Development of an efficient transgenic system of Lagenaria siceraria using seed germination pouches as a growth carrier

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Research Article

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Abstract

Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] is one of the most common horticultural and medicinal crops. Genetic transformation is a rate-limiting step in unraveling the function of genes and molecular breeding in this non-model crop. In this study, we established an optimized protocol for transformation using a seed germination pouch as a growth carrier and developed bottle gourd composite plants. Overground explants were inoculated with *Agrobacterium rhizogenes* strain R1000, which harbored a binary vector pBI121 carrying the GUS reporter gene. The integration of GUS into transgenic roots was confirmed via reverse transcription-polymerase chain reaction and histochemical staining. Furthermore, we screened for desirable genotypes from 14 bottle gourd germplasm collections based on the number of induced roots, transformation efficiency, and induction rate, and “Changguang” (transformation efficiency, approximately 36.8%) was selected as the genotype suitable for hairy root induction in bottle gourd. The optimized protocol presented here is a rapid, suitable, and efficient tool for studying the function of genes and root biology of bottle gourd in the future.

Key Message

This study developed an optimized *Agrobacterium rhizogenes*-mediated transformation protocol for developing bottle gourd composite plants and screened the desirable genotype.

Introduction

The bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] (2n = 2x = 22), which belongs to the Cucurbitaceae family, is one of the most important horticultural and medicinal crops in Africa and Asia (Whitaker 1971; Beevy and Kuriachan 1996; Erickson et al. 2005; Xu et al. 2014). Fresh bottle gourd fruits are widely consumed as a vegetable (Morimoto and Mvere 2004), and dry fruits can be used as containers, handicrafts, or musical instruments (Lee, 1994; Yetisir and Sari, 2003; Morimoto and Mvere, 2004).

As a non-model cucurbit crop, although the genome of bottle gourd has been completely sequenced (Wu et al. 2017; Wang et al. 2018; Xu et al. 2021), the challenges faced in genetic transformation considerably hinder the evaluation of gene function in this crop. Traditional genetic transformation of Cucurbitaceae spp. using *Agrobacterium tumefaciens* is complex, time-consuming, and labor-intensive (Smarrelli et al. 1986; Vasudevan et al. 2007; Ilina et al. 2012), and no successful cases have been reported to date.

Agrobacterium rhizogenes (*A. rhizogenes*), a relative of *Agrobacterium tumefaciens* (Riker 1930), can be used in an alternative genetic transformation method that has been reported to be successful in pea, cowpea, common bean, chickpea, tomato, and cotton crops (Clemow et al. 2011; Mellor et al. 2012; Khandual et al. 2014; Ron et al. 2014; Aggarwal et al. 2018; Ho-Plágaro et al. 2018; Cui et al. 2020), as well as Cucurbitaceae crops including *Cucumis sativus*, *Cucurbita pepo*, and *Cucumis melo* (Pak et al. 2009; Anuar et al. 2011; Ilina et al. 2012). Infection with *A. rhizogenes* induces the development of “hairy” roots in plants that develop from the site of infection where the root-inducing (Ri) plasmid is transferred

from bacterial cells to plant genomes rather than tumors (Chilton et al. 1982; David et al. 1988). To the best of our knowledge, no study has reported the genetic transformation of *A. rhizogenes* into bottle gourd.

The aim of this study was to determine an optimized protocol of *A. rhizogenes*-mediated genetic transformation of bottle gourd. In this study, we describe a simple, low-cost, and efficient protocol for *A. rhizogenes*-mediated genetic transformation of bottle gourds. Further, we identified a bottle gourd genotype that can develop transgenic roots efficiently, which will lay the foundation for research on root biology and root-related gene function in bottle gourd.

Materials And Methods

Plant materials and growth conditions

Fourteen bottle gourd germplasm collections with diverse genetic backgrounds described by Wu et al. (2017) were selected for this study (Table 1). The seeds were rinsed with distilled water and then immersed in 70% ethanol for 30 s. Subsequently, the seeds were immediately rinsed thrice with sterile distilled water (2 min each) and incubated in 3% (v/v) sodium hypochlorite for 10 min with gentle shaking. The seeds were then washed again with sterile distilled water at least thrice (2 min each). The sterilized seeds were first placed on petri dishes containing moistened filter paper and germinated in a growth incubator at 28°C in the dark for approximately 3 d. The germinated seeds were seeded into a sterile substrate (vermiculite: peat, 3:1) in a growth incubator at 28°C/22°C with a 16 h light/8 h dark cycle.

