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Improved production of transgenic *Dioscorea zingiberensis* (Dioscoreaceae) by *Agrobacterium tumefaciens*-mediated transformation

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ABSTRACT. The establishment of high-efficiency *Agrobacterium*-mediated transformation techniques could improve the production of *Dioscorea zingiberensis*, a medicinal species with a high diosgenin content. We co-cultivated embryogenic calli induced from mature seeds with *A. tumefaciens* strain EHA105. A binary vector, pCAMBIA1381, which contains the *gfp* and *hpt* genes under the control of the ubiquitin promoter and the CaMV 35S promoter, respectively, was used for transformation. Pre-culture, basic medium, acetosyringone, and bacterial density were evaluated to establish the most efficient protocol. The optimal conditions consisted of MS medium without $CaCl_2$ for pre- and co-cultivation, three days for pre-culture, addition of 200 µM AS, and an OD_{600} of 0.5. The transgenic plants grown under selection were confirmed by PCR analysis and Southern blot analysis. This protocol produced transgenic *D. zingiberensis* plants in seven months, with a transformation efficiency of 6%.

Key words: *Dioscorea zingiberensis*; *Agrobacterium tumefaciens*; GFP; Embryogenic calli; Transformation

INTRODUCTION

The monocotyledonous *Dioscorea zingiberensis* C.H. Wright, a perennial herbal plant widely distributed in China, is one of the most important resources of medicinal plants. It has the highest content of diosgenin of all the species in the world (Qin et al., 1996). Diosgenin is used as a precursor for the synthesis of medical compounds with wide application, such as alleviating the symptoms of coughing and pneumonia, subsidence of swelling and reducing the cholesterol level in the body. In recent years, however, the wild germplasms of *D. zingiberensis* have been exhausted by harvesting. The tubers from cultivated plants suffer from the decline of diosgenin concentration with the degeneration of the germplasm. For these reasons, it is important to breed elite cultivars with high diosgenin yield, rapid growth, and high disease and insect resistance. Jia et al. (2004) found that there are three types of *D. zingiberensis* plants, gynoecious, androecious and monoecious plants, where the three types of *D. zingiberensis* bloom at different times, thus leading to difficulties in conventional sexual hybridization for improving the quality of artificial plantings. *Agrobacterium*-mediated transformation provides a new efficient way to solve the problem. Since 2002, *in vitro* multiplication of *D. zingiberensis* has been performed by using explants such as stems (Yan et al., 2002), leaves (Mo et al., 2004), nodal cuttings (Chen et al., 2003), anthers (Yan et al., 2007), and embryos (Yuan et al., 2005). Liu and Liu (2005) found that hairy roots were induced when *D. zingiberensis* was infected with *A. tumefaciens*. However, they could not successfully establish a system for genetic transformation, and nor did they obtain the regenerated transgenic plants. Another study in *Agrobacterium*-mediated transformation of green calli from nodal stem of *D. zingiberensis* has been recently reported (Zhu et al., 2009). These studies provide a precondition for establishing a successful *Agrobacterium*-mediated transformation system of *D. zingiberensis*.

Embryogenic calli derived from mature seeds have previously been used for *Agrobacterium*-mediated transformation in several other monocotyledons and are considered to be the best target tissue for transformation, because they are actively dividing (Cheng et al., 2004). Therefore, this study was conducted with the main objective of establishing a stable transformation system for *D. zingiberensis*, and optimizing the factors that influence *Agribacterium*-mediated transformation of *D. zingiberensis* based on the rate of transient GFP expression.

MATERIAL AND METHODS

Plant material

Mature seeds of *D. zingiberensis* C.H. Wright collected from an experimental field of Huazhong Agricultural University were sterilized with 0.1% (w/v) mercuric chloride solution for 8 min, and afterward rinsed several times with sterile distilled water. The mature embryos were peeled and inoculated in Murashige and Skoog (1962) (MS) medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L 6-BA. These axenic cultures were maintained in the dark at 25 ± 2°C for 40 days. After subculture for three times, the calli were improved to whitish yellow, compact and dry embryogenic calli. These optimized cultures were used for further transformation experiments.

