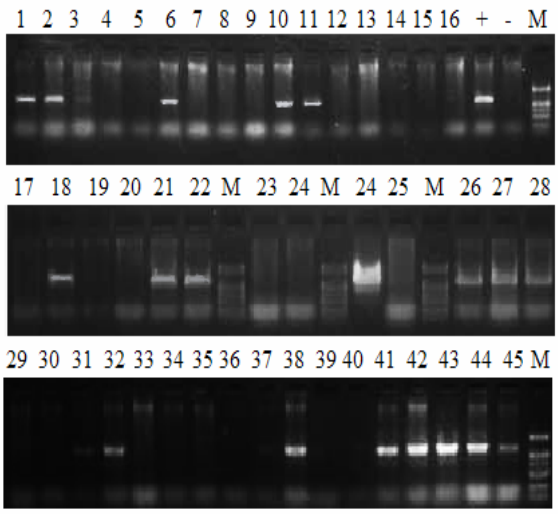

Fig. 9. PCR detection of transgenic p1301UH plants

+, Positive control; -, Negative control

1-45, Detection of transgenic plants by PCR with primers P3 and P4

Statistics of transformation efficiency: The gain rate of the transformed callus was 51.7% and 42.5%, respectively, and the regeneration rate of the resistance callus was 51.2% and 49.1%, respectively. The result of PCR identification indicated that the gain rates of positive transgenic plants were 51.7% and 42.5%, the total transformation efficiency was 16.5% and 6.2% (Table. 1), and the former was 2.66 times of the latter. The experiment led to the inference that the possibility of appearance of false positives in coalescence with the hygromycin gene is much less.

Table 1. Sampling statistical table of transformation efficiency.

Introduction plasmid	Pick-up rate of resistance callus (100%)		Regeneration rate of resistance callus (100%)		Pick-up rate of regenerated seedlings (100%)		Overall transformation efficiency (100%)
	Resistance callus	Transformed callus	Regenerated resistance callus	Resistance callus	Positive regenerated seedlings	Regenerated seedlings	
p1301UH	35	118	26	53	17	40	6.2%
p121UHH	81	130	44	86	15	29	16.5%

Discussions

This experiment successfully constructed the plant expression vector p121UHH, in which Hspa4 and Hpt gene were driven by Ubi-1 promoter. Compared with the plant expression vector p1301UH, in which Hspa4 gene was driven by Ubi-1 promoter and Hpt was driven by CaMV35S promoter. Two vectors were successfully introduced to the rice genome DNA by mediation through *Agrobacterium tumefaction*. It is well known that monocotyledons have low transformation efficiency. Several factors may be ascribed responsible for low transformation efficiency; these include materials, strain, promoter (Hiei *et al.*, 1994),tissue conditions, temperature, temperature, cultivation time, concentration of antibiotics (Chai *et al.*, 2003), and other factors (Xu *et al.*, 2009). Under the similar and optimal conditions, we finally obtained 15 transgenic p121UHH plants and 17 transgenic p1301UH plants. More importantly, the transformation efficiency of transgenic p121UHH plants was far higher than that of transgenic p1301UH plants. At the same time that the Ubi-1 promoter induced the Hpt gene, the promoter also induced Hspa4 gene in transgenic p121UHH plants. There have been no reports that the elevation of transformation efficiency was through the fusion of the resistance gene. The experiment generated the idea of increasing transformation efficiency and also helped to improve rice characteristics through genetic engineering.

Acknowledgements

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