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Transformation of Soybean by Infecting Embryonic Calli with *Agrobacterium tumefaciens* and That of Soybean and Kidney Bean by Injecting the Bacteria into Germinating Seeds

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Abstract

An effort was made to generate transgenic plants from embryogenic callus of soybean (*Glycine max* (L.) Merrill) by using *Agrobacterium tumefaciens*, strain EHA101 harbouring the binary vector pIG121, which contains neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT) and β-glucuronidase (GUS) genes. Analysis by PCR and Southern blotting analysis of the putative transgenic soybean showed that the plants carried the genes for GUS, NPTII and HPT. The germinating seeds were injected with *Agrobacterium tumefaciens*, followed by sonication and vacuum infiltration. Analysis of the progeny plants by PCR and Southern hybridization showed that the transformed plants maintained the transferred DNA. Compared with transformation of embryogenic callus, that of germinating seeds was simple and repeatable, since it required no prior tissue culture steps and produced transgenic plants more efficiently. Transgenic plants of kidney bean (*Phaseolus vulgaris* L.) were also obtained by injection with the *Agrobacterium* into germinating seeds.

Key words Transformation, *Agrobacterium*, soybean, kidney bean, sonication, vacuum infiltration

Abbreviations

GUS, β-glucuronidase. NPTII, neomycin phosphotransferase. HPT, hygromycin phosphotransferase. PCR, polymerase chain reaction.

1. Introduction

Genetic manipulation of plants has become important in efforts aimed at the improvement of crops. Transformation of plant tissues has been attempted by several methods, for example: the *Agrobacterium*-mediated transfer of T-DNA (Facciotti *et al.* 1985, Baldes *et al.* 1987, Hinchee *et al.* 1988, Delzer *et al.* 1990, Hooykaas *et al.* 1992, McKenzie *et al.* 1992, Bailey *et al.* 1994, Droste *et al.* 1994, Mauro *et al.* 1995, Thinland *et al.* 1995, Hadi *et al.* 1996, Cheng *et al.* 1997), particle bombardment (McCabe *et al.* 1988, Christou 1990, Finer *et al.* 1991, Stewart 1996), *in planta Agrobacterium*-mediated transformation (Chee *et al.* 1989, Chowrita *et al.* 1995, Bent *et al.* 1998), and sonication assisted *Agrobacterium*-mediated transformation (Trick *et al.* 1997, Trick *et al.* 1998, Santarem *et al.* 1998).

The *Agrobacterium*-mediated transfer of T-DNA has proved to be the best method available for the transfer of DNA to plant cells. However, there have been problems in the production of transgenic fertile plants, namely difficulties associated with regeneration of plants, and in the selection of transgenic plants, as well as problems related to the methylation of DNA (Uliani *et al.* 1996). Studies on the transformation of soybean have been limited and transformation efficiency was very low (Parrott *et al.* 1989). The first transgenic soybean plants were produced from germinating seeds using an *Agrobacterium* pTiT37-SE (Hinchee *et al.* 1988). Transformation of germinating seeds by injection with *Agrobacterium* has also been reported (Chee *et al.* 1989), however, only 0.07% of inoculated seeds yielded transgenic progeny plants. Although some successes have been reported with sonication assisted *Agrobacterium*-mediated transformation (SAAT), this procedure has been limited by loss of fertility of transgenic plants derived from tissue culture to produce progeny plants (Trick *et al.* 1998).

In the present studies, we attempted to improve the method for production of transgenic plants by the infection of embryogenic callus and the injection of soybean germinating seeds, and then demonstrated that this method of transformation for soybean could be applied to kidney bean. The results showed that the method for transformation of germinating seeds injected with a liquid culture of *Agrobacterium*, followed by sonication and vacuum infiltration is simple, repeatable and more efficient than that of embryogenic callus, and also useful for transformation in kidney bean.

