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TRANSFORMATION EFFICIENCIES OF THE SOYBEAN VARIETY PC 19 [*Glycine max* (L.) Merrill] USING *Agrobacterium tumefaciens* AND THE COTYLEDONARY NODE METHOD

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ABSTRACT

In the present study, the transformation efficiencies of the soybean variety PC 19 [Glycine max (L.) Merrill] using Agrobacterium tumefaciens in the cotyledonary node method were investigated. PC 19 is a popular soybean variety being grown in Vietnam. The binary vector pZY 102 /pTF 102 containing the bar gene and the gusA gene was used for transformation experiments. Glufosinate selection scheme was applied with 0, 8, and 3 mg l^{-1} of glufosinate at the first and second shoot initiation and shoot elongation stages, respectively. The results in four experiments showed the transformation efficiencies identified by Southern analysis of the T0 plants were from 1 to 3%. The analysis of T1 plants by GUS assay showed that the transmission of the transgene followed a Mendelian inheritance. The result obtained from this study implies that the popular soybean variety, PC19 could be used to develop transgenic soybean variety transformed with useful genes for the improvement of productivity and quality of soybean.

Keywords*: Agrobacterium tumefaciens*, cotyledonary node, glufosinate, *Glycine max*, transformation

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important crop as it is an economic source of both oil and protein. Developing an efficient genetic transformation technology for soybean should facilitate physiological and molecular biology studies as well as the production of transgenic cultivars for higher productivity and quality.

Transgenic soybean plants have been developed using either *Agrobacterium*- or particle bombardment-mediated transformation methods in conjunction with either shoot meristems, cotyledonary nodes or cultured embryogenic tissues (Hinchee et al. 1988; Di et al. 1996; Tricker and Finer 1998). The *Agrobacterium*-mediated transformation in the cotyledonary node method was considered more efficient in soybean transformation (Olhoft et al. 2003). In addition soybean transformation efficiency could be enhanced by adding antioxidants to the co-cultivation medium (Olhoft et al. 2003), whereas different wounding methods have also been successfully used to increase *Agrobacterium* infection into the target plant tissue (Trick and Finer 1997, Xue et al. 2006).

Our work has focused on investigating the transformation efficiency of soybean variety, PC 19 using *Agrobacterium tumefaciens* in the cotyledonary node for transferring the *bar* and *gus*A genes to the soybean plants. PC 19 is a popular variety soybean being grown in Vietnam, so the present study would help in establishing efficient procedures to transform popular soybean Vietnamese varieties with useful genes for biotic or abiotic tolerance. It is envisaged that the adoption of transgenic soybean cultivars will help increase soybean productivity in Vietnam, which is at present rather low with conventionally, bred varieties.

MATERIALS AND METHODS

Plant materials

Soybean variety PC19 [*Glycine max* (L.) Merrill] was used in this study. Seeds of soybean were surface sterilized by placing seeds into a tightly sealed chamber containing chlorine gas made by mixing 5 ml of 37% HCl (12N HCl) and 100 ml commercial bleach (5.25 % sodium hypochlorite) for 16 h. Sterilized seeds were germinated in 100 x 20 mm petri dishes, on the germination medium (GM, Table 1). Seeds were germinated and grown

in a growth chamber for 5-6 days at 25^0C under fluorescent lighting (90-150 mmol photons m^{-2} s^{-1}) in an 18/6 h (light/dark) photoperiod.

