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HIGHLY EFFICIENT *RHIZOBIUM RHIZOGENES*-MEDIATED HAIRY ROOT TRANSFORMATION FOR GENE FUNCTIONAL STUDY IN MUNG BEAN *(Vigna radiata* (L.) R. Wilczek)

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SUMMARY

Mung bean (*Vigna radiata* (L.) R. Wilczek) is one of the economically important legume crops in Asia. The demand for high quality mung bean seeds is currently increasing. Moreover, the crop can fix the atmospheric nitrogen. Those make the crop is suitable for sustainable agriculture and a major source of the plant-based protein foods. Functional genomics study in mung bean is required to comprehend the molecular mechanisms behind agronomic traits and elevate the crop trait improvement process; however, it is severely hampered due to the lacking of efficient and large-scale genetic analysis tool. *Rhizobium rhizogenes*-mediated hairy root transformation is a quick and efficient alternative to investigate root-specific processes and interactions in different species. In this study, we developed a protocol to generate hairy roots with low cost and high transformation efficiency that has not been reported in mung bean. Using the method, nearly 100% of the *R. rhizogenes* infected plants formed hairy roots and carried at least three transgenic roots harboring the desired construct per plant. It only required twenty days to complete the whole transformation process. In addition, the composite plants, that are composed transgenic roots and wild-type shoot, were used to examine with the fixing nitrogen bacteria and showed the GUS expression in nodules. In conclusion, our highly efficient *R. rhizogenes*-mediated transformation system provides high throughput genetic analysis assay for functional genetics study, and biotechnological application in mung bean.

Keywords: Hairy root transformation, mung bean, nodulation, *Rhizobium rhizogenes*

INTRODUCTION

Mung bean (*Vigna radiata* (L.) R. Wilczek) is a traditional cultivated legume in South, East, and Southeast Asia. Short duration and low input requirements are the advantages make mung bean as a suitable rotation crop for small holder farmers. Mung bean seeds are rich in protein, fiber, and contain higher levels of folate and iron than most other legumes (Keatinge *et al.,* 2011). Moreover, mung bean can fix atmospheric nitrogen via root rhizobial symbiosis, leading to improve soil fertility and texture (Graham and Vance, 2003). Therefore, mung bean is an ideal crop for sustainable agriculture and has become a major source of the plant-based protein foods.

Despite the socioeconomic importance, genomic and genetic resources for molecular breeding and crop improvement of the legume lag behind others such as *Medicago truncatula, Lotus japonicus* and soybean *(Sato et al.,* 2008; Schmutz *et al.,* 2010; Young *et al.*, 2011; Varshney *et al.*, 2013). The draft genome of mung bean sequence was recently constructed that covered 80% of the estimated genome size (Kang *et al*., 2014). The reference genome sequence is an useful resource for developing molecular markers that accelerate the mung bean breeding program (Kim *et al*., 2015). However, translational genomics, studies comparing the genome organization or gene structure and function of model versus crop species, are not sufficient to study gene function and improve mung bean crop traits due to low-quality reference genomes, and less effective molecular tools (Somta *et al.,* 2022). Genetic modification by overexpression or gene knock-out is the approach to investigate gene function, and stable genetic transformation is an efficient gene analysis tool. However, the stable transformation is labor-intensive and too inefficient to be useful on a large scale. In contrast, the transient transformation especially hairy root transformation mediated by *Rhizobium rhizogenes* (*R. rhizogenes*), provides an alternative fast, efficient, and large-scale tool to study gene function in plants. Importantly, the approach would be effective tool to investigate genes that are involved in root biology, such as those functioning in symbiotic and pathogenic interactions, biotic and abiotic stress tolerance, or nutrient uptake (Nguyen *et al.,* 2021; Jain *et al.,* 2023).

Though *R. rhizogenes*-mediated hairy root transformation is well-established in different legume species both *in vitro* and in plant inoculation method, no systematic method has been conducted in mung bean using *in vivo* inoculation approach yet (Somta *et al.,* 2022; Chen *et al.,* 2023). In this study, we established a rapid and highly efficient *R. rhizogenes*-mediated hairy root transgenic system for mung bean. The transgenic efficiencies of different mung bean genotypes were assessed based on the GUS staining assay. In addition, the approach is efficient for analyzing the symbiotic interaction between mung bean and nitrogen-fixing, *Bradyrhizobium japonicum* (*B. japonicum*) soil bacteria.