2. Materials and Methods

2.1 Formation of embryogenic callus of soybean

Soybean (*Glycine max* (L.) Merrill) seeds obtained from the province of Aceh, Indonesia, were grown in a greenhouse in Japan under 11 h of light and 13 h of darkness daily at 28 ℃ ± 5 ℃. We used five cultivars; *Orba biasa, Orba merah, Kipas putih, Kipas hitam and Lokal idi*. The cultivars are tropical, short day plants that require a minimum of about 11 h of light for the induction of flowers.

Pods were harvested 12 days after pollination. After soaking for 30 s in 70% ethanol, they were surface-sterilized in 0.6% Sodium Hypochlorite (NaClO) for 15 min and then rinsed three times in sterile deionized water for at least 5 min each time. Immature seeds, 5 mm in length, were removed from the pods. The intact seeds with embryo were placed on MS salts medium (Murashige and Skoog, 1962) supplemented with 0.25 mg l^{-1} BA and 0.1 mg l^{-1} NAA at 25℃ under light condition (3,000 lx). Explants that produced callus were subcultured every 4 weeks onto the same fresh medium. The cultured explants were maintained under the same environmental condition as immature seed's culture.

2.2 Bacterial strain and vector

Embryogenic callus was inoculated with *Agrobacterium tumefaciens*, strain EHA 101 that carried the intermediate plant transformation vector, plasmid pIG121. The binary vector contained genes for neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT) and β-glucuronidase (GUS). The plasmid contained an intron-GUS reporter gene required for the synthesis of GUS polypeptide. This intron-GUS reporter gene did not express detectable GUS activity in *Agrobacterium tumefaciens* (Ohta *et al.* 1990).

Agrobacterium was grown overnight in YEP medium containing Bacto trypton (10.0 g l^{-1}), Bacto yeast extract (10.0 g l^{-1}), NaCl (5.0 g l^{-1}), $MgCl_2$ (2 mM), Glucose (1.0 g l^{-1}), and supplemented with kanamycin (50 mg l^{-1}) and hygromycine (50 mg l^{-1}). Finally, the bacteria concentration was adjusted to an OD_{600} of 0.5.

2.3 Transformation of embryogenic callus of soybean

Embryogenic callus was cultured on pre-culture medium, MS salts medium containing 0.25 mg l^{-1} BA and 0.1 mg l^{-1} NAA hormones, under light condition (3,000 lx) at 25 ℃ for two days. Then, the calli were soaked in a suspension of the bacteria for 5 min. After placing on filter paper, the callus was co-cultured with the bacteria on the MS medium with the same hormones as the pre-culture medium. The inoculated calli were selected by culturing on the fresh medium that contained 250 mg l^{-1} kanamycin and 500 mg l^{-1} claforan (***Cefotaxime sodium***) for one month.

2.4 Transformation of germinating seeds of soybean and kidney bean

Mature seeds of soybean and kidney bean were sterilized in 0.6% Sodium Hypochlorite (NaClO) for 15 min and then rinsed three times in sterile deionized water for 5 min each time. The seeds were germinated by placing on sterile moistened paper towels in Petri dishes at 25 ℃ for 24 h in darkness. Seed coats were taken off and one of the two cotyledons of each germinated seed was removed. The seeds, with plumule, cotyledonary node, and adjacent cotyledon tissues attached, were transformed by the following procedures: a) injection, b) injection and sonication, and c) injection and vacuum infiltration.

a. Injection treatment

The germinating seeds were injected with a liquid culture of *Agrobacterium tumefaciens*, strain EHA101 grown overnight that also containing plasmid pIG121, the same plasmid used for transformation of embryogenic callus. Injections were done at two different sites with a 28.75 gauge (0.4 x 19 mm) needle in cotyledonary node and adjacent regions. A total of 100 μl of the liquid culture was injected into the seeds.

b. Sonication treatment

Injected seeds were gently resuspended and put in a float at the center of a bath sonicator (Honda W-103T model, frequency 45 kHz). The seeds were treated with the sonicator for two minutes. After sonication treatment, seeds were placed on sterile filter paper and then incubated at 25 ℃ in the light condition (3,000 lx) for 24 h and then planted on soil medium in pots.