Table 1
List of the 14 bottle gourd germplasm collections used in this study

Accession code	Area of collection
Chuangguang	Guangdong province (Southern China)
Qingxiu	Hubei province (Central China)
J104-1	Guangdong province (Southern China)
JH-8	Zhejiang province (East China)
YD-3	Guangdong province (Southern China)
HZ	Zhejiang province (East China)
Qiangyao	Zhejiang province (East China)
PS30	Former Serbia and Monteneg
G32	Anhui province (East China)
Yuanhulu	Zhejiang province (East China)
Nanqingnan	Zhejiang province (East China)
YD-4	Guangdong province (Southern China)
Shangyuduanpu	Zhejiang province (East China)
NXZ	Hubei province (Central China)

Bacterial strains

A. rhizogenes strain R1000, which contains the pRiA4b Ri plasmid derived from *A. rhizogenes* strain A4T (Moore et al. 1979; White et al. 1985), was used for transformation of bottle gourd. A binary vector pBI121 that carries the β-glucuronidase (GUS) reporter gene was transformed into *A. rhizogenes* R1000 competent cells via electroporation. Transformed agrobacterial colonies were grown on YEB plates supplemented with Rif and kanamycin (50 mg/L) for 2 d. The positive-transformed strains were inoculated into liquid YEB medium under Rif and kanamycin selection conditions and incubated in a shaker at 28°C and 180 rpm for 12 h. Subsequently, 200 µL of the *A. rhizogenes* culture was re-inoculated into 200 mL YEB medium under Rif and kanamycin selection, and incubated in a rotary shaker at 28°C and 180 rpm for 16 h, until the optical density at 600 nm (OD_{600}) reached 0.8–1.2 (OD_{600} of 1.0 = 5×10^8 cells/mL).

Plant transformation

The roots of six-day-old bottle gourd seedlings were cut off at a 45° angle close to the root using a sterile razor blade to obtain overground explants of bottle gourd. The activated *A. rhizogenes* cells were

collected via centrifugation at 4,000 × *g* for 10 min and resuspended in liquid callus-inducing medium (CIM) (20% Murashige and Skoog medium containing 0.4 mM acetobutanone). Subsequently, the bacteria were incubated in a rotary shaker at 28°C and 180 rpm for 1 h until the OD_{600} reached 0.8. Wounded overground explants of bottle gourd were incubated with 20 mL R1000 bacterial solution containing the binary vector pBI121, which harbored the GUS reporter gene, in a small beaker (50 mL) for 1 h. Inoculated overground explants of bottle gourd were inserted into a sterile medium-containing seed germination pouch (Beijing Qiwei Yicheng Technology Co., Ltd., Beijing, China), which was previously soaked in 20 mL of CIM. The inoculated bottle gourd overground explants were placed in the dark in a growth incubator (21°C) for co-culturing and allowed to dry to moderately control the wilting of plant materials. Three days later, the plant materials were transferred to a growth incubator with 60% light at 22°C with a 16 h light/8 h dark regime. For the remainder of the induction period, the germination pouches were re-watered with CIM when necessary and checked periodically.

Histochemical GUS staining

The roots of five plants from each accession were randomly selected for staining using the GUS Histochemical Stain Kit (MM1001-1KIT; MKbio, Shanghai, China) according to the manufacturer's instructions, and three replicates were performed. Root samples were incubated in the dark at 37°C for 6–7 h. After pouring out the staining solution, the roots were soaked in absolute ethanol for 2 min thrice to decolorize the false-positive color.

Polymerase chain reaction (PCR) analyses

Total RNA was extracted from transformed hairy roots using the RNA Prep Pure Plant kit (Tiangen Co., Beijing, China) according to the manufacturer's instructions. RNA was reverse transcribed using the PrimeScript RT Reagent Kit (TaKaRa, Kyoto, Japan). The PCR reaction mix (10 μL) contained DNA samples (1 μL, 50 ng/μL), primer mixes (2 μL, 10 μmol /L), 10× PCR buffer (1 μL), ddH_2O (5 μL), and Taq enzyme (DR100A, TaKaRa, 1 μL). The PCR cycling procedure was as follows: 3 min at 95°C, followed by 34 cycles at 95°C for 30 s, annealing at 55°C for 30 s, and 72°C for 40 s, with a final elongation step at 72°C for 10 min.

The primer pair used was GUS-F: 5'-CAACGAACTGAACTGGCAGA-3' and GUS-R: 5'-AGAGGTTAAAGCCGACAGCA-3'.

Statistical analyses

The data were statistically analyzed using Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) and SPSS software (IBM, Armonk, NY, USA). The number of induced roots, transformation efficiency, and induction rate of 14 bottle gourd germplasm collections were analyzed.

Results

Optimization of protocol for generating bottle gourd composite plants

The essential ex vitro composite plant induction method was based on previous methods (Collier et al. 2005), and has been applied to several species, including pea, cowpea, common bean, and squash (Clemow et al. 2011; Mellor et al. 2012; Ilina et al. 2012; Khandual et al. 2014). Based on these studies, we improved the method and performed modifications appropriate for bottle gourd and proposed an optimized protocol for generating bottle gourd composite plants (Figs. 1 and 2). Bottle gourd seeds were sterilized and germinated in sterile glass dishes containing filter paper at 28°C in the dark for approximately 3 d until germination (Fig. 2A). The germinated seeds were seeded onto sterile substrates containing appropriate proportions of vermiculite and peat and were cultivated in a growth incubator for 6 d. Subsequently, the vigorous plants were cut off at a 45°C angle close to the root and placed in a small beaker containing R1000 bacterial solution harboring the binary vector pBI121 for 1 h. Subsequently, the excised overground explants were inserted into a sterile seed germination pouch containing liquid CIM (Fig. 2B), clamped to a foam board (Fig. 2C), and sealed to maintain moisture levels (Fig. 2D). The inoculated overground explants of bottle gourd were placed in a dark growth incubator (21°C) for co-culturing and were transferred to a normal growth incubator after 3 d. Approximately 9 d later, the first hairy roots emerged (Fig. 2E), which were discarded because they were not transgenic roots (Fig. 2F). The transgenic roots emerged after another 10 d of co-culture (Fig. 2G, H).

