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A robust in-vitro and ex-vitro *Agrobacterium rhizogenes*-mediated hairy root transformation system in mungbean for efficient visual screening of transformants using the *RUBY* reporter

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Abstract

Background Mungbean is one of the most economically important grain legume crops in Asia. Functional genomics studies in mungbean are necessary to understand the molecular mechanisms behind agronomic traits, to advance the crop improvement. However, this progress is significantly impeded by the absence of effective and extensive genetic analysis tools. *Agrobacterium rhizogenes*-mediated hairy root transformation has become a powerful tool for studying gene function and an efficient alternative for investigating root-specific interactions and processes in different species, due to its quick and simple methodology. *Agrobacterium*-mediated plant transformation, however, is known to be difficult in legumes, especially in mungbean.

Results In this report, we developed an *Agrobacterium rhizogenes*-mediated mungbean transformation system using both in-vitro and ex-vitro approaches, with *RUBY* employed as a reporter gene. We optimized various parameters, including mungbean genotypes, explant age, optical density of the bacterial culture, co-cultivation medium, and acetosyringone concentration. Our findings indicated that in-vitro transformation was more efficient than ex-vitro in terms of hairy root induction percentage and the proportion of transformed hairy roots expressing the *RUBY* reporter gene. However, the ex-vitro transformation technique was faster and less complex than the in-vitro method. The highest transformation efficiency for *RUBY* expression was achieved using 5-day-old cotyledonary nodal explants of cv. *K-851*, inoculated for 30 min with A4 *Agrobacterium* cells resuspended in full-strength MS medium at an OD_{600} of 0.5 and supplemented with 100 µM acetosyringone. A total of 60 composite plants were generated and evaluated through PCR, resulting in a transformation efficiency of 6.13%. These optimized parameters also led to the highest percentage of *RUBY* expression using the two-step ex-vitro hairy root transformation method.

Conclusion We have developed a simple, rapid, low-cost, and labor-efficient *Agrobacterium rhizogenes*-mediated mungbean transformation protocol using both in-vitro and ex-vitro approaches, with *RUBY* as a reporter gene. This method enables the generation of composite mungbean plants that are easier to handle, exhibit higher

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transformation efficiency, and can be effectively used for root specific functional genomics studies. We expect this technology to be widely adopted for investigating root-related processes in mungbean and other plant species.

Keywords Agrobacterium Rhizogenes, RUBY, Mungbean, Gene functional studies, Visual detection, Hairy root transformation, Composite plant

Background

Mungbean (*Vigna radiata* L.) is the third most important short-duration grain legume, cultivated in Southeast Asia, valued for its supply of easily digestible protein. It serves as an invaluable dietary source, rich in protein and free from flatulence-causing factors, thus meeting the nutritional needs of a large population globally. Mungbean is grown on approximately seven million hectares (mha), primarily in tropical and subtropical regions of India, with an average productivity of 750 kg/ha [1, 2]. In recent years, the demand for mungbean production has significantly increased due to rising domestic consumption and export potential. This growing demand also presents opportunities to enhance the income and livelihoods of smallholder mungbean farmers.

In contrast to other legume crops such as *Glycine max*, *Medicago truncatula*, and *Lotus japonicus*, genomic and genetic resources for mungbean remain limited, despite its significant socioeconomic importance [2–6]. Recently, a draft genome covering approximately 80% of the estimated mungbean genome was developed [7], providing a valuable resource for molecular marker development and facilitating the acceleration of mungbean breeding programs [8]. However, due to the limited quality of available reference genomes and the inefficiency of current molecular tools, translational genomics studies—such as those comparing genome organization, gene structure, and gene function between model and crop species remain inadequate for advancing gene function analysis and trait improvement in mungbean [9].

Genetic modification through gene knockout or overexpression is an effective strategy to study gene function, with stable genetic transformation being a powerful tool for such analyses. However, stable transformation in mungbean is labor-intensive and inefficient, limiting its large-scale application. In contrast, transient transformation methods—particularly *Agrobacterium rhizogenes*-mediated hairy root transformation offer a faster, more efficient, and scalable alternative for plant functional genomics studies. This approach is especially useful for investigating genes involved in various root-mediated physiological processes, including symbiotic and pathogenic interactions, nutrient uptake, and responses to abiotic and biotic stresses [10, 11]. *Agrobacterium rhizogenes*-mediated hairy root transformation has been well-established in various legume species using both in-vitro and ex-vitro inoculation techniques [9, 12, 13]; however, only limited reports are currently available for mungbean.

Agrobacterium-mediated transformation—using Ti or Ri plasmids—is based on the integration of the transfer DNA (T-DNA) into the host genome. Confirmation of transgene insertion is therefore a critical step, which can be achieved through molecular biology techniques such as PCR [14], Southern blotting [15], and,, whole genome sequencing (WGS) [16]. Detection of selectable marker genes, typically conferring resistance to herbicides or antibiotics, is a common method for identifying transformed cells [17]. Determining the optimal concentration of the selectable agent is critical, as it must effectively inhibit non-transformed cells while minimizing the risk of escape plants. This requires thorough experimentation to establish the appropriate dosage for efficient selection. While these factors are essential for generating stable transformants, they can also present significant bottlenecks, particularly due to the reliance on selectable marker genes [18]. An alternative approach for identifying transgenic materials involves the use of reporter genes, which enable visual inspection of transformed cells. Reporter genes such as *luciferase* (*LUC*) and *β-glucuronidase* (*GUS*) have been widely used for screening transformants. However, these methods are considered destructive and require specialized equipment or the addition of exogenous substrates to detect fluorescence activity [19]. Other reporter genes, such as *green fluorescent protein* (*GFP*), are among the most extensively used in genetic transformation studies. *GFP* is non-destructive, does not require exogenous substrates, and its expression is cell-autonomous, independent of cell type or tissue location [20]. Despite these advantages, *GFP* has been reported to induce deleterious effects on transformed cells and may hinder regeneration from transformed tissues [21]. *DsRed2*, another fluorescent reporter gene, offers several advantages. It shows no detrimental effects on plant growth or fertility and is easier to detect than *GFP*, owing to the minimal autofluorescence in the red spectrum [22]. The detection of red fluorescent protein is generally considered more reliable than *GFP* because of the faint autofluorescence under red light [23]. In a comparative study, [24] evaluated the screening efficiency of herbicide resistance (Basta) versus *GFP* fluorescence and concluded that *GFP* fluorescence was more consistently correlated with confirmed transgenics,

as verified by PCR analysis. Based on their findings, the authors also suggested that reporter genes are more reliable than selectable marker genes for identifying true transgenics and eliminating false-positive (chimeric) events that may escape antibiotic selection [24].