c. Vacuum infiltration

Injected seeds were placed on filter paper in a

perti dish and the opened petri dish was placed inside a vacuum desiccator. Infiltration was conducted by a vacuum pump (GP-50 model, Nakamura Seisakusho, Co. Ltd. Tokyo, Japan) with the following conditions: free air displacement; 50 l min^{-1}, ultimate (ballast valbe) open; 10^{-2}, vacuum shutt; $x10^{-4}$ Torr, and revolution; 450 rpm for 2 min with occasional swirling. All of treated seeds were incubated at 25 ℃ under the light condition (3,000 lx) for 24 h and then planted on soil medium in pots.

2.5 Analysis of transgenic plants

Transformed plants (R_0) were analyzed by two methods. GUS expression was assayed by placing tissue, leaves, flowers, and young pods in a GUS assay solution containing 10 mM $Na_2EDTA.H_2O$, 0.1% Triton X-100, 0.1 M NaH_2PO_4, 0.5 M K_3Fe (CN) and 250 $\mu g\, ml^{-1}$ X-Glu. For amplification by PCR, we selected two specific sequences of the *Nos*-NPTII gene for identification of this gene in the genomic DNA from the putative transformed plants. Primer A (5'-CCCCTCGGTTCCAATTAGAG-3') was located in the Nos promoter region 33 bp from site of initiation of translation (ATG) in the 5' direction and primer B (5'-CGGGGGGTGGGC-GAGGAACTCCAG-3') corresponding to a sequence in the 3'-flanking region of the NPTII gene, at a distance of 150 bp from the signal for termination of translation in the 3' direction, respectively. These primers were tested by amplifying a characteristic 1.0 kb region of the *Nos*-NPTII gene with 10 ng of pIG121 as template as a positive control. The conditions for PCR were 35 cycles of 94 ℃ for 45 s, 50 ℃ for 30 s, and 72 ℃ for 1.5 s.

Progeny plants (R_1) were analyzed by PCR and Southern hybridization. DNA was prepared from leaf tissue using the procedure described by Honda and Hirai (1990). Southern hybridizations were performed with a non-radioactive DNA labeling and detection kit (Boehringer Mannheim, FRG). The GUS probe was prepared by digesting the DNA of plasmid pIG121 and labeled with digoxigenin into a DIG High Primer kit (Boehringer Mannheim, FRG). The DNA of plasmid pIG121 was digested with *Sal*I and *Sac*I for detection of DNA fragment of GUS. Probes were also prepared by PCR, using the primers of NPTII (Primer A and Primer B) for detection gene of NPTII, and using the primers of HPH for detection gene of HPT. The primers of HPH were hph (A): 5'-CGA CAG CGT CTC CGA CTG-3' and hph (B): 5(-CGG CCG CGC TTC TGC GGG CG-3'.

3. Results and Discussion

3.1 Transformation by infection of embryogenic callus of soybean

When immature seeds were cultured on medium that contained BA (0.25 mg l^{-1}) and NAA (0.1 mg l^{-1}), somatic embryos developed upon the cotyledons within four weeks (**Fig. 1-A**). Immature seeds formed embryogenic callus (**Fig. 1-B**) completely when some cultivars were cultured for five months. The rates of regeneration were 19.2% for the *Orba biasa* and 2.2% for the *Orba merah* cultivars (**Table 1**). We also tested the response of the cultivars on MS medium containing 2,4-D (0.25~1.0 mg l^{-1}) and BA (0.25~1.0 mg l^{-1}), but no embryogenic callus were formed.