Bacterial strains and plasmids

The *Agrobacterium tumefaciens* strain EHA105, harboring the modified binary vector

pCAMBIA1381, was used in this study. The T-DNA regions of the modified pCAMBIA1381 contain a green fluorescent protein gene (*gfp*) under the control of a ubiquitin promoter (*ubi*) and a gene encoding *hpt* (hygromycin phosphotransferase) as a selectable marker under the control of cauliflower mosaic virus 35S promoter (Figure 1). Both EHA105 and pCAMBIA1381 were kindly provided by Dr. Yongjun Lin.

Figure 1. Structure of the T-DNA region in plasmid pCAMBIA1381-GFP (LB = left border; RB = right border; *gfp* = green fluorescence protein; *htp* = hygromycin phosphotransferase; ubi-promoter = ubiquitin promoter; CaMV35S = 35S promoter).

Transformation and regeneration of embryogenic calli

The *A. tumefaciens* strain EHA105 (pCAMBIA1381) was grown for two days on LB solid medium supplemented with 50 mg/L hygromycin and 50 mg/L kanamycin. The *Agrobacterium* cultures were transferred to liquid MS medium (without $CaCl_2$) supplemented with 2.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 500 mg/L CH, 400 mg/L Gln, 600 mg/L Pro, and 200 µM acetosyringone (AS), and shaken at 200 rpm 2-3 h at 28°C until an OD_{600} of 0.5. The *D. zingiberensis* tissues described above were cultured on pre-cultivation media at 25° ± 2°C in the dark for three days. The pre-cultured explants were surface-dried briefly on sterile filter paper and immersed in the bacterial suspension for 30 min, blotted on sterile filter paper, and then placed on filter paper on a Petri dish containing 25 mL solid MS medium (without $CaCl_2$) with 2.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 500 mg/L CH, 400 mg/L Gln, 600 mg/L Pro, and 200 µM AS. After co-cultivation at 20°C in the dark for three days, the explants were thoroughly rinsed in sterile water containing 500 mg/L carbenicillin. The explants were briefly dried on sterile filter paper again and then cultured on co-cultivation medium with 400 mg/L carbenicillin at 25° ± 2°C in the dark for seven days to eliminate superfluous *A. tumefaciens*. Afterwards, the explants were cultured on MS selection medium with 250 and 35 mg/L hygromycin at 25° ± 2°C in the dark. After 14 days, the explants were planted on selection medium containing 250 mg/L carbenicillin and 70 mg/L hygromycin.

After a two-month selection, the hygromycin-resistant calli were transferred onto MS medium supplemented with 0.2 mg/L NAA, 2.0 mg/L 6-BA, 1000 mg/L CH and 70 mg/L hygromycin to regenerate shoots at 25° ± 2°C under a 16/8-h light/dark cycle with light intensity of 2000 lux for two months. The primary regenerated shoots from the calli were then transferred to 1/4 MS supplemented with 0.5 mg/L IAA and 70 mg/L hygromycin for induction of roots. Rooted plantlets were transferred to plastic pots containing a sterilized soil mixture (1:1 vermiculite:perlite), placed inside polyethylene bags to maintain high humidity, incubated at 25° ± 2°C in a growth chamber for two weeks, and then transferred to the greenhouse.

Assays of different factors

In order to increase the efficiency of the transformation system, we investigated the

effects of different factors that could influence T-DNA transfer. The optimized conditions consisted of the duration of precultivation of calli before infection, the parameters of infection such as duration of submerging and OD_{600}, the basic media in pre- and co-cultivation, and presence or absence of AS. Transient transformation efficiency was compared based on the percentage of GFP+ calli three days after co-cultivation. GFP expression was observed with a stereomicroscope (Olympus Ac Adapter) with a green channel. The green fluorescence indicated the GFP expression level.