Identification of transgenic roots

To determine whether the developed transgenic roots were positive transgenic roots, PCR using GUS-specific primers was performed; the expected size of the amplified PCR product was 750 bp, and the binary vector pBI121 was used as a positive control. The cDNA of seven transgenic roots was randomly selected for amplification and four PCR products of an expected size were obtained; however, no PCR product was obtained from control root and ddH_2O samples (Fig. 3A), excluding the contamination of the cDNA. Further, the four putative positive transgenic roots and control roots were analyzed via GUS histochemical staining. GUS stain (indigo) was observed in the four putative positive transgenic roots, but not in the control roots (Fig. 3B). These results indicate the successful integration of the binary vector pBI121 carrying the GUS reporter gene into the genome of the four putative positive transgenic roots.

Screening for desirable genotypes

Using the aforementioned optimized protocol for generating bottle gourd composite plants, 14 bottle gourd germplasm collections with diverse genetic backgrounds were selected to screen for desirable genotypes. Based on the criteria of obtaining at least one positive transgenic root, composite plants were successfully obtained from all of the tested 14 bottle gourd germplasm collections. The number of induced roots, defined as the average number of emerged transgenic roots from five composite plants, ranged from 1.05 ± 0.33 to 9.05 ± 0.10. The number of induced roots from accession PS30 was the

highest, followed by those of G32, NXZ, and YD-3, and the lowest average number of roots induced was observed in Nanqingnan (Fig. 4). Transformation efficiency, defined as the ratio of the number of positive transgenic roots to the number of emerged putative transgenic roots, ranged from approximately 1.28–36.81%. We observed that Changguang showed the highest transformation efficiency followed by Qingxiu and J104-1, which showed comparatively high transformation efficiency. A comparatively low transformation efficiency was observed in G32, Yuanhul, Nanqingnan, YD-4, and Shangyuduanpu with a transformation efficiency of ≤ 10%, and the lowest transformation efficiency was observed in NXZ (Fig. 5). The induction rate, defined as the ratio of the number of positive transgenic plants to the number of plants infected with *A. rhizogenes*, ranged from approximately 6.7–73.3%. The highest induction rate was observed in Changguang, with an induction rate of approximately 73.3%, and the lowest induction rate was observed in Shangyuduanpu and NXZ, with an induction rate of approximately 6.8% (Fig. 6). The results show that based on the highest transformation efficiency, highest induction rate, and moderate number of induced roots, the accession “Changguang” is the optimal genotype suitable for hairy root induction in bottle gourds.

Discussion

Bottle gourd is a common horticultural and medicinal crop. Challenges faced in genetic transformation considerably hinder the evaluation of gene function in this crop. In our study, we developed an optimized protocol for transformation of bottle gourd plants and screened for a desirable genotype for generating bottle gourd composite plants. To develop a more efficient transformation procedure for generating bottle gourd composite plants, we examined and optimized various methods, including hairy root culture medium, age of the seedlings, explants, site for inoculation, temperature of co-cultivation, vectors, and genotypes, most of which were not mentioned in this study since they have been investigated in several studies (Collier et al. 2005; Clemow et al. 2011; Mellor et al. 2012; Ilina et al. 2012; Khandual et al. 2014) and a breakthrough was not made in the present study.

The most notable improvement in our study was the use of a sterile seed germination pouch as a growth carrier, in which the excised stems of vigorous plants were further incubated with liquid CIM (Fig. 2B–F). Previous studies report that cut shoots are inserted into *A. rhizogenes*-containing Fibrgro® cubes or rockwool, which can be easily contaminated, and it is difficult to observe the growth of hairy roots in these media (Collier et al. 2005; Clemow et al. 2011; Mellor et al. 2012; Khandual et al. 2014). Moreover, it is easy to damage hairy roots when removing Fibrgro® cubes or rockwool. The sterile seed germination pouch is a plant growth bag specifically used to observe root growth. The liquid moves upward through a paper core in the pouch via capillary action to support plant growth. In this regard, the CIM added to the seed germination pouch should be appropriate and prevent plants from getting soaked in CIM. The other challenge addressed in the culture of bottle gourd composite plants was that the leaves of the explants tended to turn yellow. In this case, the CIM was poured out and the explants were rinsed several times with sterile ddH2O.

Although the essential ex vitro composite plant induction method was similar to methods described previously (Collier et al. 2005), the observed transformation efficiency varied between species (Clemow et al. 2011; Mellor et al. 2012; Ilina et al. 2012; Khandual et al. 2014). Approximately 2–4 transformed roots per transformed plant are obtained with a transformation efficiency of approximately 80% in soybean (Collier et al. 2005), and 1–4 transformed roots per transformed plant are obtained with a transformation efficiency of approximately 70% in pea (Clemow et al. 2011). The achieved transformation efficiencies in cowpea and squash are approximately 80% and 85%, respectively (Mellor et al. 2012; Ilina et al. 2012). However, in bottle gourd, due to the poor efficiency of genetic transformation in diploid plants, the highest transformation efficiency obtained was 36.81% (Fig. 5), with induction rates ranging from approximately 6.7–73.3% (Fig. 6), and the number of induced roots was approximately 1–9 (Fig. 4). Nonetheless, the transformation efficiency was considerably higher than that of traditional *A. tumefaciens-mediated* transformation. A higher transformation efficiency can be achieved by screening for excellent genotypes from more bottle gourd germplasm collections in the future.

