Journal of Life Science **2004**, Vol. 14. No. 2. 368~373 ©JLS

Expression of Phosphinothricin Acetyltransferase Gene in Transgenic Rice Plants

Soo In Lee[1] and Sung-Ho Lee*

Division of Applied Life Science (BK21 program) and Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea
[1]Genomics Division, National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea

Received April 1, 2004 / Accepted April 14, 2004

We have obtained fertile transgenic rice plants resistant to the broad spectrum herbicide Basta® (active ingredient phosphinothricin, PPT) by PEG-mediated transformation procedure. The plasmid pCaMV35S:: Bar was used to deliver the *bar* gene into embryogenic suspension culture-derived protoplasts of rice (*Oryza sativa* L.). Transformed plants were regenerated and selected on medium containing 15 mg/l of phosphinothricin. Stable integration and expression of the *bar* gene in transgenic rice plants was confirmed by Southern and Northern blot analysis. Transgenic R_1 plants were also confirmed by assays for phosphinothricin acetyltransferase (PAT) activity. The *bar* gene was expressed in the primary transgenic rice plants and in the next generation progeny, in which it showed a 3 : 1 Mendelian inheritance pattern. Transgenic R_1 and R_2 plants were resistant to the herbicide Basta® when sprayed at rates used in field practice.

Key words – herbicide, *Oryza sativa*, protoplast, transformation

The tissure culture techniques for rice are some of the best elaborated among the Gramineae. Several groups also have gone even further toward the goal of transforming cereals by regenerating plants from rice protoplasts isolated from suspension cultures [5,8]. Selectable marker genes facilitate selection of apparently transformed cells from a large population of nontransformed cells. To aid rice transformation, several selectable marker genes have been tested so far. The most widely used markers are kanamycin, G418, hygromycin and phosphinothricin (PPT). In this report, we show that the *bar* gene, which confers resistance to PPT, the active ingredient in the broad-spectrum herbicide Basta®, can be used effectively to produce transgenic rice plants that are also resistance to the herbicide. The *bar* gene, cloned from *Streptomyces hygroscopicus*, encodes the enzyme phosphinothricin acetyltransferase (PAT), which acetylates the NH_2-terminal group of PPT, abolishing its herbicidal activity. This gene in conjunction with bialaphos or phosphinothricin has been shown to be a effective selectable marker in obtaining transgenic plants of many species including maize, wheat and rice [5]. Bialaphos is a tripeptide which is composed of PPT, an analogue of glutamic acid, and two L-alanine residues. PPT, released by the action of peptidases on bialaphos, is a powerful inhibitor of glutamine synthetase. It is believed that inhibition of glutamine synthetase leads to ammonia accumulation resulting in death of the plants. Chimeric constructs consisting of the CaMV 35S promoter fused to the *bar* gene have been transferred into tobacco, tomato, potato [4], and rape [3] through *Agrobacterium*-mediated transformation, and such transgenic plants were resistant to 4-10 times than the field rate of application of the both PPT and bialaphos. In the case of rice (*Oryza sativa* L.), Dekeyser *et al.* [5] reported that the *bar* gene could be used as a selectable for obtaining transgenic calli from rice protoplasts. However this study was limited to the production of PPT-resistant calli and plant regeneration from these calli was not shown. Christou *et al.* [2] regenerated transgenic rice plants using CaMV 35S promoter-*bar* chimeric gene by electric discharge particle acceleration. Here we report the expression of the *bar* gene in transgenic rice plant. We show that transgenic plants expressing PAT are completely resistant to high doses of the commercial formulations of Basta®.

***Corresponding author**
Tel : +82-55-751-5949, Fax : +82-55-754-0086
E-mail : leesh@gsnu.ac.kr

Materials and Methods

Plant materials

Mature seeds of the rice (*Oryza sativa* L.) cultivar Nagdongbyeo were used for the iniation of callus and embryogenic cell suspension culture for protoplast isolation [8]. Nurse cells used for protoplast propagation were derived from a prolonged suspension culture of the rice

cultivar C5924 [1], kindly provided by Dr. Shimamoto.

Plasmid

Plasmid expression vector pBY505 [14] containing the bacterial phosphinothricin acetyltransferase gene as the selectable marker was a generous gift from Dr. J. G. Kim (Myung Ji Univ.). Plasmid pCaMV35S::Bar was constructed by inserting fragment a 1.1 kb *Pst*I/*Eco*RI containing the Basta® resistance (*bar*) gene from pBY505 into the pBlueScriptII SK(+) (Stratagene, La Jolla, CA). Plasmid was amplified in *E. coli* strain XL1-Blue. Plasmid DNA was isolated as descibed by Maniatis *et al.* [9] and purified by cesium chloride gradient centrifugation.