Infected and co-cultured embryogenic callus (**Fig. 1-C**) formed shoots and initiated roots on MS medium without hormones (**Fig. 1-D**). Regeneration of the soybean immature seeds showed that embryogenic callus can be used as the target explant for transformation of soybean because the somatic embryos that have both shoots and root axes are competent to produce a mature plant. After transformation and selection of callus, one plant was eventually regenerated. The rate of transformation was 3.5% (**Table 1**). The plant showed normal morphology and formed flowers and produced seeds (**Fig. 1-E**). The other cultured embryogenic callus did not form shoots or roots and

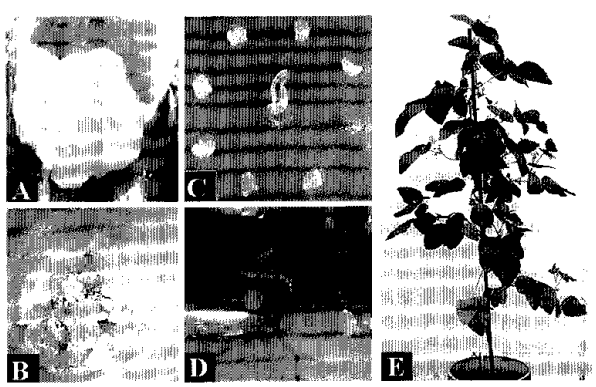

Fig. 1. Transformation of soybean by infection of embryogenic callus with *Agrobacterium tumefaciens*.
A, Callus derived from immature embryo. **B,** Embryogenic callus formed after culture on MS medium containing BA 0.25 mg l^{-1} and NAA 0.1 mg l^{-1}. **C,** Shoots that produced from embryogenic callus which was selecting on MS medium containing 250 mg l^{-1} kanamycin and 500 mg l^{-1} claforan. **D,** Regenerated shoot which formed roots and leaves. **E,** The transgenic plant that was obtained from the *Orba biasa* cultivar.

Table 1. Efficiencies of the transformation of five Indonesian cultivars of soybean using *Agrobacterium tumefaciens* pIG121 and of regeneration of transformed plants (R_0).

Cultivar	No. of seeds[a]	No. of calli[b]	No. of regenerants[c]	No. of transgenic plants[d]
Orba biasa	50	47(94%)	29	1(3.5%)
Orba merah	50	46(92%)	2	0
Kipas putih	50	46(92%)	0	0
Kipas hitam	50	44(88%)	0	0
Lokal Idi	50	36(72%)	0	0

a number of immature seeds cultured. **b** number of immature seeds produced callus, %(No. of calli) b/a x 100. **c** number of independent embryogenic calli generated from the callus that had been co-cultured with *Agrobacterium*. **d** number of transgenic plants produced from the embryogenic callus after selection in the presence of kanamycin (250 mg l^{-1}) and claforan (500mg l^{-1}), % (No. of transgenic plants) d/c x 100.

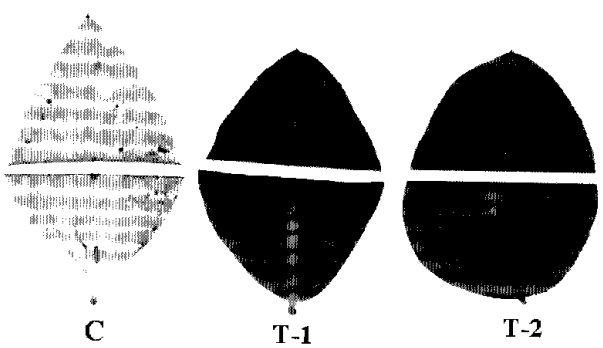

Fig. 2. Detection of GUS activity in the transgenic plant derived from the transformed soybean plants.
C, Leaf from a control plant (not treated plant). **T-1,** Leaf from a putative transgenic plant (R_0) that was produced by infection of embryogenic callus. **T-2,** Leaf from a transgenic progeny plant (R_1) that was produced by injection of germinating seeds.