PCR analysis

Genomic DNA was extracted from both transgenic and wild *D. zingiberensis* leaves using the modified CTAB method. Specific primers for the *htp* gene: 5'-ATG TTG GCG ACC TCG TAT T-3' and 5'-ATC GTT ATG TTT ATC GGC ACT-3' were designed to amplify an internal 517 bp of the *hpt* gene. The total volume of the reaction mixtures was 20 µL, including 1 ng genomic DNA, 0.5 µL of each primer (50 µM), 0.5 µL dNTP mix (10 mM each), 2 µL Taq DNA polymerase, the corresponding reaction buffer (0.5 U/µL) and 1.5 µL Mg^{2+}. For positive controls, 1 ng plasmid DNA was added to the reaction mix. Cycling parameters began with an initial hot start at 94°C for 5 min, then 35 cycles of denaturation (94°C; 30 s), annealing (60°C; 35 s), and extension (72°C; 1 min), followed by a final extension of 10 min at 72°C. PCR amplification products were visualized on a 1.0% agarose gel stained with ethidium bromide.

Southern blot analysis

A Southern blot analysis was also performed to further confirm the stable transgene integration. Genomic DNA (20 µg) from transformed and non-transformed control plants was digested with *Eco*RI and separated on a 0.8% agarose gel. The DNA was capillary-blotted onto a nylon membrane. A 517-bp fragment containing the *hpt* gene fragment amplified from plasmid pCAMBIA1381 was labeled with DIG-dUTP using a DIG-High Prime DNA Labeling and Detection kit (Roche, Germany). The nylon membrane was incubated in prehybridization solution and shaken at 40 rpm for 3 h at 42°C; the digoxin-labeled probe was then added to the hybridization solution and the mixture shaken at 40 rpm for 24 h at 42°C. Washing and detection were performed according to manufacturer instructions (Roche).

RESULTS AND DISCUSSION

Preparation for the embryogenic calli

Establishing an efficient regeneration system is fundamental for the genetic manipulation of any species. In this study, approximately 90% of the calli were induced when mature seeds were planted on MS medium supplemented with 2.0 mg/L 2,4-D, and 0.5 mg/L 6-BA. However, most of the calli were watery and sticky, and were not suitable for transformation. Therefore, embryogenic calli were induced from the non-embryogenic calli by subcultivation on MS medium supplemented with 2.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 500 mg/L CH, 400 mg/L Gln, 600 mg/L Pro for 3-4 months. Catlin (1990) also obtained a high frequency of induced embryogenic calli using a modified MS medium supplemented with additional amino acids

(termed RVIM). In this study, embryogenic calli (Figure 2a) were whitish yellow, compact, and dry. A previously published protocol for *D. zingiberensis* transformation used green calli as the targets (Zhu et al., 2009). However, this protocol is genotype-dependent and cannot be readily used in new varieties. In this study, we established a relatively genotype-independent protocol for generating embryogenic calli, as well as a system to transform plants from embryogenic calli (Figure 2a, d, e, and f).