Other interesting and reliable reporter genes have also been identified, enabling the visual identification of transformed cells with the naked eye and eliminating the need for fluorescence or light-based imaging. This system leverages the biosynthesis of colorful compounds such as betalains [25] and anthocyanins [26, 27], allowing real-time, in vivo detection without additional substrates or equipment. Recently, *RUBY* has emerged as a novel reporter gene that enables the visual identification of transformed cells with the naked eye. *RUBY* is an artificial open reading frame that encodes the necessary enzymes for betalain biosynthesis and has proven to be an effective visible reporter in both monocot and dicot plant species [25, 28, 29]. It is a promising tool for plant transformation studies, particularly in systems where non-destructive, substrate-free detection is desirable.

In this study, we have developed a rapid and highly efficient *Agrobacterium rhizogenes*-mediated hairy root transformation system in mungbean, using both in-vitro and ex-vitro approaches. We also investigated the visibility of the *RUBY* reporter gene during the early stages of hairy root formation and evaluated its effectiveness as a visual marker across seven mungbean genotypes with diverse genetic backgrounds. Additionally, this approach offers potential for efficient analysis of symbiotic interactions between mungbean and nitrogen-fixing soil bacteria.

Materials and methods

Plant materials

Seven mungbean cultivars cv. *K-851*, *PUSA-105*, *MUM2*, *ML-267*, *SML668*, *PDM139*, and *PUSA RATNA* were used as plant materials to establish both in-vitro and ex-vitro hairy root transformation protocols. The seeds were obtained from the National Seed Corporation (NSC), New Delhi, India. For the in-vitro assay, mungbean explants were prepared by germinating surface-sterilized seeds in magenta boxes containing 50 mL of full-strength MSB_5 medium [30], following the protocol described by Kumar et al. [31]. Seed germination was carried out in a plant growth chamber set at 28 °C with a 16-h light/8-h dark photoperiod and 60% relative humidity.

For the ex-vitro experiments, sterilized seeds were germinated on filter paper placed inside a plant growth chamber under the same conditions (28 °C, 16-h light/8-h dark, 60% relative humidity). Seeds were irrigated daily with sterilized distilled water for five days or until germination [32]. The germinated seedlings were then transferred to pots in green house containing vermiculite and maintained under similar environmental conditions. Regular irrigation was provided using a mixture of tap water supplemented with one-fourth strength Hoagland solution, as described by [32].

Binary vector construct and mobilization into *Agrobacterium rhizogene* cells

The binary plasmid construct *35S:RUBY* (Addgene #160908), which harbors the *RUBY* reporter gene under the control of the *CaMV35S* promoter, was used in this study (Fig. 1). This construct is hereafter referred to as the

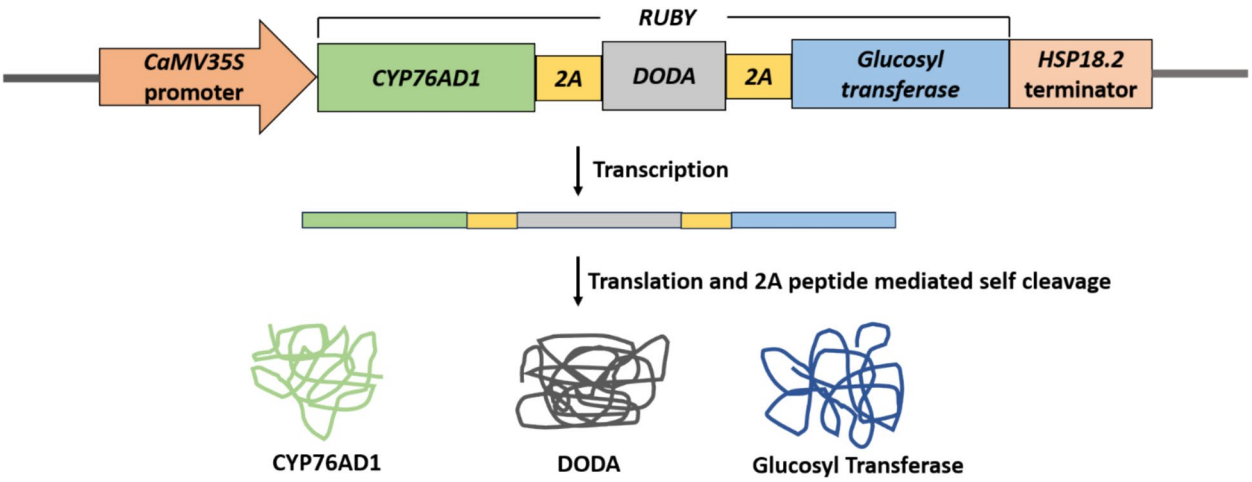

Fig. 1 Schematic strategy for expressing the whole betalain biosynthetic pathway genes in a single cassette. All three betalain biosynthetic genes were fused into a single ORF, and regulated through *CaMV35S* enhanced promoter and *HSP18.2* terminator. In between the genes, sequences that encode *2 A* peptides were placed. The 2 A peptides undergo self-cleavage, thus releasing the individual enzymes for betalain biosynthesis. The ORF of 2 A-linked betalain biosynthesis genes is named as RUBY (ref: Addgene #160908)

RUBY binary vector (RBV) [25]. The RBV was introduced into *Agrobacterium rhizogenes* strain A4 competent cells using the freeze-thaw method, following the protocol described by [33]. Transformed colonies were selected on Yeast Extract Mannitol (YEM) agar plates supplemented with 50 mg/L spectinomycin. A parallel glycerol stock was prepared by inoculating a single transformed colony into YEM broth containing 50% (v/v) glycerol and storing it at −80 °C for long-term preservation. The presence of the *RUBY* gene in transformed colonies was further confirmed by PCR using *RUBY*-specific primers, and the expected gene fragment was successfully visualized.