Protoplast transformation, selection and plant regeneration

Rice protoplasts were isolated from embryogenic cell suspension, treated with plasmid DNA in the presence of 25% (w/v) PEG, and then cultured using nurse culture procedure as described by Lee *et al.* [8]. Fourteen-day-old microcalli were transferred to selection medium [11] containing 15 mg/l phosphinothricin. After 14 days of selection, microcalli were propagated on soft agarose medium for 7 to 8 days. Transgenic rice plants were regenerated from resistant calli as described by Lee *et al.* [8] and transferred to soil in the glasshouse. They subsequently flowered and produced seeds.

Molecular analysis

Southern blot analysis

Total genomic DNA was extracted from leaf blades of transgenic rice plants according to Dellaporta *et al.* [6] and quantified after RNase treatment. Genomic DNA (10 μg per lane) was loaded onto agarose gel with or without digestion with *Pst*I/*Eco*RI endonuclease [13]. Following electrophoresis in an 0.8% agarose gel, DNA was transferred to Hybond-N membrane (Amersham), fixed to the membrane by UV-crosslinking and hybridized with a ^{32}p-labeled radioactive probe according to a standard protocol [12]. The radioactive probe used for hybridization to detect a *bar* gene was 1.1 Kb *Pst*I/*Eco*RI fragment of pCaMV35S::Bar.

Northern blot analysis

Total RNA was isolated from young leaves of R_0 plants and prepared for blot analysis as described by Sambrook *et al.* [12]. Twenty microgram of total RNA was size-fractionated by formaldehyde agarose gel electrophoresis. Northern blot was performed with *bar* cDNA as a probe. The sizes of hybridized RNA species were estimated based on the positions of 18S and 28S rRNA seperated in the same agarose gel.

PAT assay

Plants of PPT-resistant R_1 progeny were tested for the presence of PAT using a modification of De Block's [4] procedure. Leaves (50-100 mg) were homogenized in mortar containing extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 1 mg/l bovine albumine). After centrifugation for 10 min at 13,000 rpm, 20 μl of supernatant (25 μg protein in 20 μl extract) was added to 2.5 μl of PPT solution (0.166 M PPT in extraction buffer) containing 2 μl (0.02 μCi) of [1-^{14}C]acetyl-CoA (50-60 mCi/mmol; Amersham). Tubes were incubated at 37℃ for 30 min and centrifuged at 13,000 rpm for 30 second. The supernatant (7 μl aliquots) were spotted onto silica gel TLC plates. Ascending chromatography was carried out in a 3 : 2 (v/v) mixture of 1-propanol and NH_4OH (25% NH_3). Plates were dipped in a solution containing 0.4% (w/v) diphenyl oxazole in 1-methylnaphthalene and dried. ^{14}C-labeled compounds were detected by flurography on X-ray film (Fuji) after on overnight exposure.

Herbicide application

Resistance to herbicide was examined in the R_1 and R_2 generation plants. The R_1 and R_2 seeds were planted in pots and germinated in growth chamber. After 1 month, rice leaves from untransformed and transformed plants were sprayed with 1 %(v/v) Basta® in a growth chamber (Conviron). Changes in the morphology and pigmentation of leaves were monitored for 3 to 7 days.

Results and Discussion

Protoplast transformation and regeneration of transgenic rice plants

A map of the transformation vector, pCaMV35S::Bar, containing a phosphinothricin acetyltransferase gene under control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter is shown in Fig. 1A. Three independent protoplast-transformation of the japonica variety, Nagdongbyeo, were performed with pCaMV35S::Bar as described in the experimental protocol, and summarized in Table 1. The protoplasts were cultured using agarose embedding culture