MATERIALS AND METHODS

Plant materials

Mature seeds of different mung bean cultivars including DX1, DX2 and DX3 were provided as gifts from Dr. Tran Thi Truong, at the Legumes Research and Development Center, Vietnam Academy of Agricultural Sciences, Hanoi, Vietnam. These cultivars

have been widely cultivated in the North provinces of Vietnam and showed high yield and adaptation.

Bacterial strains and plasmids

The *R. rhizogenes* K599 harboring the pZY102 plasmid and microsymbiotic nitrogen-fixing, *B. japonicum* USDA110 were provided as gifts from Prof. Gary Stacey at University of Missouri- Columbia, MO, USA.

Seed sterilization and plant growth conditions

Mung bean seeds were surface sterilized with 70% ethanol for 1 min, followed by 10% Clorox for 10 min. They were then rinsed four - five times with sterile deionized water and germinated on trays that contain 72 pots and were filled with 1:1 (v/v) mixture of sterilized perlite and vermiculite. Trays were watered regularly with plant nutrient solution B&D, supplemented with 1mM KNO_3 (Broughton and Dilworth, 1971) and maintained in an environmentally controlled plant growth chamber (16:8 h light/dark photoperiod, 27°C, 100% humidity). Microsymbiotic nitrogen-fixing, *B. japonicum* USDA110 was cultured in HM medium (Cole and Elkan, 1973) with 0.004% chloramphenicol and grown at 28-30°C for 3 days. After 3 days of culture, bacteria were pelleted and diluted in sterile water to an optical density, OD_{600} of 0.02 for inoculation. Bacterial solution was inoculated directly onto the composite mung bean plants.

***R. rhizogenes*-mediated mung bean hairy root transformation**

The binary plasmid pZY102 was used in this study (Zeng *et al.,* 2004). The *GUS* gene that contains an intron and driven by CaMV 35S promoter was used as a selection marker. Mung bean hairy root induction and transformation procedures were developed based on previous reports in soybean (Nguyen *et al*., 2018; Nguyen *et al*., 2021) with some modification. Briefly, the single colony of *R. rhizogenes* K599 harboring the pZY102 plasmid was picked, streaked on the LB plates containing 50 mg/L spectinomycin, and incubated at 28^{o}C for one day. A day before inoculation, fresh bacterial culture of K599 was resuspended in 1 mL liquid LB medium, then spread on the LB plates containing appropriate antibiotics. The plates were incubated at 28^{o}C overnight, and fresh bacterial paste was used to inoculate into the 2.5-3 days old seedlings. The day of inoculation, the fresh bacteria were collected by scraping on the surface of the plates. Bacterial mass was stabbed through the hypocotyl proximal to the cotyledon using needles. Infected seedlings were covered with a sterile transparent lid to keep 100% humidity, and maintained in plant growth chamber (16:8 h light/dark photoperiod, 27°C). Twelve days after infection, hairy roots about 0.5-1 cm in length were observed and counted.

For mung bean nodule formation, transgenic composite plants (mung bean plant with hairy roots) were then inoculated with *B. japonicum* USDA110. Nodules produced on transgenic roots were harvested at 25 dpi, confirmed for GUS expression.

DNA extraction and PCR assay

Chromosomal DNA was isolated using CTAB method (Doyle, 1990), following routine isolation techniques from hairy roots for genotyping assay. Hairy roots that

formed at the inoculated site of the infected plant were collected, pooled, and used for DNA extraction. The PCR amplification was performed using *GUS* gene-specific primers with PCR master mix (K0172, Thermo Scienctific, USA). Specific primer pairs used for genotyping were GUS-F: TGGTGGGAAAGCGCGTTACAAG; GUS_R: ACCTGCCAGTCAACAGACGCGTG were designed based on the *GUS* gene sequence and synthesized by Phusa Genomics, Can Tho, Vietnam. The PCR products were analyzed by electrophoresis on 1% agarose gel.