Transformation of soybean

The binary vector pZY 102 /pTF 102 (Frame et al. 2002) containing the *bar* gene and the *gus*A gene was used for transformation experiments (Fig. 1). This binary vector was introduced into *Agrobacterium tumefaciens* strain EHA 101 (Hood et al. 1986) and used for transformation. *A. tumefaciens* stock of EHA 101 (pZY 102) was made by streaking on solidified YEP medium (An et al., 1988) containing 100 mg l^{-1} spectinomycin, 100 mg l^{-1} streptinomycin, 50 mg l^{-1} kanamycin and 25mg l^{-1} chloramphenicol and incubating at 28^0C for 3 days. The day before explant inoculation, 3 ml liquid YEP medium containing 100mg L^{-1} spectinomycin, 100 mg L^{-1} streptinomycin, 50 mg L^{-1} kanamycin and 25 mg L^{-1} chloramphenicol was inoculated with a single colony and shaken at 220 rpm, 280C for 8 hours. Then 3 ml of A. *tumefaciens* culture was added to 100 ml liquid YEP medium containing 100 mg L^{-1} spectinomycin, 100mg L^{-1} streptinomycin, 50mg L^{-1}kanamycin and 25 mg L^{-1} chloramphenicol and shaken at 220 rpm, 28^0C for 16-18 hours or until OD_{650} reached 1.2. Before inoculation, 50 ml aliquots of the *Agrobacterium tumefaciens* culture were centrifuged for 10 min at 5000 rpm at room temperature to pellet the cells. The *A. tumefaciens* pellet was subsequently re-suspended in 50 ml liquid co-cultivation medium (CCM 1, Table 1) and shaken at 220 rpm for 30 min before inoculation. Cotyledonary nodes explants were prepared from 5-6 day old seedlings and infected with *Agrobacterium a*s described by Olholf et al. (2003) with some modifications.

From a single seedling, two explants were obtained by removing the roots and the majority of the hypocotyl appro. 3-5 mm below the cotyledonary node on the hypocotyl, separating the cotyledons, and finally cutting vertically through the remaining hypocotyl with a number 15 sterile surgical blade (Kehr Surgical Pvt.Ltd., India). Wounding of the explant was achieved by making 7-10 slices on the cotyledonary explants. Approximatlely 50 explants were then inoculated in the 50 ml co-cultivation/*Agrobacterium tumefaciens* suspension for 30 min with shaking 50-70 rpm. After this time, five explants were cultured per 100 x15 mm petri dish and the explants were positioned with the adaxial side on a sterile 70 mm Whatman #1 filter paper (Whatman International, Maidstone, UK) placed on solid CCM (Table 1). Stacks of five Petri dishes were wrapped with parafilm "M" (American National Can. Chicago, Ill) and incubated at 25^0C for 5 days in the light.

Selection and plant regeneration

After co-cultivation, the explants were briefly washed in liquid shoot induction medium (SIM, Table 1). The explants were cultured on solidified SIM without glufosinate with the hypocotyl and cotyledonary node placed within the medium to stimulate shoot induction for the first 14 days, and incubated in a growth chamber averaging 25^0C under fluorescent lighting (90-150 µmol photons m^2 s^{-1}) in 18/6 h light. After this time, explants were sub-cultured to fresh SIM conatining 8 mg L^{-1} glufosinate and incubated for the second 14 days to select of transformed shoots. Big shoots that may have developed from primary shoot were cut and discarded. Cotyledons were excised from the callus/shoot pad after a total of 28 days on SIM and the callus was trimmed before transferring to shoot elongation medium (SEM) containing 3 mg L^{-1} glufosinate for selection. Subculture to fresh medium was done every two weeks. The elongated shoots (3-5 cm) were excised and basal portions were dipped in 1 mg L^{-1} indole 3- butyric acid for 1-3 min. The elongated shoots were then placed into rooting medium (RTM)

Screening of T_0 plants

One to two weeks after transfer to soil, T_0 plants with two trifoliates were screened for putative transformants that expressed the *bar* gene. The upper surface of a leaf was painted with 100 mg L^{-1} glufosinate along the midrib using a cotton bud. To make 100 mg L^{-1} glufosinate, 20 µl Liberty (concentration of active ingredient glufosinate was 200 mg/ml; AgrEvo, USA) was added to 40 ml of H_2O plus 40 µl Tween 20. Plants were scored based on the tolerance of the leaf tissue at 3 to 5 days after painting. Glufosinate-resistant T_0 plants were grown in the greenhouse until maturity

Southern blot analysis

Leaves of T_0 plants were used to isolate genomic DNA following the method of Dellaporta et al. (1983) with some modifications. Approx. 10 µg DNA was digested with Pvu II as single cut and with *Hin*d III plus *Xba*I as double cut. Southern blot analyses were carried out following standard protocols (Sambrook and Russell 2001). The hybridisation probes (*gus*A) were DIGdUTP-labelled by PCR using a PCR DIG probe synthesis kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions.

GUS assay

We selected randomly 20 T1 seeds of the event 1 and event 5. The seeds were surface sterile and put on GM medium for 10 days at 25^0C under fluorescent lighting (90-150 mmol photons m^{-2} s^{-1}) in an 18/6 h (light/dark) photoperiod. Roots and shoots of T1 plants were stained GUS as described by Jefferson et al. (1987) to evaluate the transgene transmission to the progeny.