In-vitro hairy root transformation

A single colony of *Agrobacterium rhizogenes* harboring the *RUBY* binary vector (RBV) was inoculated from a freshly streaked plate into 100 mL of YEM broth (HiMedia, Mumbai) containing 50 mg/L spectinomycin and incubated at 28 °C with shaking at 180 rpm for 14 h, as described [32]. Prior to harvesting, the optical density (OD) of the bacterial culture was measured at 600 nm using a spectrophotometer. The cells were then harvested by centrifugation at 6,000 rpm for 10 min at 4 °C and resuspended in liquid plant growth medium (MS medium without growth regulators) [30], adjusted to pH 5.5, and supplemented with acetosyringone. The bacterial suspension was diluted to the desired OD_{600}, and incubated at 22 °C with shaking at 90 rpm for 30 min prior to explant inoculation [34].

Cotyledonary nodes were excised from 5-day-old mungbean seedlings and individually submerged in the bacterial suspension. The explants were incubated at 22 °C, 90 rpm for 30 min, then briefly dried on sterile blotting paper and placed on Whatman filter paper discs moistened with liquid plant growth medium supplemented with acetosyringone. The explants were co-cultivated for 3 days at 22 °C in the dark, following the method described [31]. After co-cultivation, explants were washed 5–6 times with sterile distilled water and once with distilled water containing 500 mg/L cefotaxime for 10 min with gentle agitation to remove excess *Agrobacterium* from the surface. The explants were dried again on sterile blotting paper and cultured on MS medium solidified with 2.5 g/L phytagel and supplemented with 500 mg/L cefotaxime. Cultures were maintained in a growth chamber at 28 °C under a 16-h light/8-h dark photoperiod with 60% relative humidity, as previously described [31]. Hairy root induction percentage and transformation efficiency were assessed by visually observing red-colored roots after two weeks of culture. Three independent in-vitro experiments were conducted to evaluate the impact of different parameters on *Agrobacterium rhizogenes*-mediated hairy root transformation in mungbean.

Ex-vitro hairy root transformation

We also evaluated the hairy root response under ex-vitro inoculation conditions and calculated the percentage of response in seven mungbean genotypes. Seven-day-old mungbean seedlings, with both cotyledons intact, were used for the study. Seeds were germinated in small cups containing vermicompost for 7 days and maintained at 28 °C with a 16 h/8 h (light: dark) cycle and 60% relative humidity in a plant growth chamber. The mungbean seedlings of all seven genotypes were inoculated with *Agrobacterium rhizogenes* A4 strain. The fully grown bacterial culture harboring the *RUBY* binary vector, streaked on YEM medium containing 50 mg/L Spectinomycin, was used to inoculate the cotyledon site by pricking with a toothpick. The cotyledons were detached before inoculation, following the method described by [35]. The experiment was conducted in triplicate. After inoculation, the mungbean seedlings were placed in a humid chamber and irrigated with a 1/4 strength Hogland nutrient solution. Hairy root induction percentage and transformation efficiency were calculated by visually observing *RUBY* expression on the 10th day post-inoculation, using the equations described in the section "Parameters for *Agrobacterium rhizogenes*-mediated Hairy Root Transformation."

Parameters used to assay effect of *Agrobacterium rhizogene* on hairy root transformation

Three parameters—bacterial cell density, inoculation duration, and co-cultivation temperature—were assessed for their effect on hairy root induction efficiency. The experiments were conducted in triplicate for each of the three factors: A: three cell densities (OD_{600} = 0.3, 0.5, and 0.7); B: three inoculation durations (20, 30, and 45 min); C: three co-cultivation temperatures (22 °C, 25 °C, and 28 °C). For each parameter set, a minimum of 15 explants were used per replicate. Hairy root induction and transformation efficiency percentages were calculated accordingly.

$$\mathbf{HR}\ (\%) = \mathbf{N_{hr}}/\mathbf{N_T} \times 100$$

$$\mathbf{TE}\ (\%) = \mathbf{N_{RUBY}}/\mathbf{N_{hr}} \times 100$$

Where **HR** denotes the hairy root induction percentage, **Nhr** stands for the number of cotyledonary nodes that developed hairy roots with an approximate length ≥1 cm, $\mathbf{N_T}$ represents the total number of inoculated cotyledonary nodes, **TE** indicates the transformation efficiency, $\mathbf{N_{RUBY}}$ refers to the number of cotyledonary nodes that produced at least one hairy root and showed RUBY gene expression [36, 37].

Influence of plant genotype and explant age on response to *Agrobacterium rhizogene*

We studied the interaction between *Agrobacterium rhizogenes* and various mungbean genotypes cv. (*PUSA-105*, *PUSA-RATNA*, *K-851*, *SML668*, *ML267*, *MUM2*, and *PDM139*), on the response to *Agrobacterium rhizogenes*, along with the effect of explant age (3-, 5-, and 7-day-old seedlings). Cotyledonary nodes were prepared from all seven genotypes as previously described, and the transformation procedure followed the in-vitro hairy root transformation protocol detailed earlier. Hairy root induction percentage and transformation efficiency were assessed by visually observing *RUBY* expression on the 14th day of culture, as described in the preceding section.

Molecular confirmation of A4 Rhizogene cells and analysis of putative transgenic hairy roots

Agrobacterium rhizogenes (A4) cells were confirmed through PCR analysis using *rolA*, *rolB*, and *mas1*-specific primers, as listed in Table 1. The *35S:RUBY* vector construct was agro-mobilized using the freeze-thaw method and confirmed by PCR before plant inoculation (Fig. 2). The transgenic mungbean hairy roots (red roots) from all seven genotypes were molecularly analyzed through PCR and RT-PCR to confirm T-DNA integration and gene expression. Genomic DNA was extracted from the transgenic hairy roots and evaluated for the presence of the *rol* and *RUBY* genes by PCR using the primers outlined in Table 1. Untransformed mungbean root genomic DNA was used as a control for this experiment.