GUS histochemical staining and calculation of transformation frequency

Eighteen days after inoculation with K599, fifteen to thirty plants per treatment that formed hairy roots were randomly collected, immersed in the GUS staining solution and incubated at 37^{o}C overnight (Blázquez, 2007). Samples were then transferred into ethanol 70% for clearing and storage. The hairy root transformation frequency was calculated by numbers of plants produced hairy root per total inoculated plants, and the transgenic roots frequency was based on the numbers of GUS-stained roots per total induced hairy roots per plant.

Statistical analysis

Each experiment with 50 seedlings was a biological replicate and repeated two additional times. Sample means between genotypes or treatments were compared using one-way ANOVA followed by Tukey's multiple range test. All statistical analyses and graphs were performed using Graphpad Prism v9.0 (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Genotype-dependent in *R. rhizogenes* mediated hairy root transformation in mung bean

R. *rhizogenes* strain K599 causes hairy root disease and effectively induces hairy root formation in different plant species including several legume species (Boisson-Dernier *et al.,* 2001; Kereszt *et al.,* 2007; Geng *et al.,* 2012; Aggarwal *et al.,* 2018; Nguyen *et al.,* 2021). Moreover, the composite plants that contain induced hairy roots are effective material to study root biology (Kereszt *et al.,* 2007). Therefore, we developed an efficient, simple and rapid *R. rhizogenes*-mediated hairy root transformation *in vivo* in mung bean using strain K599. Three mung bean cultivars; DX1, DX2, and DX3 were tested with *R. rhizogenes* K599, and all genotypes produced hairy root at the infected site at twelve days after inoculation (Figure 1a-c). However, hairy root induction frequency was different among genotypes. While nearly 100% of the tested seedlings from the cultivars DX1 and DX2 produced hairy roots, this frequency was significantly reduced in the DX3, 79.55% (Figure 1d). Not only the hairy root induction frequency was low in the DX3, numbers of hairy root per plant produced by the DX3 were reduced to a half as compared to other cultivars (Figure 1e). These data suggested the hairy root induced by *R. rhizogenes* K599 in mung bean was dependent on genotype, and the DX1, DX2 cultivars performed better than the DX3 in transformation efficiencies (98.85±1.15%, 98.75±1.25% and 79.55±6.15%) and numbers of root hair formation (7.1±0.37, 6.5±0.4, and 2.7±0.38). Induced hairy roots transformation through *R. rhizogenes* has been considered as genotype-independent

approach to generate transgenic root that no significant difference in hairy root induction frequency were observed among tested cultivars (Fan *et al.,* 2020; Cheng *et al.,* 2021; Zhou *et al.,* 2022). However, several reports recently indicated that genotype is a critical factor effect the transformation efficiency in *R. rhizogenes* mediated hairy root transformation (Aggarwal *et al.,* 2018; Xu *et al.,* 2020; Niazian *et al.,* 2023). Thus, our results suggested that genotype is a critical factor should be considered to examine before establish *R. rhizogenes* mediated hairy root transformation in mung bean.