RESULTS AND DISCUSSION

Transformation method

Cotyledonary node excised from 5-day-old *in vitro* seedlings was used as explant for *A. tumefaciens* infection. The use of cotyledonary node has an advantage of yielding higher rate of regeneration with multi-shoots. The production of some phenolic compounds by wounding the cotyledonary node can stimulate the infection of *A. tumefaciens* and the incorporation of the single stranded T-DNA to the plant genome (Olhoft et al. 2003). The techniques to make wounds should also be optimal. Very deep wounds and very high number of wounds could cause death to the cells and limited the infection of *A. tumefaciens*. In addition to wounding, Olhoft et al. (2003) reported that the supplement of thiol to the co-cultivation medium could stimulate the infection of *A. tumefaciens* and the transfer of T-DNA to the plant cells. The excision of shoot tips and lateral shoots helped increase the number of transformed multi-shoots. The number of multi-shoots regenerated is dependent genotype. Some varieties could produce 5-6 shoots per cotyledon node infected (Tran Thi Cuc Hoa, 2007). In this study, the variety, PC19 produced more than four shoots per cotyledonary node.

For selection with glufosinate, in the present study, we used 8 mg L^{-1} in the shoot initiation medium to select shoots after their development in the SIM for 14 days after *A. tumefaciens* infection and 3 mg L^{-1} in the shoot elongation (SE) medium. Zeng et al. (2004) obtained high transformation efficiency in the variety Williams 82, when using 8 mg L^{-1} glufosinate in the SIM and 3-4 mg L^{-1} in the SE.

Tran Thi Cuc Hoa (2008) reported that the use of 5 mg L^{-1} in the SE was optimal for Maverick, while the concentration of glufosinate in the SE should be lower, *i.e.* 3-4 mg L^{-1} for Willams 82, MTĐ 176 and HL 202.

The use of high concentration of glufosinate (10 mg L^{-1}) in the SIM was suitable to most of the soybean varieties (Tran Thi Cuc Hoa, 2008) but Zhang et al. (1999) reported that the regeneration of shoots was suppressed at 10 mg L^{-1} glufosinate for A 3237.

Table 1. List of components in media used for the soybean cotyledonary-node transformation method

Component	GM (germination medium)	CCM (co-cultivation medium)	SIM (shoot induction medium)	SEM (shoot elongation medium)	RM (rooting elongation medum)
MS Salts (Duchefa)	-	-	-	1X	1X
B5 salts (Duchefa)	1X	1/10X	1X	-	-
B5 vitamins (Duchefa)	1X	1X	1X	1X	1X
MES (Duchefa)	-	20 mM	3 mM	3 mM	3 mM
Sucrose (Duchefa,w/v)	2%	3%	3%	3%	2%
Agar, (Sigma, w/v)	-	0,35%	-	-	-
Phytagel (Sigma, w/v)	0.28%	-	0.28%	0.28%	0.28%
pH	5.8	5.4	5.7	5.7	5.6
Asparagine (Sigma, w/v)	-	-	-	50 mg l^{-1}	50 mg l^{-1}
Glutamine (Sigma, w/v)	-	-	-	50 mg l^{-1}	50 mg l^{-1}
Indole-3-acetic acid (Sigma)	-	-	-	10 mg l^{-1}	-
Zeatin riboside (Duchefa)	-	-	-	0.1 mg l^{-1}	-
6-benzyl- aminopurine (Sigma)	-	1,67 mg l^{-1}	1,67 mg l^{-1}	-	-
Gibberrellic acid (Sigma)	-	0,25 mg l^{-1}	-	0.5 mg l^{-1}	-
L-Cysteine (Sigma)	-	2,2 mM	-	-	-
Acetosyringone (Sigma)	-	0,2 mM	-	-	-
Sodium thiosulfate (Sigma)	-	1,0 mM	-	-	-
DTT (Invitrogen)	-	1,0 mM	-	-	-
Ticarcillin (Duchefa)	-	-	60 mg l^{-1}	60 mg l^{-1}	-
Cefotaxime (Duchefa)	-	-	100 mg l^{-1}	100 mg l^{-1}	-
Vancomycine (Gold-Biotech/Duchefa)	-	-	50 mg l^{-1}	50 mg l^{-1}	-
Glufosinate-ammonium (Sigma)	-	-	8 mg l^{-1}	3 mg l^{-1}	-