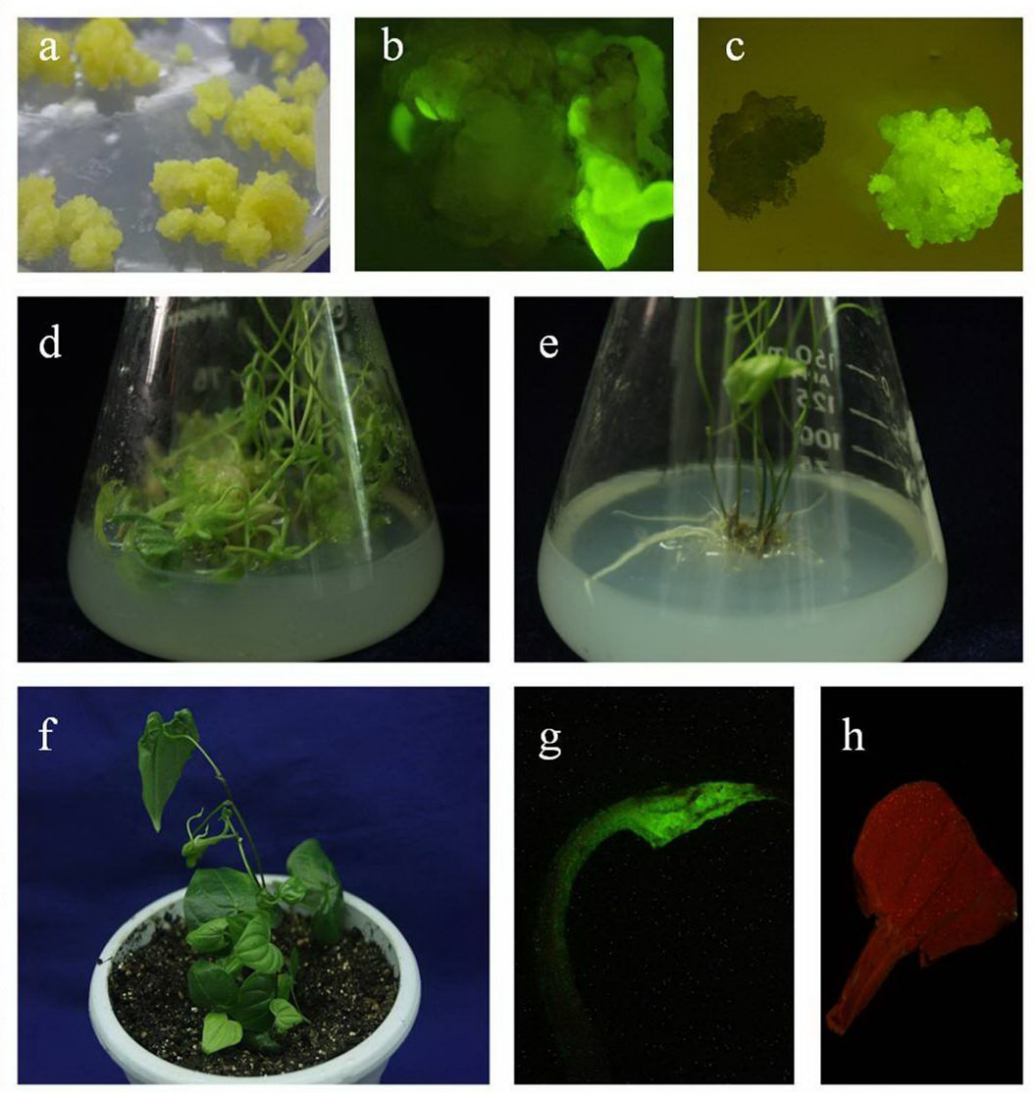

Figure 2. a. Embryogenic callus from mature seeds. **b.** GFP transient expression in embryogenic calli. **c.** GFP stable expression in transgenic and non-transgenic calli. **d.** Formation of shoots from hygromycin-resistant embryogenic calli. **e.** Development of roots from shoots of transgenic plants. **f.** Well-grown transgenic plant in pot. **g.** Leaves of transgenic plants showing GFP expression. **h.** Leaves of non-transgenic plants.

Selection and regeneration of the transgenic plants

Prior to selections, the minimum lethal hygromycin concentration of wild-type calli at the shoot regeneration stage was determined. Calli were completely growth-inhibited and eventually died after three weeks, when the concentration of hygromycin was 70 mg/L (data not shown). Based on this result, 70 mg/L hygromycin was chosen for selection.

After co-cultivation at 20°C in the dark for three days, transient GFP expression was observed in most explants (Figure 2b). After two months of selection, the hygromycin-resistant calli that expressed stable GFP fluorescence (Figure 2c) were transferred to MS medium supplemented with 0.2 mg/L NAA, 2.0 mg/L 6-BA, 1000 mg/L CH, and 70 mg/L hygromycin. After three months, they gradually regenerated into shoots (Figure 2d). The primary regenerated shoots were transferred to 1/4 MS supplemented with 0.5 mg/L IAA and 70 mg/L hygromycin, and complete development of roots was observed within two months (100% rooting) (Figure 2e). Thus, a total of 10 putative transgenic plants were obtained. Compared to non-transgenic, no morphological differences in any of the regenerated transgenic plants were observed (Figure 2f), except for the GFP expression in leaves between transformation plants and non-transformation plants (Figure 2g and h). Thus, we can conclude that the *gfp* gene was successfully inserted into the genome of *D. zingiberensis*.

Table 1 shows the effects of pre-cultivation, medium in pre- and co-cultivation, OD_{600}, and concentration of AS on GFP transient expressions.

Table 1. Effects of pre-cultivation, medium in pre- and co-cultivation, OD_{600}, and concentration of acetosyringone (AS) on GFP transient expressions.