The optimized protocol presented here for generating bottle gourd composite plants is an efficient, rapid, and widely applicable tool for studying gene expression or gene function associated with roots and root-associated responses (salt stress, nutrient absorption, lateral root, or root biology), and interactions of shoots and roots in bottle gourd. This tool has significant potential for enabling development in bottle gourd research.

Conclusions

Using seed germination pouches as growth carriers, an optimized protocol was established to generate bottle gourd composite plants from bottle gourd explants via infection with the *A. rhizogenes* strain R1000. Fourteen genotypes of bottle gourd were screened based on the number of induced roots, transformation efficiency, and induction rate, and “Changguang” (transformation efficiency ~ 36.8%) was selected as an excellent genotype suitable for hairy root induction using our system. The optimized system provides a useful tool for studying gene function associated with roots and root-associated responses, and the interactions of shoots and roots in bottle gourd in the future.

Declarations

Author Contributions YW, GL, and XW (Xiaohua Wu) designed the experiment. LH helped conducting the experiment. YL, ZF, ZM, JW, XW (Xinyi Wu), BW, and ZL assisted in performing the experiments. YW wrote and revised the manuscript. GL supervised the project. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest the authors declare no conflict of interest.

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Figures

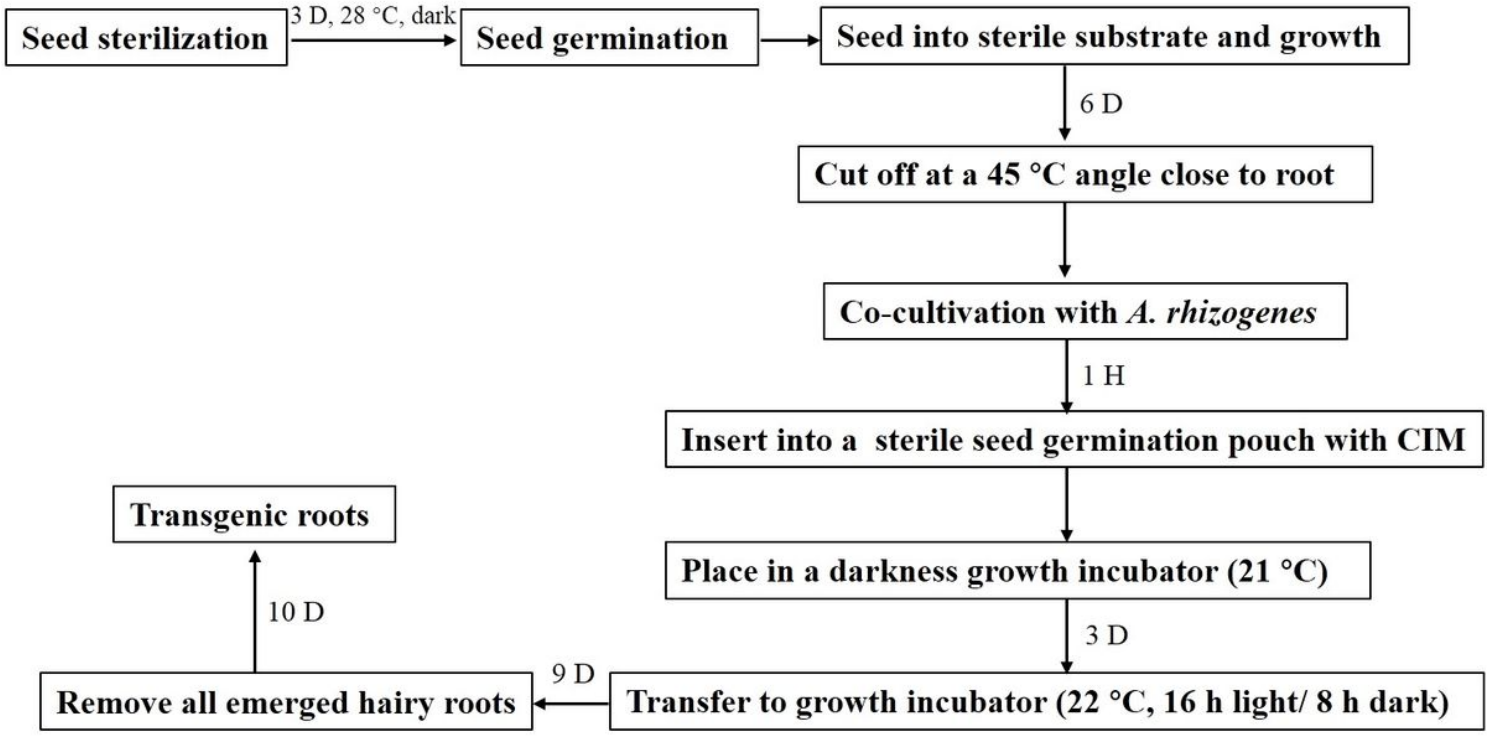

Figure 1

Schematic representation of the timeline required for the generation of bottle gourd composite plants under the optimized conditions.