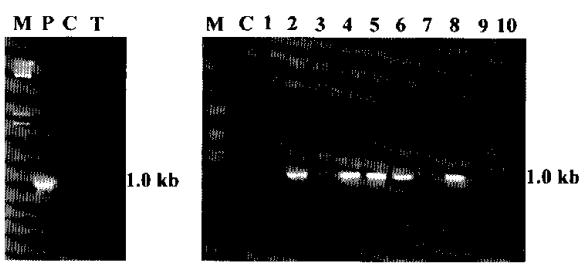

Fig. 3. Amplification by PCR of the *Nos*-NPTII gene from DNA isolated from the transformed plants derived from embryogenic callus of *Orba biasa*.
A, Transformed plants (R_0). **B,** Progeny plants (R_1). Lane **M**; markers *Hind*III digested of DNA, **P;** DNA of plasmid pIG121, **C;** DNA of non-treated plant (control), **T;** DNA of transformed plant (R_0), **2, 4, 5, 6, 8;** DNA of transgenic progeny plants (R_1) that contained the NPTII gene, **1, 3, 7, 9, 10;** non-transformed plants that did not contained the T-DNA fragment.

therefore did not regenerate into complete plant.

We analyzed the distribution of GUS activity histochemically in transformed plants of soybean. After histochemical staining with 5-bromo-4-chloro-3-indolyl-D-glucuronide cyclohexyl-ammonium salt (X-glu), light microscopy revealed that the transgenic plant derived from the *Orba biasa* cultivar had blue coloration in its leaf tissue (**Fig. 2 T-1**). Thus, the gene for GUS appeared to have been transferred into the plant genome and to be expressed in the intact of transgenic soybean plants. Analysis of the putative transgenic plant by PCR revealed that the plant generated from embryogenic callus contained the *Nos*-NPTII gene (**Fig. 3-A**). In ten R_1 plants of soybean, four of ten plants showed GUS activity. Analysis by PCR showed that five of ten R_1 plants of soybean contained the Nos-NPTII gene (**Fig. 3-B**).

3.2 Transformation by injection of germinating seeds of soybean and kidney bean

Surviving plants (R_0) of soybean and kidney bean were identified by NPTII assay. The presence of NPTII was determined by amplification by PCR. In transformation of germinating seeds by injection, transgenic plants were produced at a rate of 12%. Injection and sonication produced transgenic plants at a rate of 16%. Injection and vacuum infiltration produced transgenic plants at a rate of 24%. Transformation of germinating seeds of kidney bean was also achieved successfully at a rate of 8% by injection, 8% by injection+sonication, and 12% by injection+vacuum infiltration (**Table 2**).

Table 2. Efficiencies of the transformation of soybean and kidney bean plants (R_0) by injection (I), injection+sonication (I+S) and injection+vacuum infiltration (I+V) of germinating seeds

Method	No. of injected seeds[a]		No. of surviving plants[b]		No. of transgenic plants[c]	
	S	K	S	K	S	K
I	25	25	9(36%)	9(36%)	3(12%)	2(8%)
I+S	25	25	8(32%)	6(24%)	2(8%)	4(16%)
I+V	25	25	9(36%)	6(24%)	3(12%)	6(24%)
Total	75	75	26	21	8	12

a, number of injected seeds with *Agrobacterium*. **b**, number of surviving plants after injection. % (No. of surviving plants), b/a x 100. **c**, number of transgenic plants that were identified by PCR and Southern hybridization. % (No. of transgenic plants), c/b x 100. **S**, soybean. **K**. kidney bean.

The progenies (R_1) of the soybean and kidney bean plants that produced by the method of injection combined with vacuum infiltration were analyzed by amplification of genomic DNA by PCR and Southern hybridization. For example, analysis of eight R_1 plants of soybean showed that four of the plants contained the *Nos*-NPTII gene (**Fig. 4-A**). In R_1 plants of kidney bean, one of four R_1 plants was a transgenic plant (**Fig. 4-B**). We also found the GUS activity in soybean progeny plants that produced by injection method of transformation (**Fig. 2 T-2**).