Treatment	No. of explants treated	No. of transient expression explants	Transient expression rate (%)
Pre-culture (days)[a]			
3	102	50	47.4[a]
0	98	37	36.3[a]
Medium in pre-cultivation and co-cultivation [b]			
I	110	30	30.3[b]
II	113	51	44.2[a]
III	115	59	54.3[b]
OD_{600} [c]			
0.3	110	35	32.3[a]
0.5	113	57	51.7[a]
0.8	113	59	54.2[b]
AS (μM)[d]			
0	137	45	37.2[a]
200	113	56	51.3[a]

I = MS + 2.0 mg/L 2,4-D + 0.5 mg/L 6-BA + 500 mg/L CH + 400 mg/L Gln + 600 mg/L Pro. II = 1/4 MS + 2.0 mg/L 2,4-D + 0.5 mg/L 6-BA + 500 mg/L CH + 400 mg/L Gln + 600 mg/L Pro. III = MS (without $CaCl_2$) + 2.0 mg/L 2,4-D + 0.5 mg/L 6-BA + 500 mg/L CH + 400 mg/L Gln + 600 mg/L Pro. a = calli were submerged with *Agrobacterium* OD_{600} of 0.5, and then co-cultivated for 3 days in medium III with 200 μM AS. b = calli were submerged with *Agrobacterium* OD_{600} of 0.5 with 200 μM AS. c = 3 days of pre-cultivation and co-cultivation in medium III with 200 μM AS. d = 3 days of pre-cultivation and co-cultivation in medium III and OD_{600} of 0.5. Mean values for three experiments. Mean values within columns followed by the same superscript letters are not significantly different ($P < 0.05$) according to the Tukey multiple comparison test.

Necessity of the pre-culture step

Since the first transgenic plants of dicotyledonous species obtained by *Agrobacterium*-mediated transformation in the early 1980s, much effort has been made to extend the host range of *Agrobacterium* (Cheng et al., 2004). These parameters include the plant species, explant types, bacterial strains, use of virulence inducers such as AS in the co-cultivation medium, bacterial density, the inoculation, composition of co-cultivation medium, and pH value.

In order to determine the effect of pre-culture on transient expression rate of transformed *D. zingiberensis* calli, we cultured calli on MS medium (without $CaCl_2$) supplemented with 2.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 500 mg/L CH, 400 mg/L Gln, 600 mg/L Pro, 200 μM AS for zero and three days. It has been reported that pre-culture prior to inoculation with *Agrobacterium* improved the transformation efficiency in *Brassica oleracea* ssp *capitata* (Lee et al., 2000), but decreased in *Perilla frutescens* (Kim et al., 2004). In this study, as shown in Table 1, the transient expression rate was higher (47.4%) with pre-cultivation for 3 days, compared to those not pre-cultivated (36.3%). Therefore, the application of the pre-culture step led to higher transformation efficiency of *D. zingiberensis*, which may be due to the presence of AS.

MS without $CaCl_2$ identified as the optimal basic medium

In this study, MS, 1/4 MS and MS without $CaCl_2$ were used respectively for pre-cultivation and co-cultivation media. Pre-cultured calli were transferred to each co-cultivation medium I, II or III after 30 min of *Agrobacterium* inoculation. The percentage of GFP-positive calli was calculated to evaluate the efficacy of the selected medium after three days of co-cultivation. The results indicate that MS without $CaCl_2$ was the optimal basic medium for both pre-cultivation and co-cultivation (Table 1). This is probably due to the role of calcium, which may accelerate the formation of cell walls. Some wounds created in the experiment produced firm protective cell walls and even browned in the presence of Ca^{2+}. Thus, *Agrobacterium* was difficult to inject into the plant cells through the firm protective cell walls. The effective interface between bacteria and plant cells diminished, which led to the low transformation efficiency. Contrarily, cell walls were not stubborn without Ca^{2+}. Wounds created in the operation just produced wafer-thin cell walls and were not brown. Thus, *Agrobacterium* could infect plant cells easily and relatively raise the transformation rates.