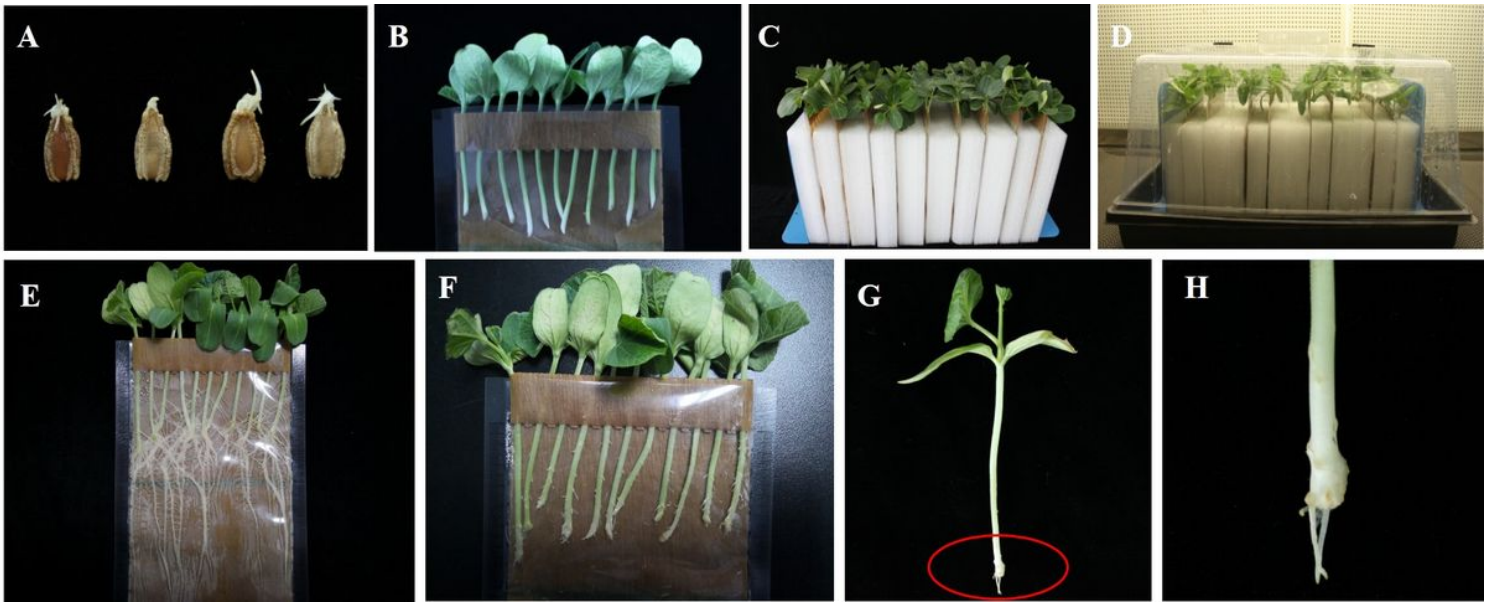

Figure 2

Illustrations of the main steps of the procedure for the generation of bottle gourd composite plants. **(A)** Germinated seeds of bottle gourd. **(B)** The six-day-old bottle gourd excised plants were inserted into a sterile seed germination pouch after *Agrobacterium rhizogenes* infection. **(C)** The seed germination pouches were fixed with a foam board. **(D)** The bottle gourd plants were placed in a growth chamber and

sealed to maintain optimal moisture levels. **(E)** Hairy roots emerged 12 d after infection with *A. rhizogenes*. **(F)** The first hairy roots were discarded since they were not transgenic roots. **(G)** Transgenic roots emerged after another 10 d of co-culture. **(H)** Magnified view of the selected area in G.