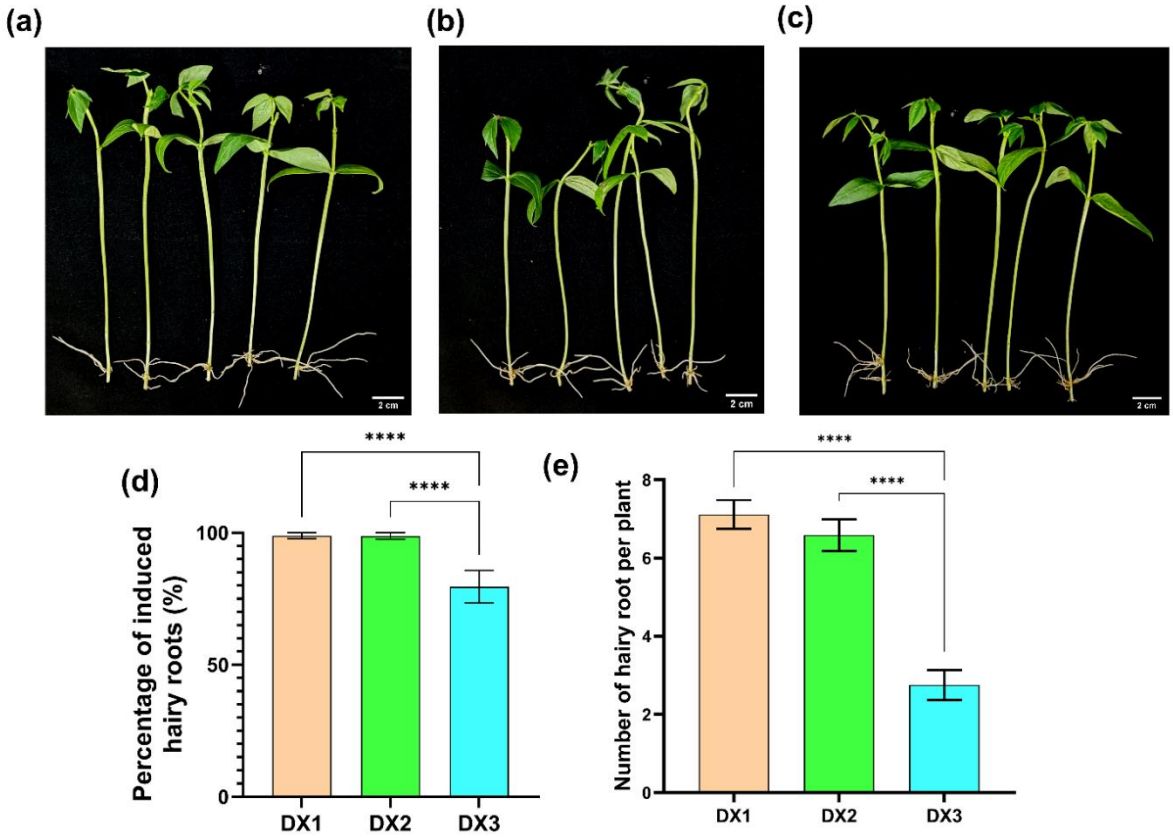

Figure 1. Hairy roots induction in three Vietnamese mung bean cultivars. Representative photographs of mung bean plants: (a) DX1, (b) DX2, (c) DX3 produced hairy roots at 18 days post injected with *R. rhizogenes* strain K599. (d) Hairy roots induction rate and (e) numbers of hairy roots produced per plant at 18 days after injected with *R. rhizogenes* strain K599. Data represent means ± SEM, n > 44. Statistical analysis was done by one-way ANOVA followed by a post-hoc Tukey's multiple range test, **** $p < 0.0001$.

To determine whether the induced hairy roots are transgenic or not, we performed GUS staining and PCR analysis for independent infected plants. More than fifteen hairy roots from each cultivar were randomly selected for GUS staining. Consistence with low transformation efficiency, the transgenic root ratio per plant was also genotype dependent. Whereas 50-53% of transformed roots from the cultivars DX1 and DX2 were transgenic roots, only 29.08±6.29% of hairy roots produced by the

DX3 cultivar were carrying the *GUS* gene (Figure 2a,b,c,e). PCR analysis further confirmed that the induced hairy roots from these cultivars were indeed transgenic roots, contained *GUS* gene (Figure 2f).

Belongs to legume species, mung bean can establish a symbiosis with *rhizobia* (Graham and Vance, 2003). Therefore, to evaluate the reliability of the approach to study root biology in mung bean, we inoculated the composite mung bean plants with nitrogen-fixing soil bacteria, *B. japonicum* strain USDA110. Twenty-five days after inoculation, nodules were formed in the induced hair roots of the composite plants. In addition, GUS staining showed nodules and the hairy roots carrying the nodules were positive transgenic roots and nodules (Figure 2d). Thus, the result indicates the method we developed here is reliable and useful for root functional study in mung bean.