Effect of acetosyringone

Plant phenolic compounds that induce the expression of *Agrobacterium* vir genes are important for gene transfer (Stachel et al., 1985). Phenolic compounds such as acetosyringone have been reported as the key component in the transformation of rice (Hiei et al., 1994) and wheat (Cheng et al., 1997). Therefore, we compared the transient GFP expression rates when AS at a concentration of 200 μM was excluded from the cultivation medium. In the present study, the results (Table 1) show that the highest transient expression rate was achieved when the AS concentration was 200 μM. In our analysis, the difference in the requirement of acetosyringone for successful plant transformation may be due to the difference in the duration of inoculation and co-cultivation, as well as in the competence of target tissues.

Optimal bacterial concentration

Optical density is considered to be an important factor in *Agrobacterium*-mediated transformation. We previously observed that the bacterial concentration was dependent on the cultivar (Dutt and Grosser, 2009). Decreasing OD_{600} values were also co-related with improved regeneration and transformation frequency (Yu et al., 2002). In this study, we found that transient GFP expression rates increased with the OD_{600} of the bacterial suspension (Table 1). The highest transient GFP expression rate occurred with an OD_{600} of 0.8, but at this OD_{600}, bacterial colonization and calli necrosis was prominent, which affected the regeneration of the transgenic cells. Therefore, further experiments restricted the OD_{600} to 0.5.

Molecular analysis of the transgenic plants

To verify the insertion of the *hpt* gene, 10 putative transgenic plants and an untransformed control plant were analyzed by PCR amplification. The expected 517-bp *hpt* gene fragment was found in the positive control (pCAMBIA1381) as well as in all analyzed plants (Figure 3). No PCR products were detected in non-transgenic control plants. This indicates that all putative transgenic plants were positive for hygromycin-resistance.

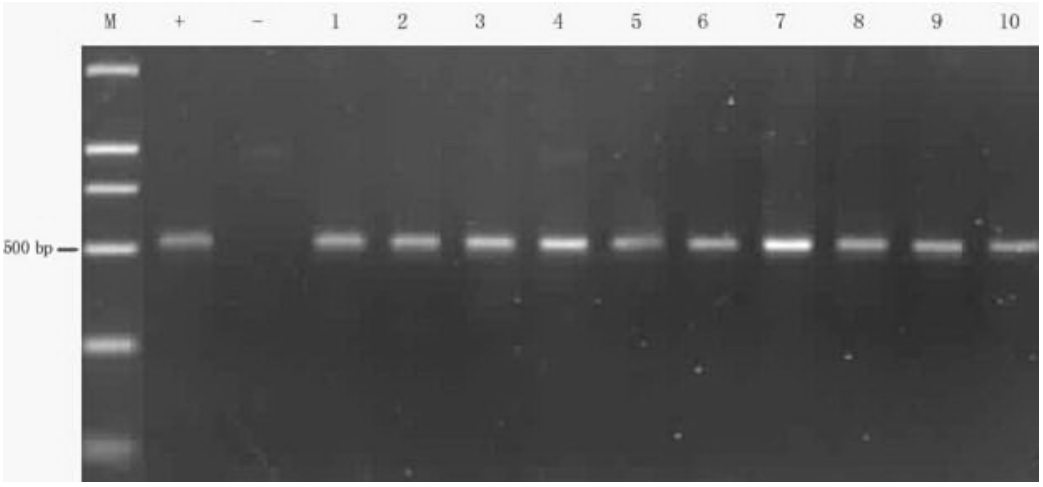

Figure 3. PCR analysis of *Dioscorea zingiberensis* transformed by *Agrobacterium tumefaciens* strain EHA105. Amplification of a 517-bp fragment of the *hpt* gene. *Lane M* = DNA marker; *lane* + = plasmid pCAMBIA1381, used as the positive control; *lane* - = the untransformed plant, used as the negative control; *lanes 1-10* = transgenic plants.

Southern hybridization of DNA from three transgenic and a non-transformed control plant digested with *Eco*RI revealed that T-DNA was integrated into the *D. zingiberensis* genome in one transgenic plant with a single-copy, while the two other transgenic plants harbored no transgene copy (Figure 4).