To exclude the possibility of minor contamination of the genomic DNA or of the reagents used for PCR, we analyzed the genomic DNA from the transformed plants by genomic blot hybridization. The genomic DNA was digested with *Sal* I and *Sac* I for detection of the gene of GUS. The results revealed that the progeny plants contained 2.0 kb of the appropriate T-DNA fragment insertion. The presence of the identical size of the band for the digest suggests that R_1 plants of soybean (**Fig. 5-A**) and R_1 plants of kidney bean (**Fig. 5-B**) were derived from a single transformation event in the parent. The identity of the amplified NPTII and HPT genes, PCR products, were also confirmed by Southern analysis (**Fig. 6**). The NPTII (1.0 kb) and HPT (1.1 kb) genes were found as hybridizing fragments in each lane that had been loaded with genomic DNA from R_1 plants. These fragments proved genes of pIG121 had been integrated into the genome of the progeny plants.

The above results showed that transformation of seeds by injection might be an alternative method for obtaining transformed soybean and kidney bean more efficiently, because it does not require the use

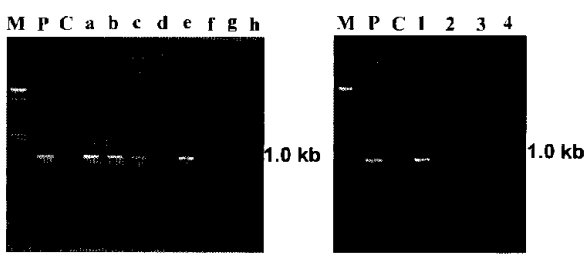

Fig. 4. Amplification by PCR of the *Nos*-NPTII gene from DNA isolated from the transgenic plants derived from seeds transformed by injection of germinating seeds.
A, Progeny plants of soybean (R_1)
Lane **M**; markers *Hind*III digested of DNA, **P**; DNA of plasmid pIG121, **C**; DNA of non-treated plant (control), **a, b, c, e**; transgenic progeny plants (R_1) that contained the NPTII gene fragment, **d, f, g, h**; DNA of non-transformed progeny plants (R_1) that did not contain the T-DNA fragment.
B, Progeny plants of kidney bean (R_1)
Lane **M**; markers *Hind*III digested of DNA, **P**; DNA of plasmid pIG121, **C**; DNA of non-treated plant (control), **1**; DNA of transgenic progeny plants that contained the NPTII gene fragment, **2, 3, 4**; non-transformed progeny plants (R_1) that did not contain the T-DNA fragment.

of any tissue culture steps. Sonication is a new and potentially more efficient method for introduction of *Agrobacterium* to plant target tissue. Sonication involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Scanning electron revealed that the sonication pro-

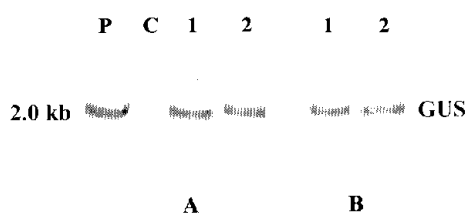

Fig.5. Southern hybridization analysis of GUS gene from transgenic soybean plants and kidney bean plants (R_1) transformed by injection of germinating seeds.

A, Analysis of DNA from soybean plants, using the GUS gene of the plasmid pIG121 as a probe that digested with *Sac* I and *Sal* I. Lane **P;** DNA of plasmid pIG121, **C;** DNA of non-treated plant (control), **1, 2 ;** DNA of transformed progeny plants of soybean (R_1) that contained the genes of GUS (2.0 kb).

B, Analysis of DNA from kidney bean, using the gene of the plasmid pIG121. Lane **P;** DNA of plasmid pIG121, C; DNA of non-treated plant (control), **1, 2;** DNA of transformed progeny plants of kidney bean (R_1) that contained the genes of GUS (2.0 kb).

duced a small channel throughout the tissue, permitting *Agrobacterium* to infect and subsequently transform tissue (Trick and Finer, 1998).

3.3 Comparison of transformation methods

Regeneration of legume plants has met with considerable difficulties. Although regeneration of tissue culture had been demonstrated for soybean (Ranch *et al.*, 1986) and kidney bean (Mohammed *et al.*, 1993), the efficiency of regeneration was too low to attempt *Agrobacterium* transformation. Trick and Finer (1998) reported that transgenic plants (R_0) were obtained from transformed embryogenic suspension culture in soybean, but no progeny plant (R_1) was recovered. However, our results showed that transgenic plants of soybean were obtained successfully by injection of germinating seeds with a liquid culture of *Agrobacterium* (**Table 2**). We also demonstrated that the method of transformation for soybean could be applied to kidney bean. This is the first report of the applicability of this method to kidney bean.