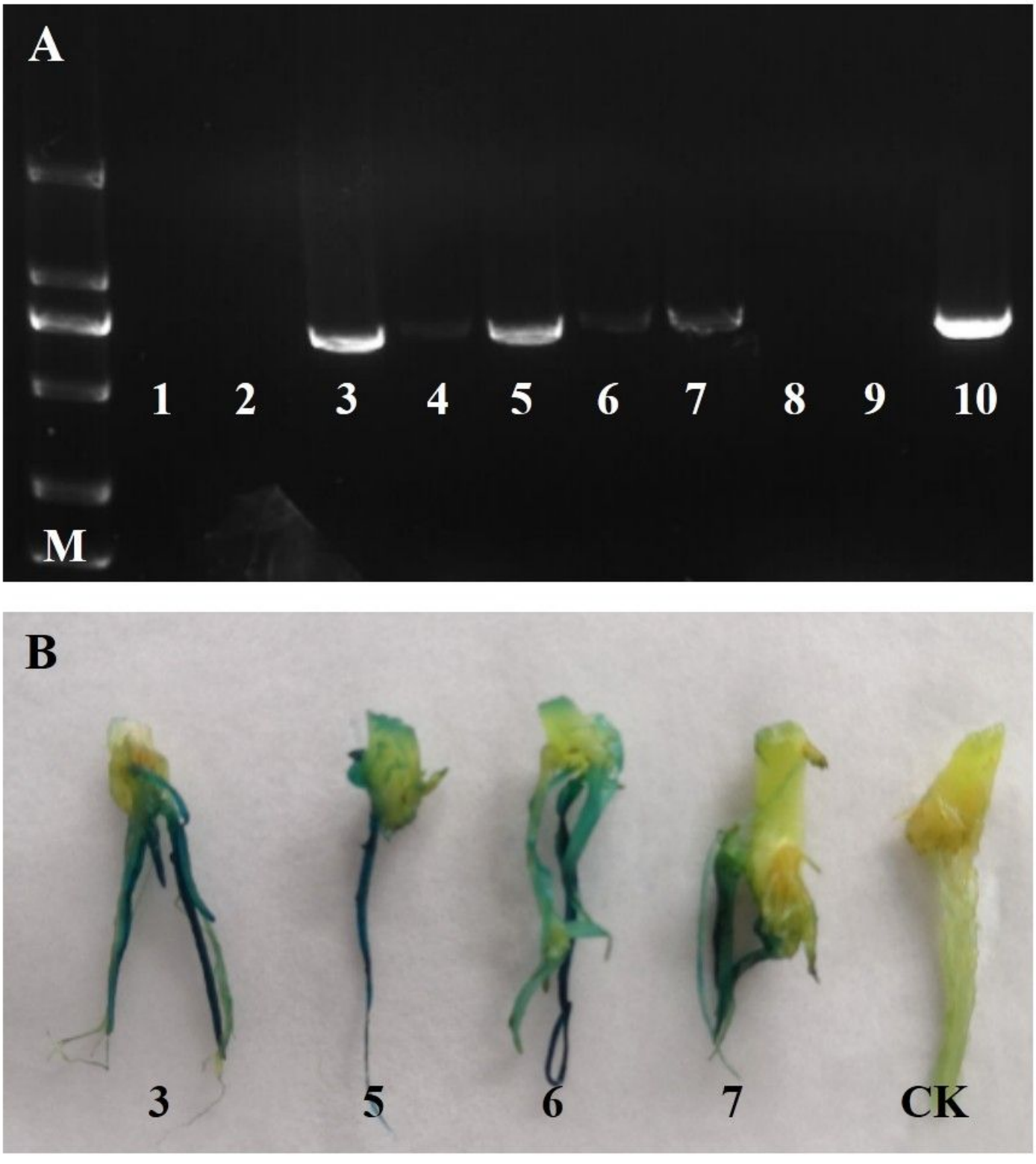

Figure 3

Identification of the transgenic roots. (A) Polymerase chain reaction analyses of bottle gourd composite plants. M, DL 2000 DNA molecular marker (the reference bands are 2,000, 1,000, 750, 500, 250, 150, and 100 bp from top to bottom, with a brightest 750 bp band); Lanes 1–7, DNA from different transgenic roots; Lane 8, negative control 1 (DNA of wild-type roots); Lane 9, negative control 2 (water used as a template); Lane 10, positive control (binary vector pBI121 plasmid DNA). (B) GUS histochemical staining of bottle gourd composite plants. 3, 5, 6, 7, four putative positive transgenic roots in (A); CK, negative control (DNA of wild-type roots); GUS, β-glucuronidase.