RUBY gene expression in the hairy roots was also evaluated by semi-quantitative RT-PCR. Total RNA was extracted from control and *RUBY*-transformed roots using TRIzol® reagent (Thermo Fisher Scientific) and purified. First-strand cDNA was synthesized using the cDNA Synthesis Kit for RT-PCR (Promega, USA). Semi-quantitative RT-PCR was performed using the cDNA as a template in a PCR system (Applied Biosystems) with the green master mix (Promega, USA) and RUBY gene-specific primers listed in Table 1. The experiment was conducted in three biological replicates, each taken from three different parts of the red roots. A putative β-tubulin

Table 1 Sequences of primers used for the study

Name and purpose	Primer name	Sequence (5′ → 3′)
Rhizogene Ri plasmid specific	rolA Fw	ATGCCTCACCAACTCACCA
	rolA Rv	ATGCCTCACCAACTCACCA
	rolB Fw	ATGGATCCCAAATTGCTATTCCTTCCACGA
	rolB Rv	TTAGGCTTCTTTCTTCAGGTTTACTGCAGC
	Mas1 Fw	CGGTCTAAATGAAACCGGCAAAC
	Mas1 Rv	GGCAGATGTCTATCGCTCGCACTCC
Real Time PCR	*Vr-tubulin* Fw	TCAGTTGAGGCCGAAGAAGA
	Vr-tubulin Rv	AAACCAGTCCCAGTCCCAAA
RUBY	Ruby Fw	AAGGGATCCTCGAGATCCTCGTG
	Ruby Rv	AGGGTACCAAGCTTTGGCTCCGT

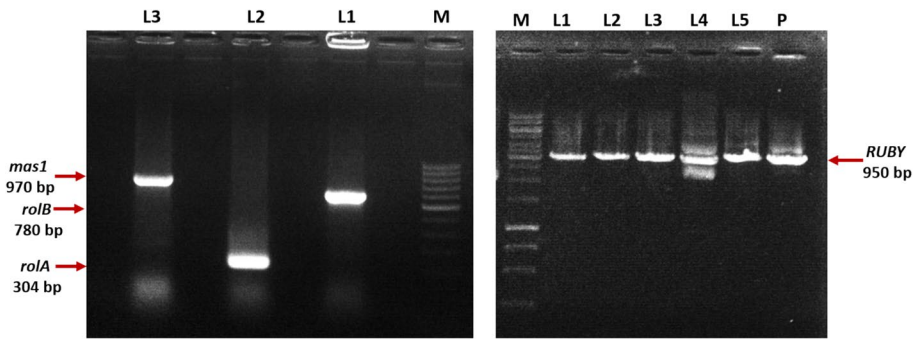

Fig. 2 PCR confirmation of *Agrobacterium rhizogene* (A4) cells using *rolA*, *rolB* and *mas1* gene specific primers and agromobilization confirmation of *35:RUBY* plasmid construct into A4 electrocompetent cells. **a** Lane L3: 970 bp PCR fragment of *mas1* gene using mas1 specific primers, Lane L2: 304 bp PCR fragment of *rolA* gene using *rolA* specific primers, Lane L1: 780 bp PCR fragment of *rolB* gene using *rolB* specific primers, **b** 950 bp fragment observed in PCR using *RUBY* gene specific primers after mobilization into A4 electocompetent cells. Lane L1-L5: 5 independent clones used for PCR confirmation during agromoblization. Lane P: positive control plasmid *35S:RU*BY, Lane M: 1 kb Marker

gene served as an internal control. The primer sequences are provided in Table 1.

Statistical analyses

All assays were biologically replicated at least three times for each treatment (combination of parameters) mentioned above in the methods. All data were analyzed using the Statistical Package for the Social Sciences (SPSS 16.0) and Excel. Significant differences between mean values were determined using Bonferroni analysis at $p = 0.05$, as described earlier [31].

Results

in-vitro hairy root induction and detection of *RUBY* gene expression

After culturing on MS medium for 7 days, the *Agrobacterium rhizogenes*-infected cotyledonary node explants produced white calli at the site of the nodal junction (Fig. 3a). On the 10th day of culture, elongation of the hypocotyl and emergence of the hairy roots were observed (Fig. 3b). By the 15th day, elongated transformed roots were observed emerging from the white calli (Fig. 3c). On the 20th day of culture, a long red hairy root, approximately 2–3 cm, expressing the *RUBY* gene was observed (Fig. 3d). A bunch of *RUBY*-expressing hairy roots was observed on the 25th day of culture in MS medium containing 500 mg/L cefotaxime (Fig. 3e). Composite plants (with transgenic roots and wild-type shoots) with a bunch of red hairy roots were seen after 25 days of culture (Fig. 3f). No red roots were observed in the control inoculated with only the A4 strain; only white hairy roots were seen (Supplementary Fig. 1). The composite plants were acclimatized in vermicompost and maintained in a plant growth chamber.

Hairy root induction and expression of *RUBY* gene via ex-vitro method

Hairy root induction was observed 2 weeks after infection with *Agrobacterium rhizogenes* at the point of wounding on the nodal region of the seedling. The infected seedlings were maintained in a humid chamber, and sterilized distilled water was regularly sprayed over

Fig. 3 The in-vitro hairy root induction and *RUBY* gene expression in mungbean. **a** Emergence of white calli at the nodal site of cultured cotyledonary node (bar = 5 mm) **(b)** The emergence of first hairy roots at the basal and nodal region of cotyledonary node after 10th day of culture (bar = 5 mm). **c** At the 15th day of culture, the elongated transformed roots were observed from the white calli (bar = 2 cm). **d** long red hairy roots approx. 2–3 cm expressing *RUBY* gene was observed at the 20th days after culture (bar = 3 cm). **e** bunch of *RUBY*-expressing hairy roots was observed on the 25th day of culture **(f)** composite plant with transgenic root and wild type shoot was observed with a bunch of red hairy roots were seen after 25th days of culture (bar = 3 cm)

the surface of the seedlings every 2 days. Red-colored hairy roots, expressing the *RUBY* gene, were visible in the bunch of hairy roots that emerged (Fig. 4a-c). The hairy roots elongated to 2–3 cm after the third week of inoculation (Fig. 4d). No red-colored roots were observed in the control inoculated with only the A4 strain; only white-colored hairy roots were seen (Supplementary Fig. 2). Non-transformed white hairy roots were removed, and the mungbean composite plants were transplanted into pots containing vermicompost and irrigated with 1/4 strength nutrient solution (Fig. 4e).