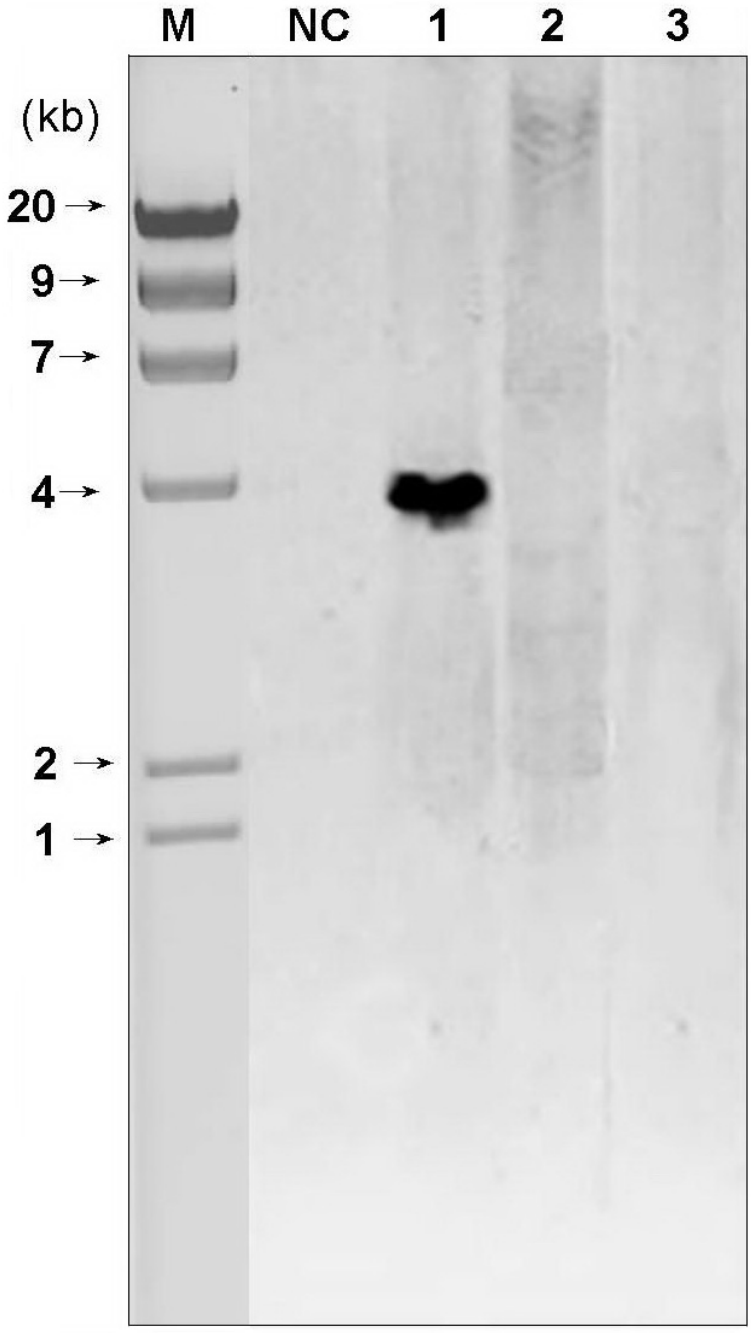

Figure 4. Southern blot analysis of the transgenic plants. *Lane M* = DNA marker; *lane NC* = non-transformed plant, used as the negative control; *lanes 1-3* = transgenic plants. DNA was digested with *Eco*RI and hybridized with the DIG-labeled *hpt* gene probe.

CONCLUSIONS

In this study, *D. zingiberensis* was successfully transformed by *Agrobacterium*-mediated transformation. The transformation was performed according to the following method. Pre-culture was carried out for three days on solid MS medium (without $CaCl_2$) with 2.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 500 mg/L CH, 400 mg/L Gln, 600 mg/L Pro, and 200 µM AS, submerged in the *Agrobacterium* inocula with OD_{600} of 0.5 for 30 min, and then co-cultured with the *Agrobacterium* for 3 days on the same medium, followed by transfer to the same medium with 500 mg/L carbenicillin for 7 days and then by transfer to MS selection medium with 250 mg/L carbenicillin and 35 mg/L hygromycin in the dark at 25° ± 2°C. After 14 days, the explants were planted on the selection medium containing 250 mg/L carbenicillin and 70 mg/L hygromycin for two months. Under the optimized conditions, overall transformation efficiency was 6.00%. Thus, our results show that there may still be room for further modification and improvement of transformation efficiency in *D. zingiberensis*.

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