Transformation efficiency

In this study, four experiments were carried out each starting with 100 cotyledonary nodes infecting with *A. tumefiences*. The number of regenerated shoots ranged from 93-97 and the recovered plants after selection with glufosinate were from 5-8 (Table 2). The T_0 plants tolerant to glufosinate were identified by painting the leaves with Liberty - a commercial herbicide at a concentration of 100 mgl^{-1} glufosinate. The leaf painting with Liberty was proved to be a rapid and sensitive method to evaluate glufosinate resistant plants. The results showed that in the Experiment 1, 3 plants were resistant to glufosinate, in the experiment 2, 1 plants were resistant, and in the experiment 3 and 4, each had 3 resistant plants.

To confirm the presence of the transformed gene in the genome of the plants resistant to glufosinate, the Southern blot analysis was done using the *gus*A gene as a probe for the transgenes. The presence of *gusA* gene in the transformed plants exhibited an expected band of 2215 bp (Fig. 2). Most of the events showed a simple integration of the transgene into the plant genome. The number of the transformed plants (T_0) showing the presence of *gus*A gene were two, one, three and two in Experiment 1, 2, 3 and 4, corresponding to the transformation efficiency of 2%, 1%, 3% and 2%, respectively. On the average, the transformation efficiency of the variety, PC19 in our study was 2%. Zeng et al. (2004) reported that transformation efficiency in the soybean variety, Williams 82 varied from 0.1 to 5.9% using *A. tumefaciens* in the cotyledonary node method. Paz et al. (2004) obtained transformation efficiencies

in two soybean cultivars, Willams 79 and Williams were from 2.0 to 6.3% by *A. tumfaciens*-mediated transformation in cotyledonary node method and glufosinate selection. Following similar procedures, Tran Thi Cuc Hoa (2008) reported that the transformation efficiency in soybean ranged from 1 to 5%. Paz et al. (2006) reported that by using "half seed" explants, the transformation efficiencies could be higher than those in the cotyledonary node method could. Half seed was derived from mature seed of soybean following an overnight imbibition while the cotyledonary node was derived from 5-7-day old seedlings. Liu et al. (2008) obtained high transformation efficacies of 3.8 - 11.7% in five Chinese soybean varieties using *A. tumefaciens*-mediated transformation and hygromycin selection. Also using hygromycin selection, Olhoft (2003) increased the transformation efficiency in the soybean variety, Bert from 0.7% to 16.4% by adding mixtures of the thiol compounds, L-cysteine, dithiothreitol and sodium thiosulphate to the co-cultivation medium.

In this study, among 10 plants resistant to Liberty, eight plants were truly transformed confirmed by Southern analysis indicating that the escape percentage in transformation were 20%. The escape was seen in the Experiment 1 and 4 and there was no escape in the Experiment 2 and 3. The assay by leaf painting with Liberty has eliminated many plants recovered from selection by glufosinate. To minimize the escape, the selection procedure by glufosinate should be optimized. The concentration of glufosinate in the SE medium should be increased to 4-5 mg l^{-1} instead of 3 mgl^{-1} as used in this study. Flores et al. (2008) used a new selection scheme, namely: 0, 10, and 5 mg L^{-1} of glufosinate at the first, second shoot initiation and shoot elongation stages, respectively.

Progeny screening of T_1 generation seedlings was performed with GUS staining of the event 1 and the event 5 found the *gus*A gene were transmitted to the progeny in a normal Mendelian fashion (Fig. 3). Southern blot analyses of T_0 plants showed the event 1 and the event 5 had single integration of the transgene into the plant genome (Fig. 2).

It demonstrated that soybean variety PC 19 and other popular varieties being grown in Vietnam can be transformed with useful genes for insect resistance or abiotic tolerance leading to the production of transgenic soybean cultivars for commercial application to help increase soybean productivity in Vietnam.

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Table 2. Efficiency of A. *tumefaciens*-mediated soybean transformation (PC 19 cultivar) using cotyledonary node method (vector/construct pZY102).