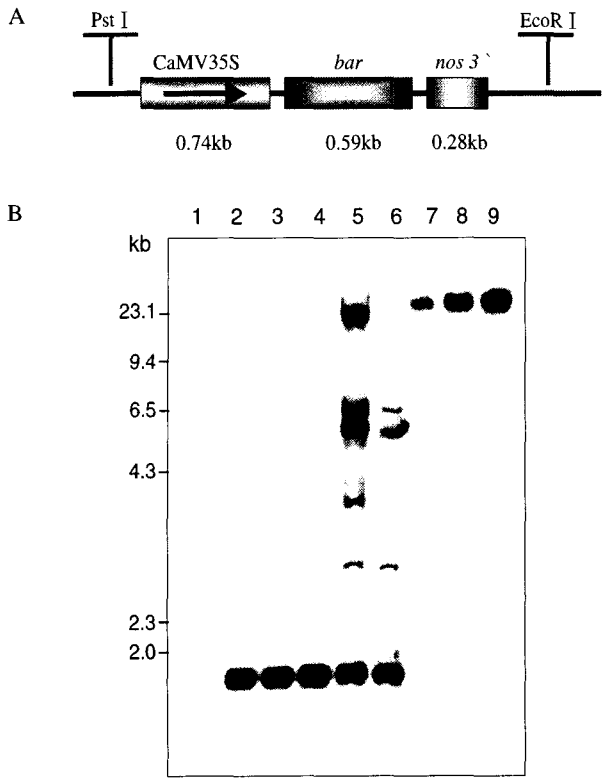


Fig. 1. (A) Structure of the plasmid pCaMV35S::Bar containing the bacterial phosphinothricin acetyltransferase (*bar*) gene. (B) Southern blot hybridization analysis of rice transformants. DNA from individual plants transformed with pCaMV35S::Bar was digested *Pst*I/*Eco*RI (lane 2-6) and undigested (lane 7-9), and hybridized with a ^{32}P-labeled *Pst*I/*Eco*RI fragment containing the coding region of the *bar* gene. DNA from untransformed control (lane 1) is shown.

procedure with nurse cells. Visiable phosphinothricin-resistant colonies were obtained from protoplasts treated with plasmid in fresh liquid protoplast culture medium containing 15 mg/l of phosphinothricin after 4 weeks of culture. In control, 15 mg/l of phosphinothricin inhibited significantly the formation of microcolonies from protoplasts not treated with plasmid in presence of PEG solution. From the total of 124 phosphin-othricin-resistant calli, 39 green plants were regenerated. Most of the plants were derived from independent calli. From 39 plants that were transferred to the glasshouse, 31 were fertile (Table 1). The number of seeds were produced in the range of 20-80 per plant.

Integration and expression of *bar* gene in transgenic rice plants

The physical presence of the *bar* gene in the transformants was confirmed by Southern hybridization with *bar* fragment from pCaMV35S::Bar. When DNA samples from the phosphinothricin-resistant primary plants (R_0) were digested with *Pst*I/*Eco*RI and hybridized with probe representing the *bar*-coding region, the presence of an intact *bar*-coding region was detected in transformed plants (Fig. 1B, lanes 2-6). This band was not detected in untransformed plants (Fig. 1B, lane 1). Evidence that the *bar* sequence is integrated into the genomic DNA of the rice cells is provided by the comigration of the *bar*-hybridizable band with the high molecular weight fragments of ethidium bromide-stainable DNA present in undigested samples (Fig. 1B, lanes 7-9). No hybridizable band was present in DNA from untransformed plants (Fig. 1B, lane 1).

To examine the transcription of the introduced foreign gene in R_0 plants, total RNA was extracted from five phosphinothricin-resistant plants, and *bar* mRNA was analyzed by Northern blot using a *bar*-coding region as a probe. In transgenic plants, *bar* mRNA was detecating expression of *bar* gene in the rice plants (Fig. 2, lanes 2-6). Although all of these plants contain the same gene, they showed variation in the accumulation of *bar*-specific mRNA. No *bar* mRNA was detected in untransformed control plants (Fig. 2, lane 1). These results indicated that *bar* gene was integrated into rice chromosome and transcribed correctly in the transgenic rice plants under the control of CaMV 35S promoter. Analysis of the *bar* gene expression demonstrated that there is a substantial variation between independent transforments in the level of expression, as has been

Table 1. Summary of rice transformation experiments

Experiment	Plasmid	Antibiotic (15 μg/ml)	No. PPT^R clones	No. plants regenerated	No. fertile plants
1	pCaMV35S::Bar	PPT	33	15	12
2	〃	PPT	41	5	4
3	〃	PPT	50	19	15
Sum 1-3	〃	PPT	124	39	31
C	None	PPT	-	-	-