We observed significant variation in hairy root induction, ranging from 10 to 90%, depending on the plant genotype and age of the seedlings. In the in-vitro experiment, 5-day-old mungbean seedlings of cv. *K-851* exhibited the highest efficiency, resulting in a greater percentage of hairy root induction (Fig. 5a). In the ex-vitro experiment, the 7-day-old cv. *K-851* genotype also showed the best performance for hairy root induction, while genotypes such as cv. *PUSA RATNA*, *PUSA 105*, *SML668*, *ML267*, *PDM139*, and *MUM2* were less efficient, with induction percentages ranging from 10 to 54% (Fig. 5b).

Optimization of *Agrobacterium rhizogenes* parameters

We observed the highest mean percentage of hairy root induction, 80%, at $OD_{600} = 0.5$, while at $OD_{600} = 0.3$, the induction was 40%, and the least induction, about 20%, was observed at $OD_{600} = 0.7$ (Fig. 6a). Similarly, the highest percentage of hairy root induction and transformation efficiency, 80%, occurred when the explants were inoculated for 30 min, while the least induction, 50% and 30%, occurred at inoculation durations of 20 and 45 min, respectively (Fig. 6b). Explants co-cultivated at 22 °C for 72 h responded most favorably, resulting in the highest induction of hairy roots (85%), whereas the lowest induction, 24%, was observed at 25 °C for 72 h (Fig. 7a-b). The highest mean percentage of hairy root induction and transformation efficiency (90%) was achieved with the treatment combination of OD_{600}/Co-cultivation time/Co-cultivation temperature at 0.5/30 min/22 °C, and the least, 0.7/45 min/25 °C. We found that transformation efficiency was variable depending on the co-cultivation time, co-cultivation temperature, and bacterial density used for *Agrobacterium rhizogenes* inoculation.

Fig. 4 The ex-vitro hairy root induction and *RUBY* gene expression in mungbean using hypocotyl stabbing. **a** hairy root induction observed after 1 weeks of infection at the infection sites of hypocotyls (red arrow) (bar = 1 cm). **b-c** hairy root induction observed after 2–3 weeks of infection (bar = 1 cm). **d-e** Red color hairy roots, expressing the *RUBY* gene observed in the bunch of hairy roots emerged at the sites of hypocotyls (red arrow) (bar = 1 cm)

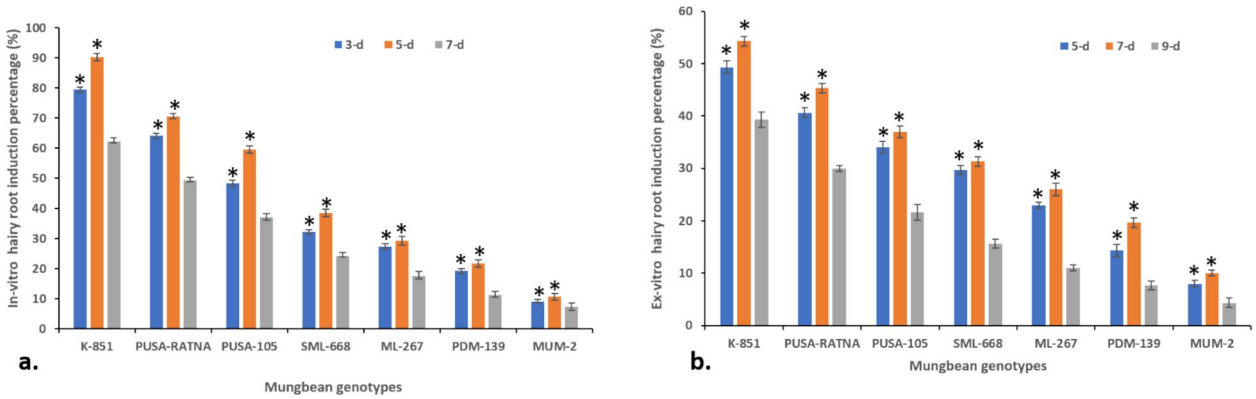

Fig. 5 Effect of mungbean seedling age on in-vitro and ex-vitro hairy root induction percentage in *Agrobacterium rhizogene* mediated plant transformation**. a** in-vitro hairy root induction percentage in seven mungbean genotypes using 3-d, 5-d and 7-d old seedling as explant. **b** ex-vitro hairy root induction percentage in seven genotypes of mungbean using 5-d, 7-d and 9-d old seedlings. The data shows the mean ± S.E of three replicate samples. *Indicates significant differences from the 7-d in in-vitro and 9-d in ex-vitro at $P < 0.05$