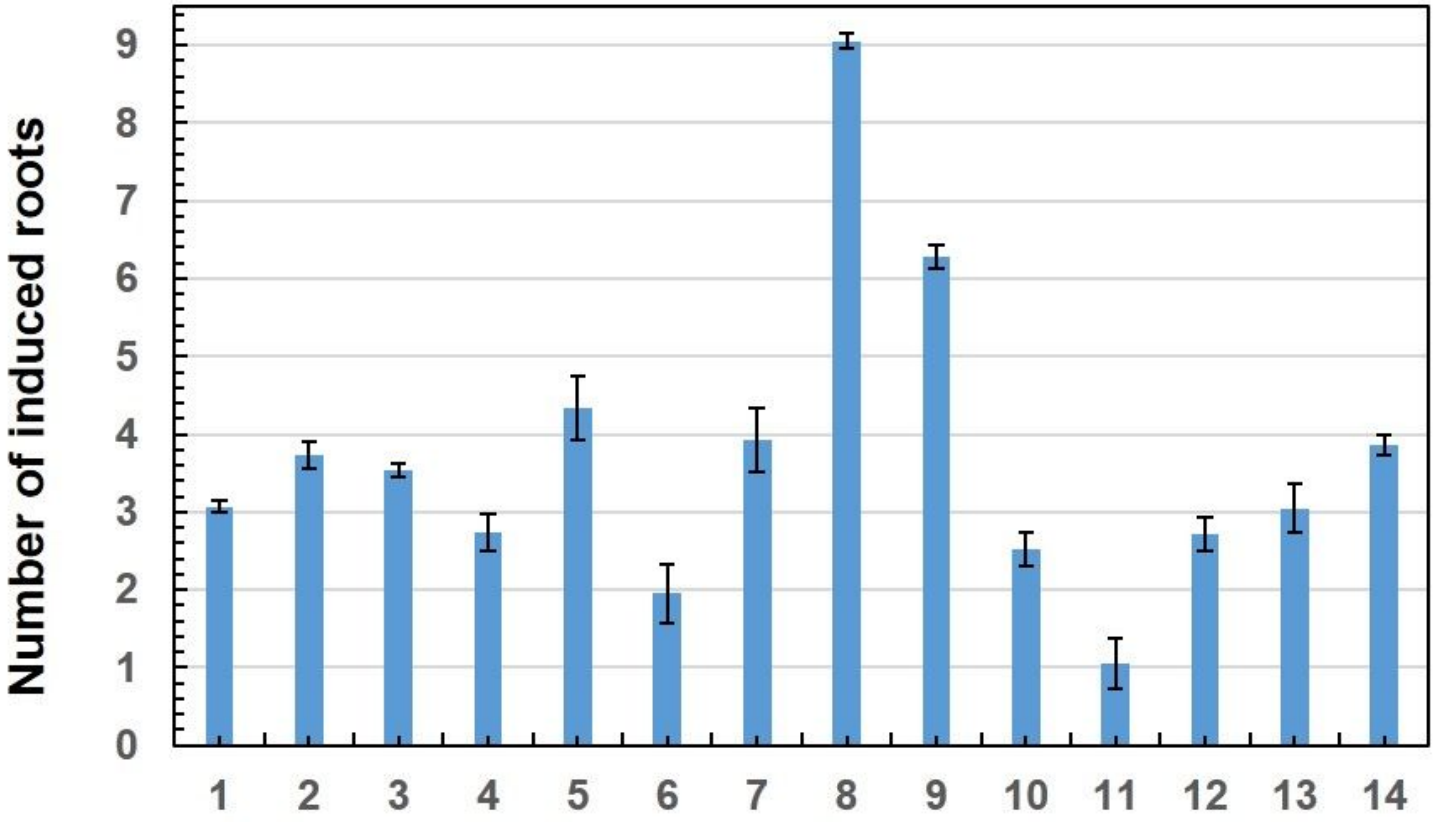

Figure 4

Average root numbers of 14 bottle gourd germplasm collections. 1–14 represent acces-sion Chuangguang, Qingxiu, J104-1, JH-8, YD-3, HZ, Qiangyao, PS30, G32, Yuanhulu, Nanqingnan, YD-4, Shangyuduanpu, and NXZ, respectively.