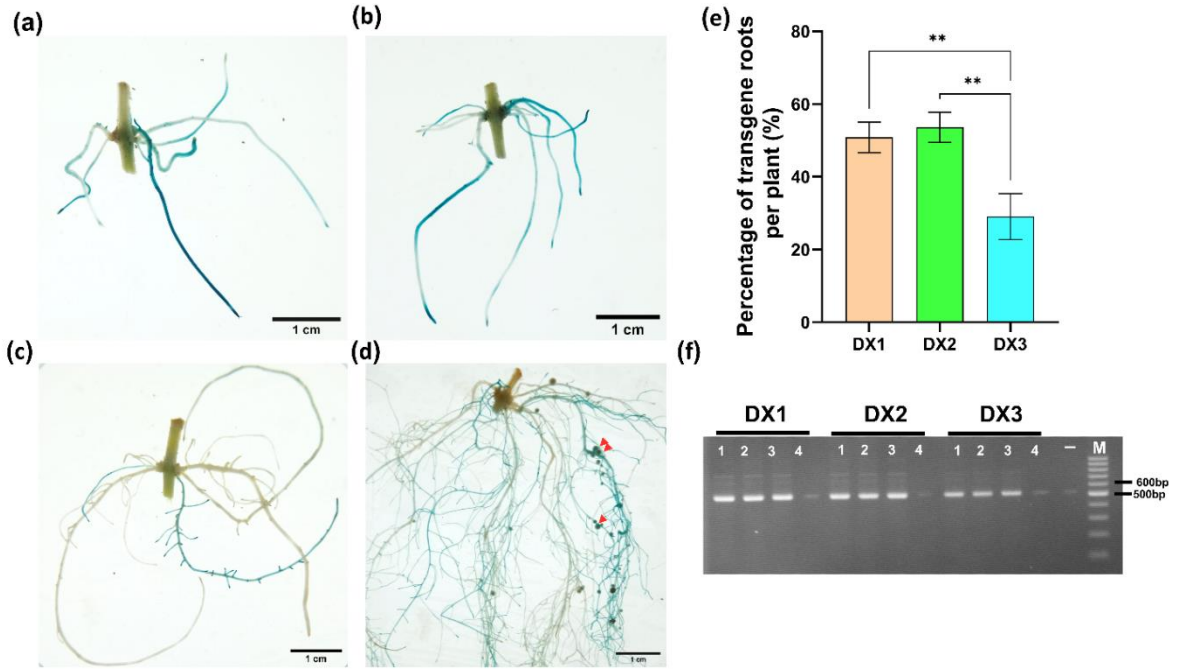

Figure 2. Verification of the transgenic hairy roots. (a-c) GUS staining assay of the 18dpi hairy roots from DX1, DX2 and DX3 respectively. (d) Transgenic hairy roots carrying nodules at 25 days after inoculation with *B. japonicum* USDA110 showed GUS expression. Red arrows indicate GUS-stained nodules. (e) Positive hairy root transformation frequency from different mung bean genotypes. Values are mean ± SEM, n > 15. Statistical analysis was done by one-way ANOVA followed by a post-hoc Tukey's multiple range test, ** $p < 0.01$. (f) PCR analysis of *GUS* gene in transgenic hairy roots. 1-3: Hairy roots induced by K599; 4: non transgene root; -: negative control.

Detailed *R. rhizogenes*-mediated hairy root transformation protocol for generating composite mung bean plants

The pipeline steps of *R. rhizogenes*-mediated hairy root transformation in mung bean are shown in Figure 3. The method described here is time and cost-efficient approach to obtain transgenic roots that can expect 80–100% of the *R. rhizogenes*-infected plants, develop 6–7 hairy roots with at least three transgenic roots carrying the

desired gene construct within twenty days.

Briefly, mature mung bean seeds were surface sterilized using ethanol 70% for one minute, followed by 10% Clorox for ten minutes. Seeds were then washed with four-five times sterilized deionized water and placed into sterilized perlite: vermiculite with ratio 1:1 and watered with the sterilized BD medium supplemented 1 mM KNO_3. The tray containing seeds were covered with a transparent lid sterilized with 70% ethanol, and maintained in a growth chamber at 27ºC.

On the same day as the sterilization of the seeds, *R. rhizogenes* K599 harboring the desired construct was streak on the LB plate containing appropriate antibiotics and incubated at 28ºC for 2 days. The single colony was resuspended in 1 mL liquid LB medium, incubated at 28ºC with orbital shaking at 200 rpm for 2 h. About 200 µL of the bacterial suspension were spread on the LB plate containing the appropriate antibiotics and incubate at 28ºC overnight.