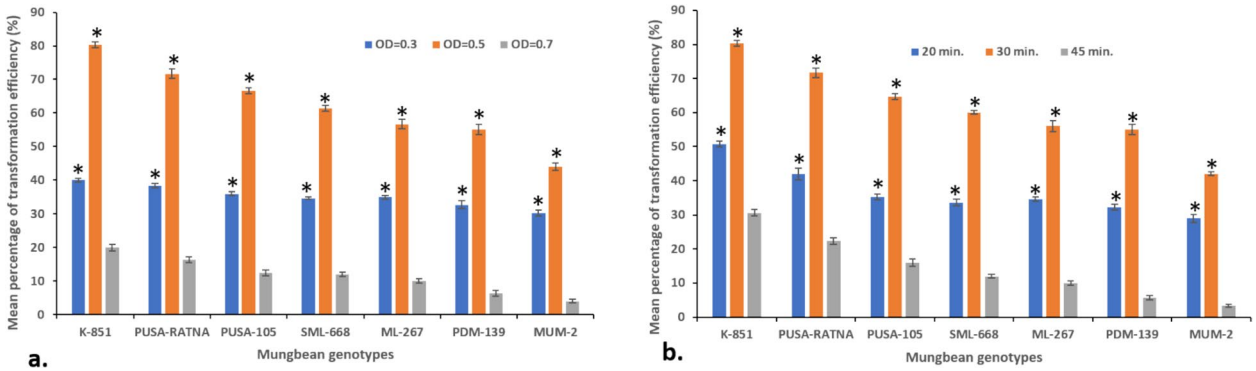

Fig. 6 Effect of bacterial optical density (O.D) and inoculation duration on in-vitro hairy root induction percentage in *Agrobacterium rhizogene* mediated plant transformation**. a** in-vitro hairy root induction efficiency percentage in seven mungbean genotypes using three various bacterial OD. 0.3, 0.5 and 0.7 at OD.600. **b** in-vitro hairy root induction percentage in seven genotypes of mungbean using three various inoculation durations of 20 min, 30 min, and 45 min. The data shows the mean ± S.E of three replicate samples. *Indicates significant differences from the OD. 0.7 and inoculation duration of 45 min. at $P < 0.05$

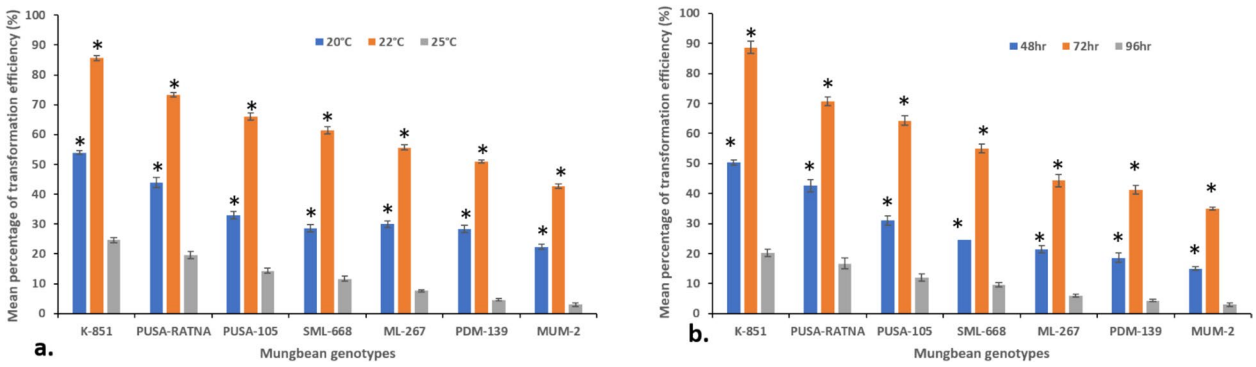

Fig. 7 Effect of Co-cultivation temperature and co-cultivation duration in in-vitro hairy root induction percentage in *Agrobacterium rhizogene* mediated plant transformation**. a** in-vitro hairy root induction percentage in seven mungbean genotypes using three temperatures ranging from 20 °C-25 °C.**b** in-vitro hairy hairy root induction percentage in seven genotypes of mungbean using three various co-cultivation duration 48 h –96 h. The data shows the mean ± S.E of three replicate samples. *Indicates significant differences from the co-cultivation temperature 25 °C and co-cultivation duration of 96 h. at $P < 0.05$

Additionally, we evaluated various concentrations of acetosyringone (50–150 µM) on hairy root generation and transformation efficiency. The optimal response occurred at 100 µM, resulting in 76% transformation efficiency, while the least induction (24%) was observed at a concentration of 150 µM (Fig. 8).

Influence of plant genotype on response to *Agrobacterium rhizogenes*

We studied the effect of plant genotype, and explant age on *Agrobacterium rhizogenes* transformation efficiency and hairy root induction percentage (Fig. 5). We observed a variation in root induction percentage, ranging from 10 to 90%, depending on the treatment. The genotype cv. *K-851* responded most efficiently to in-vitro hairy root induction when infected with *Agrobacterium rhizogene*s A4 strain. The 5-day-old mungbean cotyledonary node explants were found to be more efficient than both 3-day-old and 7-day-old explants (Fig. 5a). The data revealed that the highest induction of 90% of hairy roots occurred when inoculating 5-day-old cotyledonary nodes of cv. *K-851*, while the lowest induction (10–72%) was observed in cv. *PUSA RATNA*, *PUSA105*, *SML668*, *ML267*, *PDM139*, and *MUM2* when infected with *Agrobacterium rhizogenes* A4 strain. Mungbean cv. *K-851* exhibited the highest in-vitro transformation percentage, while cv. *MUM-2* showed the lowest (Fig. 5a). The 5-day-old mungbean cotyledonary nodes, particularly with cv. *K-851*, resulted in the highest mean transformation percentage (82%).

We also evaluated the percentage of hairy root transformation efficiency using three different media compositions: half-strength MS (1/2MS), full-strength MS, and B_5 media. We observed that full-strength MS media was more effective than 1/2MS and B5 media, leading to a higher percentage of hairy root formation efficiency (58%) in cv. *K-851*. In contrast, lower percentages of transformation (ranging from 47 to 12%) were observed in cv. *PUSA RATNA*, *PUSA105*, *SML668*, *ML267*, *PDM139*, and *MUM2*, as validated through PCR (Fig. 8a).

Molecular analysis of in-vitro and ex-vitro transformants

The in-vitro and ex-vitro mungbean putative transgenic roots (red roots) were molecularly analyzed for the presence of the *RUBY* gene through PCR amplification. We observed a 950 bp amplification product of the *RUBY* gene from the genomic DNA of transformed roots. No amplification product was detected in the untransformed control or in the transformed white roots (Fig. 9a). PCR amplification of the rol gene was also conducted on the transformed transgenic and control roots, revealing the expected 304 bp fragment. This confirmed that the tested roots were indeed hairy roots, not control roots (Fig. 9b). A total of 60 composite plants were generated and evaluated through PCR, yielding a transformation efficiency of 6.13% (Table 2). Semi-quantitative RT-PCR analysis demonstrated *RUBY* gene expression in both ex-vitro and in-vitro transformed hairy roots at different time points, compared to the control. No significant difference was observed in the expression levels between the initial, middle, and terminal parts of the RUBY-expressing red hairy roots, when compared to both the untransformed control and transformed white-colored hairy roots (Fig. 9c).