C; Control experiment

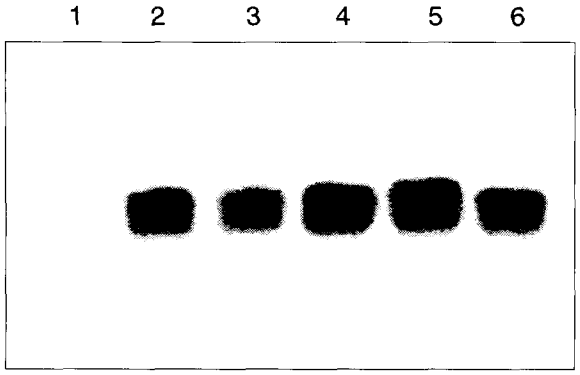
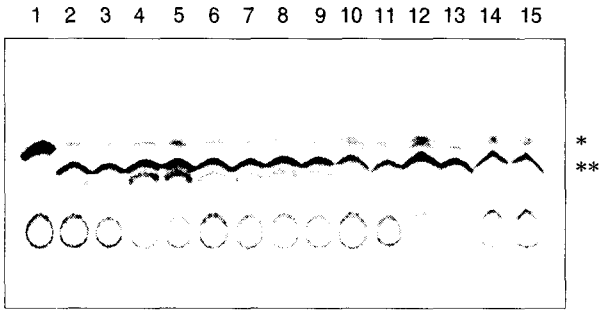


Fig. 2. Northern blot analysis of transgenic rice plants. Total RNA (20 μg) was electrophoresised in a 1.5% agarose-formaldehyde gel and blotted to a nylon membrane. A *Eco*RV fragment from pCaMV35S::Bar representing the *bar* gene-coding region was used as a hybridization probe. Lane 1, RNA from untransformed control plant. Lanes 2 to 6, RNAs from five independent R_1 plants.

observed in other case [4,7].

Analysis of phosphinothricin acetyltransferase activity

The presence of a functional *bar* gene was confirmed by demonstration of PAT activity in all phosphinothricin-resistant R_1 lines. Total soluble protein was extracted from young leaves of R_1 plants and untransformed rice plant. The *bar* gene encodes phosphinothricin acetyltransferase (PAT), an enzyme that inactivates the herbicidal compound phosphinothricin by acetylation [10]. PAT activity was only observed in an enzymatic assay in which ^{14}C-labelled acetylated PPT was detected after separation by TLC in transgenic R_1 plants (Fig. 3, lanes 2-15) and not in nontransformed plant (Fig. 3, lane 1).



* Acetyl coA
** Acetylated PPT

Fig. 3. Expression of the *bar* gene was measured as PAT activity in the leaves of R_1 transgenic plants (lane 2-15). Each lane shows the reaction products obtained with 25 μg of leaf protein. The single asterisk denotes the position of the acetyl-CoA substrate, and two asterisks denote the position of acetylated PPT product. Lane 1 contains extract from leaves of untransformed rice plant.

Inheritance of PPT resistance

To examine the inheritance of the introduced foreign genes, seeds (R_1 generation) of transgenic plants were disinfected and allowed to germinate in the medium containing 15 mg/l of phosphinothricin. The number of plants resistant or sensitive to the drug were recorded after 2 weeks. Approximate ratios of phosphinothricin resistant plants to non-resistant ones are shown in Table 2 Transformants S1, S2 and S3 showed 3 to 1, which implies that the *bar* gene is present at a single locus in the genome. However, transfomant S4 showed 5 to 1. This transformant might contain a *bar* gene a more than one locus. This result veries transmission of the introduced *bar* gene to the progeny.

Basta® tolerance in transgenic rice plants

Spraying with the commercial herbicide in a growth chamber (Conviron) clearly demonstrated that plants transformed with the *bar* gene under control of the CaMV35S promoter was fully protected against the herbicide Basta®. First damage symptoms in the controls due to the herbicide treatment with Basta® were observed within 3 days after spraying, and after 7 days the plants were completely killed. The transformed lines did not show visible damages and survived against the spraying of the herbicide (Fig. 4). The results described in this paper indicate that herbicide tolerance is an extremely useful research tool for rice biotechnology. Genetic engineering can contribute to alternative weed control by the development of herbicide resistance and the improved biosynthesis of microbial toxins. Herbicide resistance is expected to allow greater use of nonleachable and/or rapidly degraded herbicides that are not currently selective for a given crop. The science of herbicide tolerance research has made a tremendous impact

Table 2. Segregation of *bar* gene in self-progeny

Progeny of transformants	PAT activity	
	Present	Absent
S1 (38 progeny)	28	10
S2 (38 〃)	28	8
S3 (38 〃)	30	12
S4 (38 〃)	33	7