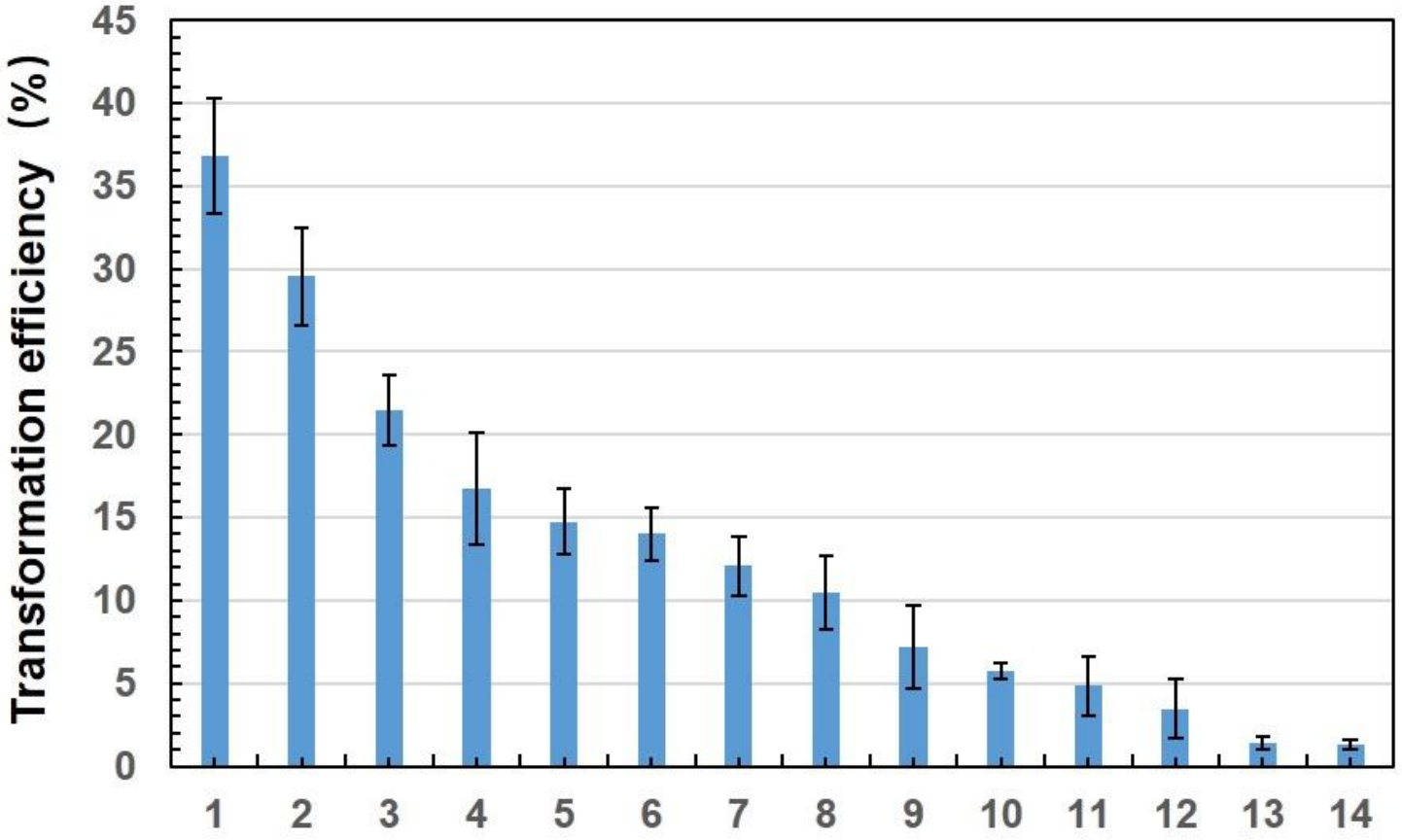

Figure 5

Transformation efficiency of 14 bottle gourd germplasm collections. 1–14 represent ac-cession Chuangguang, Qingxiu, J104-1, JH-8, YD-3, HZ, Qiangyao, PS30, G32, Yuanhulu, Nan-qingnan, YD-4, Shangyuduanpu, and NXZ, respectively.

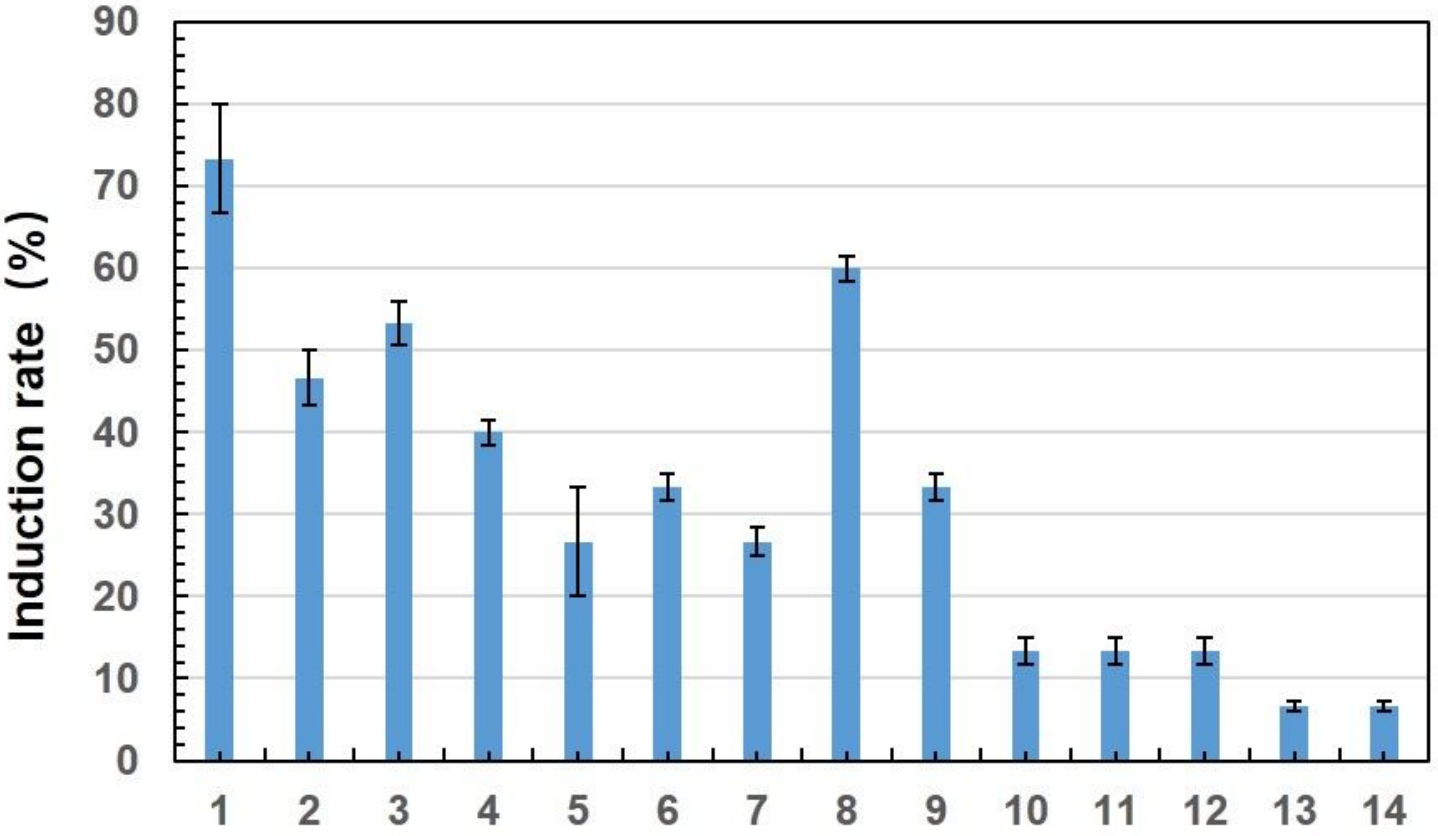

Figure 6

Induction rate of 14 bottle gourd germplasm collections. 1–14 represent Chuangguang, Qingxiu, J104-1, JH-8, YD-3, HZ, Qiangyao, PS30, G32, Yuanhulu, Nanqingnan, YD-4, Shang-yuduanpu, and NXZ, respectively.