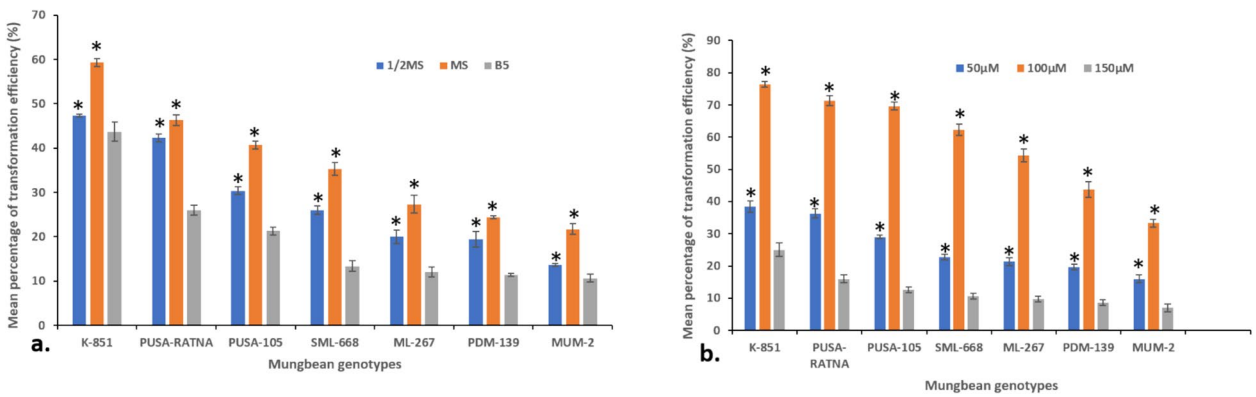

Fig. 8 Effect of culture media and acetosyrinzone concentration in in-vitro hairy root induction percentage in *Agrobacterium rhizogene* mediated plant transformation**. a** in-vitro hairy root induction percentage in seven mungbean genotypes using various media 1/2MS, MS and B5 composition **(b)** in-vitro hairy root induction percentage in seven genotypes of mungbean using three various concentrations of acetosyrinzone ranging from 50–150 µM. The data shows the mean ± S.E of three replicate samples. *Indicates significant differences from the B5 media and 150 µM acetosyrinzone concentration at $P < 0.05$

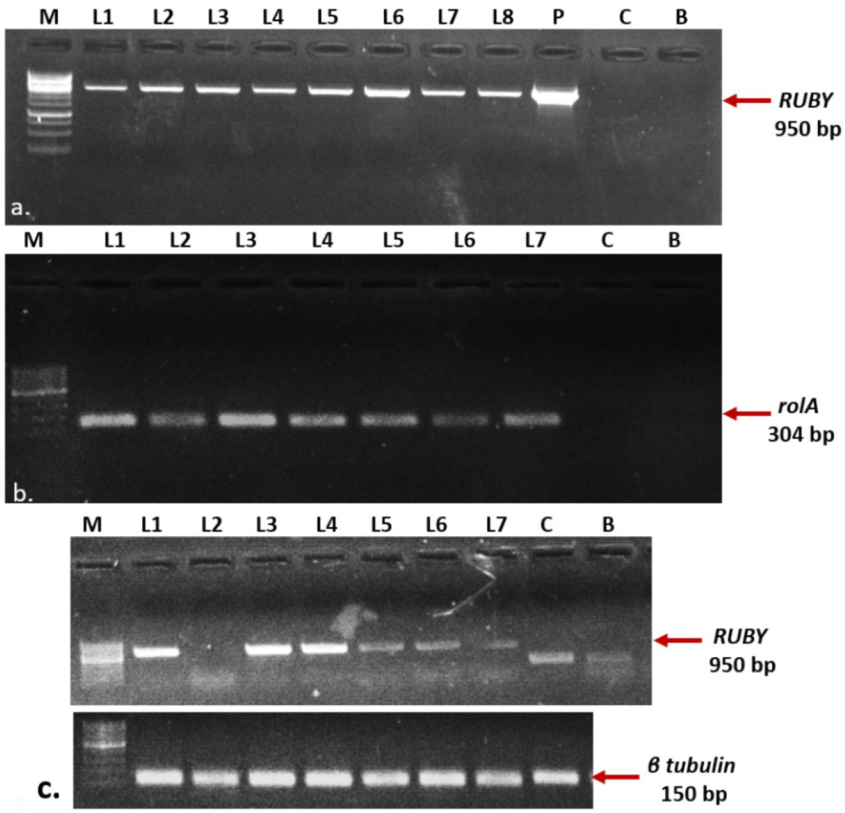

Fig. 9 Molecular analysis through PCR and RT-PCR of mungbean putative transgenic red hairy roots for the presence of the *RUBY*, *rolA* and *rolB* genes. **a** 950 bp fragment of *RUBY* gene detected in in-vitro and ex-vitro mungbean transgenic hairy roots. P: *35S:RUBY* plasmid as positive control. M: 1 kb DNA marker, Lane L1-L8: red putatively transgenic hairy roots, Lane C: white hairy roots as a negative control, B: negative control without template. **c** Semi quantitative RT-PCR based expression of the *RUBY* reporter gene in 7 random putative transgenic red hairy roots using c-DNA as template. *Vr-β tubulin* gene was used as an internal control

Table 2 Summary of the *Agrobacterium rhizogene* mediated plant transformation using 5-day-old cotyledonary node explants of *Vigna radiata* cv. *K-851* harboring *A4-35:RUBY* construct

Exp. No	No. of explants inoculated in Agrobacterium suspension	No. of explants responded with hairy root formation	No. of explants formed red roots	No. of composite plants found positive for *RUBY*/*rolA* gene by PCR	Transformation Efficiency[a] (%)
1	116	74	34	14	12.06
2	124	79	29	12	9.67
3	104	68	34	09	8.65
4	125	81	41	14	11.2
5	113	59	28	11	9.73
Total/average	582[b]	72.2[c]	33.2[c]	60[b]	6.16[a]