Compared with transformation of embryogenic callus by infection with *Agrobacterium*, that of germinating seeds was simple and repeatable. Transformation by injection of germinating seeds with *Agrobacterium* required no prior tissue culture steps, and it only involves the inoculation of cotyledonary node and adjacent cotyledon tissue of germinating seeds with *Agrobacterium*. The above

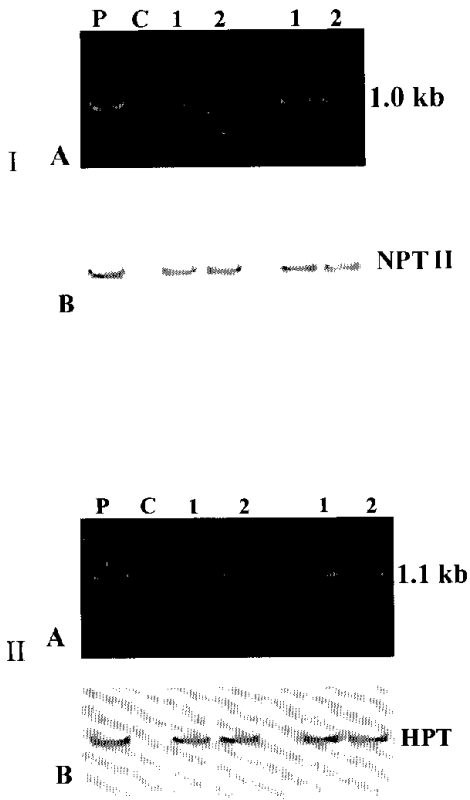

Fig. 6. Analysis by PCR of genomic DNA and Southern blotting analysis of products of the PCR, from soybean plants and kidney plants (R_1) transformed by injection of germinating seeds.

I-A, Genomic DNA was amplified by PCR using the NPTII primer (Primer A and Primer B) from a plasmid pIG121 (positive control; lane P), from a non-treated plant (lane C), from two transformed soybean plants (left: lanes 1, 2), and from two kidney bean plants (right: lanes 1, 2) that contained the gene of NPTII (1.0 kb).

I-B, Southern blotting analysis of the products of PCR using the NPTII gene as a probe.

II-A, Genomic DNA was amplified by PCR using the HPT primer: hph(A) and hph (B) from a plasmid pIG121 (positive control; lane P), from a non-treated plant (lane C), from two transformed soybean plants (left: lanes 1, 2) and from two kidney bean plants (rigth: lanes 1, 2) that contained the gene of HPT (1.1 kb).

II-B, Southern blotting analysis of the products of PCR using the HPT gene as a probe.

results show that the transgenic plants which produced progenies (R_1) were obtained efficiently by injection of germinating seeds. The rate of transformation in embryogenic callus was very low but injection of germinating seeds produced 12% transgenic plants. Transgenic plants were also produced at the frequency of 16% by injection and following sonication.

Although some successes have been reported with sonication, this procedure has been limited by loss of fertility of transgenic plants derived from tissue culture to produce progeny plants (Trick and Finer, 1998). In our study, transgenic progeny plants were produced successfully by injection and sonication of germinating seeds with *Agrobacterium*. The most efficient transformation occurred at the frequency of 24% when the method of injection followed was by vacuum infiltration (**Table 2**). The method of vacuum infiltration tremendously improved the efficiency of transformation through *Agrobacterium* infection. This might be due to large amounts of microwound in some undifferentiated cells in the plumule, cotyledonary node, or adjacent regions of germinating seeds which were caused by vacuum infiltration, which facilitated *Agrobacterium* infection. We are now using the present method for transformation in other legume plants.

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