Experiment number	No. of explants infected[a]	Regenerated plants	Recovered plants	No. of glufosinate-resistant events in T0 generation (leaf painting)[b]	GUS+ plants (Southern analysis)[c]	Transformation efficiency (%)[d]
1	100	95	7	3	2	2
2	100	97	5	1	1	1
3	100	93	8	3	3	3
4	100	95	6	3	2	2
Total	400	380	26	10	8	2

[b]Resistant to 100 mg l^{-1} glufosinate; [d]Transformation efficiency = (GUS^+/No. of explants infected) x 100

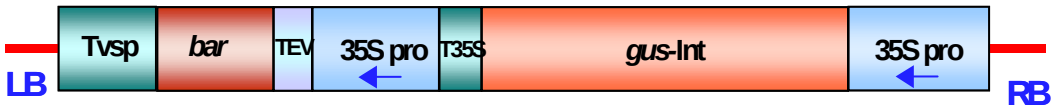

Fig 1. T-DNA region of standard binary vector pZY102 /pTF 102 (Frame et al. 2002)

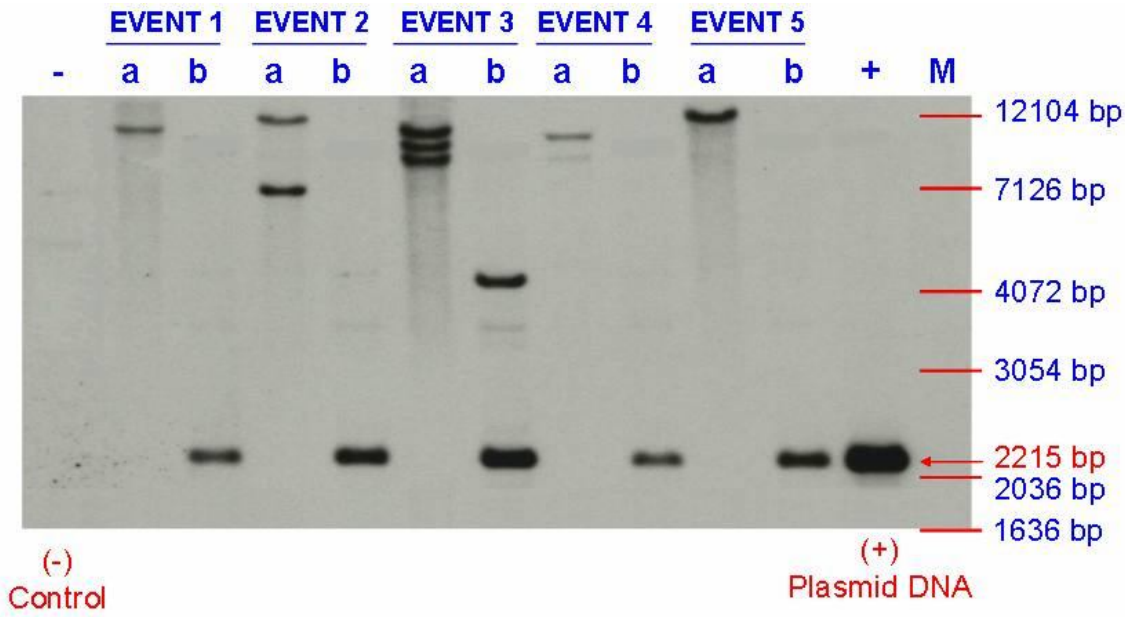

Fig. 2. Southern blot analysis of T0 plants (PC19) transformed with pZY 102
Genomic DNA digested with *Pvu*II as a single cutter (a) and with *Xba*I+*Hin*dIII as double cutters (b), probed with *gus*A using a PCR DIG probe synthesis kit . The expected transgene band sizes (2215 bp) is given with an arrow.
M: 1 kb Marker
WT: untransformed control plant
+: Plasmid DNA