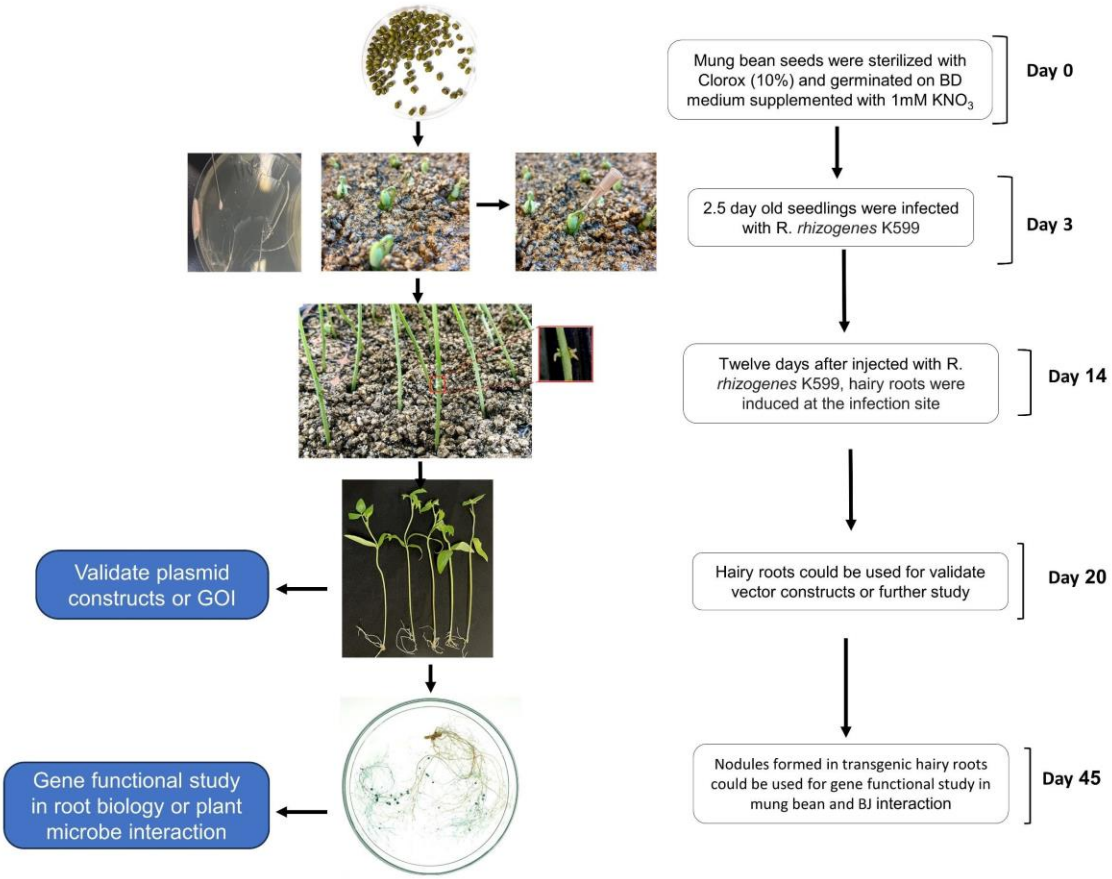

Figure 3. Workflow of the *R. rhizogenes* K599 –mediated hairy root transformation in mung bean to study root biology. GOI (gene of interest).

On day 3, fresh bacteria were collected from the plates and used for inoculation. Three days after germination, healthy seedlings with unfolded green cotyledons

were used for the transformation experiments. Bacterial paste was stabbed through the central part of the hypocotyl proximal to the cotyledon of the plantlets using needle. *R. rhizogenes* infected plants were watered with BD media, kept at high humidity by covering the transparent lids, and maintained under growth chamber condition (27ºC, 16:8 h light/dark photoperiod). Tenth days after inoculation, callus and hairy root were observed at the infected sites.

When the hairy roots were approximately 5–10 cm in length, individual or all hairy roots could be cut off for future analysis such as CRISPR efficiency, mRNA expression of gene of interest. In addition, the primary root could be removed and transferred the composite plant to new trays. Plants could be inoculated with symbiotic bacteria, *B. japonicum* USDA110 and phenotype and genotype of the hairy roots can be analyzed after three weeks.

CONCLUSIONS

In this study, we developed a simple, fast, low cost and labor-efficient protocol to generate hairy roots in mung bean for functional genomic study. The method has advantage over other approaches that it does not require aseptic condition to conduct the experiments and produce large number of composite plants within three weeks. This efficient, high-throughput transformation method makes available to analyze gene function and biotechnological applications in different mung bean cultivars.

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