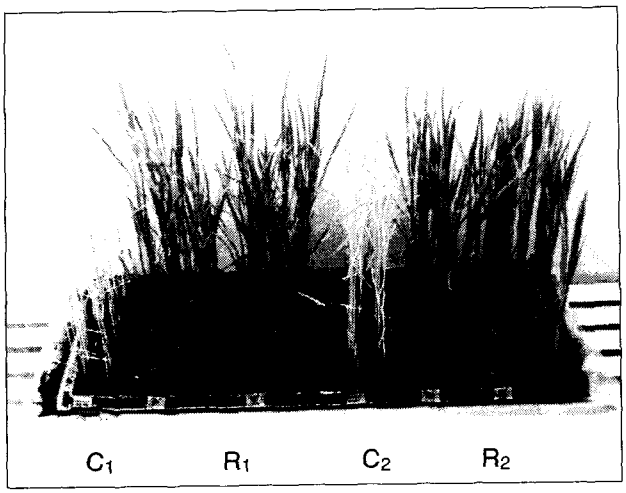


Fig. 4. Herbicide resistance in transgenic rice plants. R_1 and R_2 transgenic plants were sprayed with a 1% Basta® solution (v/v). photographic observation were recorded for 7 days after the Basta® treatment. C_1 and C_2 are untransformed control plants.

on several areas of plant enzymology, gene expression, and physiology.

Acknowledgements

This work was supported by a grant from the KOSEF/MOST to the Environmental Biotechnology National Core Research Center (grant #: R15-2003-012-02003-0) and by a grant from Kyongnam High-Tech Foundation (2004), and 'BK 21' Program from the Ministry of Human Resource Development.

References

1. Baba, A., S. Hasezawa and K. Shono. 1986. Cultivation of rice protoplasts and their transformation mediated by *Agrobacterium* spheroplasts. Plant Cell Physiol. **27**, 463-472.
2. Christou, P., T. L. Ford, and M. Kofron. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology **9**, 957-962.
3. De Block, M., D. De Brouwer and P. Tenning. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the the transgenic plants. Plant Physiol **91**, 694-704.
4. De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N. Rao Movva, C. Thompson, M. Van Montagu and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. **6**, 2513-2518.
5. Dekeyser, R., B. Claes, M. Marichal, M. Van Montagu and A. Caplan. 1989. Evaluation of selectable marker for rice transformation. Plant Physiol. **90**, 217-223.
6. Dellaporta, S. L., J. Wood and J. B. Hicks. 1983. A plant DNA minipreparation version II. Plant Mol. Biol. Rep. **1**, 19-21.
7. Jones, J. D. G., P. Dunsmuir and J. Bedbrook 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. **4**, 2411-2418.
8. Lee, S. I., H. J. Chun, C. O. Lim, J. D. Bahk and M. J. Cho. 1995. Regeneration of fertile transgenic rice plants from a Korean cultivar, Nakdongbyeo. Koean J. Plant Tissue Culture **22**, 175-182.
9. Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. Molecular cloning; A Laboratory Mannual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
10. Murakami, T., H. Anzai, S. Imai, A. Satoh, K. Nagaota and C. Thompson. 1986. The bialaphos biosynthetic genes of *Streptomyces hygriscopicus*: Molecular cloning and chracterization of the gene cluster. Mol. Gen. Genet. **205**, 42-50.
11. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant **15**, 473-479.
12. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.
13. Southern, E. M. 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. **98**, 503-517.
14. Wang, B., and R. Wu. 1995. A vector for inserting foreign genes and selection of transformed rice plants. Rice Biotcch. Q. **22**, 8.

초록 : 형질전환체 벼에서 phosphinothricin acetyltransferase 유전자 발현

이수인[1] · 이성호*
(경상대학교 생물학과, [1]농촌진흥청 농업생명공학연구원)

광범위 제초제인 Basta®에 대해 저항성을 가지는 형질전환체 벼를 개발하였다. Bar 유전자를 함유하고 있는 플라스미드 pCaMV35S::Bar를 embryogenic 현탁 배양체로부터 분리한 벼의 원형질체에 도입하였다. Phosphinotricin에 대해 저항성을 가지는 형질전환체 식물체들이 재분화되었고, 이들을 15 mg/l phosphinotricin이 함유한 배지에서 다시 선별하였다. 형질전환체 벼에서 bar 유전자의 삽입과 발현을 Southern과 Northern blot 분석으로 확인하였고, 또한 R_1 형질전환 식물체들을 PAT 활성 assay로 재차 유전자 발현을 확인 하였다. Bar 유전자는 다음 세대인 R_1 식물체에서 3:1 멘델 유전 양상을 나타내었고, 형질전환체 R_1과 R_2 식물체들은 fieild에서 살포되는 제초제 양만큼 Basta®를 살포했을 때 제초제 저항성을 나타내었다.