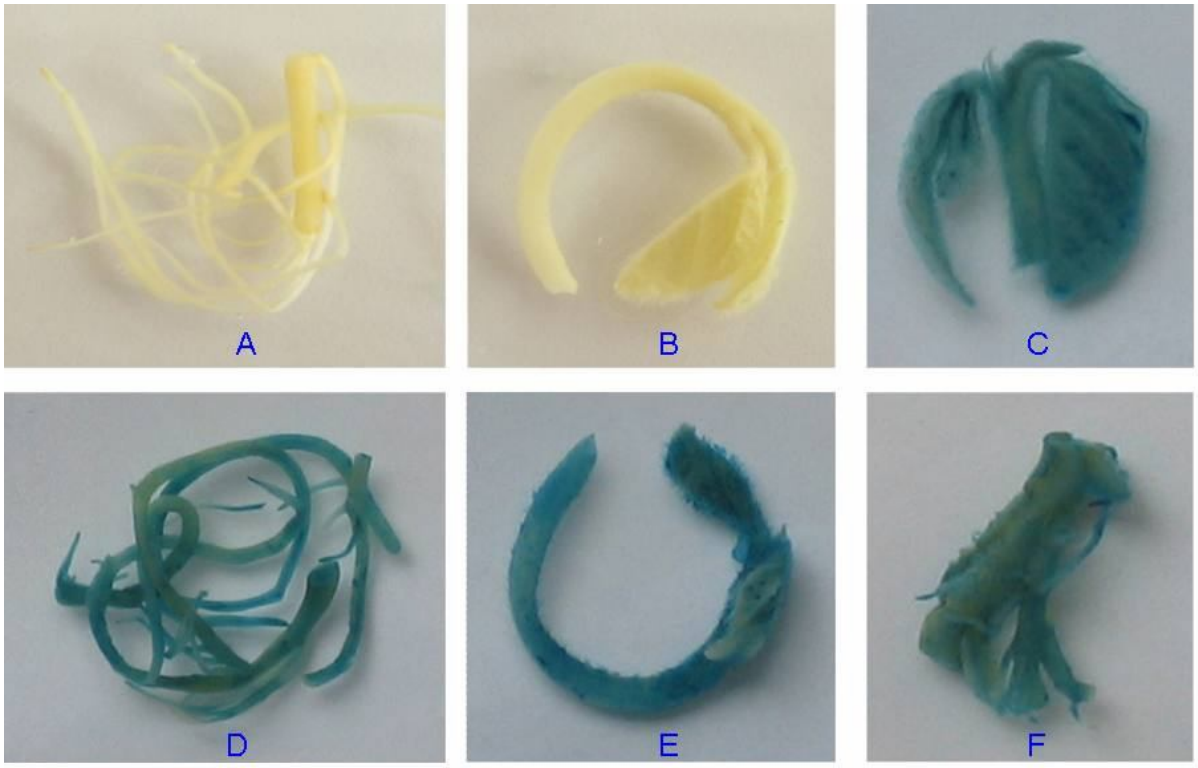

Fig 3 (A-F). GUS staining of Roots and shoots of untransformed plant and T1 plants (event 1 and 5) transformed with pZY102. (A,B: Roots and shoot of untransformed plant ; C,D: Roots and shoot of event 1; E,F: Roots and shoot of event 5).

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Hiệu quả chuyển nạp gen ở giống đậu nành PC 19 [*Glycine max* (L.) Merrill] bằng *Agrobacterium tumefaciens* và phương pháp nốt lá mầm

Trong nghiên cứu này, hiệu quả chuyển nạp gen ở giống đậu nành PC 19 [*Glycine max* (L.) Merrill] bằng *Agrobacterium tumefaciens* và phương pháp nốt lá mầm được đánh giá. PC 19 là giống đậu nành đang trồng phổ biến ở Việt Nam. Vector nhị cấp pZY 102 /pTF 102 mang gen *bar* và gen *gus*A được dùng trong các thí nghiệm chuyển nạp gen. Hệ thống chọn lọc glufosinate được áp dụng với cho 0, 8, and 3 mg L^{-1} glufosinate vào môi trường tạo chồi lần thứ nhất và lần thứ hai và môi trường vươn lóng, theo thứ tự. Kết quả cho thấy trong 4 thí nghiệm, hiệu quả chuyển nạp gen xác định bằng phân tích Southern của các cây T_0 kháng glufosinate biến động từ 1-3%. Phân tích các cây T1 bằng xét nghiệm GUS cho thấy gen chuyển nạp được truyền sang thế hệ sau theo kiểu di truyền Mendel. Kết quả của nghiên cứu này cho thấy PC19- giống đậu nành trồng phổ biến ở Việt Nam có thể được sử dụng để chuyển nạp các gen hữu dụng nhằm tăng năng suất và chất lượng của đậu nành.