[a] $N_{RUBY}/N_{hr} \times 100$

[b] Total

[c] Average response

Discussion

The *RUBY* reporter system serves as a powerful visual indicator for tracking plant genetic transformation and studying gene expression. Unlike traditional reporters such as *GUS*, *GFP*, or *LUC*, the *RUBY* system enables direct visual detection of signals without requiring expensive substrates or specialized imaging equipment. Recently, *RUBY* has been successfully applied

across a broad range of plant species, including both model organisms and crop plants. Beyond simple visual phenotyping, the quantification of betalain pigments produced by *RUBY* provides an effective means to monitor transgene presence, as well as both transient and stable gene expression across various tissues. The in-vitro method offers a rapid, efficient, and reproducible approach for generating a substantial mass of transgenic hairy roots [38]. Moreover, this system enables the swift evaluation of expression vector efficacy and facilitates the testing of various genetic components and regulatory elements under controlled conditions [39].

The effectiveness of in-vitro hairy root induction is governed by several critical factors, including plant genotype, the developmental stage of the explant, *Agrobacterium rhizogenes* strain, media composition, and the culture environment [40]. For successful in-vitro *Agrobacterium*-mediated gene delivery, additional parameters must be optimized. These include bacterial cell density, duration of inoculation, selection antibiotics, their effective concentrations for *Agrobacterium* eradication, acetosyringone concentration, and the choice of selectable marker and reporter genes [41]. Among the available strains, *Agrobacterium rhizogenes* strain A4 is widely recognized for its effectiveness in optimizing in-vitro hairy root induction [42, 43]. This is due to the strain-specific influence on hairy root induction and transformation efficiency, which are key determinants of successful gene transfer [40].

The efficiency of hairy root induction using the *Agrobacterium rhizogenes* A4 strain is significantly influenced by factors such as explant inoculation duration and bacterial cell density [44]. In the initial in-vitro experiment of the present study, we systematically evaluated the impact of these parameters on transformation efficiency. Our results revealed that an inoculation duration of 30 min combined with a bacterial cell density of $OD_{600} = 0.5$ yielded the highest percentage of hairy root induction (Fig. 6). Interestingly, lower bacterial densities produced more favorable outcomes with the A4 strain, suggesting that this strain exhibits higher infectivity compared to other strains reported in previous studies [34, 38, 45].

Two additional factors that significantly influenced in-vitro hairy root induction and transformation efficiency in this study were the type of basal root induction medium and the concentration of acetosyringone. As previously reported [38], culture media can exert a synergistic effect on in-vitro hairy root production in soybean. In our study, full-strength MS medium proved to be more effective than B_5 and half-strength MS (½MS), achieving a transformation efficiency of 58% in mungbean cv. *K-851*. In contrast, lower efficiencies ranging from 47 to 12% were observed in the other six genotypes cv. *PUSA RATNA*, *PUSA105*, *SML668*, *ML267*, *PDM139*, and *MUM2* as validated through PCR analysis (Fig. 8a).

While [38] reported the use of a constant acetosyringone concentration (40 mg/L) in root induction media, other studies have shown that the optimal concentration can vary depending on plant genotype [46]. In our investigation, we tested a range of acetosyringone concentrations (50–150 µM) and found that 100 µM was optimal, resulting in a transformation efficiency of 76%. At the highest concentration tested (150 µM), transformation efficiency dropped to 24% (Fig. 8b). This suggests that both insufficient and excessive acetosyringone levels may adversely affect transformation either by failing to activate *Agrobacterium* virulence genes effectively or by inducing toxicity in plant cells and bacteria [47].

Another critical factor influencing transformation efficiency is the plant genotype [48]. In this study, we evaluated multiple mungbean genotypes and explant ages using the A4 strain of *Agrobacterium rhizogenes*, and found that genotype had a significant impact on in-vitro hairy root transformation efficiency. Among the tested genotypes, cv. *K-851* exhibited the highest transformation rate, with 90% hairy root induction using 5-day-old seedlings, whereas cv. *MUM2* showed the lowest efficiency at 12% (Fig. 5). These findings indicate that the effectiveness of the optimized in-vitro transformation approach is highly genotype-dependent, underscoring the importance of selecting responsive genotypes for successful transformation protocols.

Another important factor influencing hairy root transformation efficiency is the age of the explant tissue [49]. Previous optimization studies in soybean reported no significant difference in transformation efficiency among cotyledons from seeds germinated for 1 to 5 days [38]. However, our findings in mungbean contrast with these observations. We observed the highest hairy root induction efficiency (90%) using 5-day-old cotyledonary node explants, while 7-day-old explants exhibited the lowest transformation efficiency (12%). This indicates that, unlike in soybean, the developmental stage of the explant plays a critical role in mungbean transformation. Our results align with previous reports suggesting that mid-aged explants (e.g., 5- to 6-day-old tissues) are generally more suitable for transformation than either very young or older tissues [31].

The ex-vitro inoculation method, particularly via hypocotyl stabbing, is a rapid and highly efficient strategy for generating composite plants, and it can be completed in either a one-step [35] or two-step procedure [50, 51]. Compared to in-vitro methods, the ex-vitro approach eliminates the need for aseptic conditions, thereby reducing both time and cost [29]. Additionally, the direct use of *Agrobacterium rhizogenes* stab cultures simplify the

protocol, as there is no requirement to prepare bacterial suspensions at defined optical densities. However, the success of the ex-vitro technique is influenced by several factors, including the bacterial strain employed, the plant genotype, and the developmental stage of the explant used.

Similar to the in-vitro approach, ex-vitro hairy root induction and transformation efficiency were significantly influenced by the *Agrobacterium rhizogenes* strain, plant genotype, and explant age. In this study, the A4 strain of *Agrobacterium rhizogenes* was employed for ex-vitro inoculation across seven mungbean genotypes. The highest hairy root formation efficiency (55%) was observed in cv. *K-851*, whereas the lowest (13%) was recorded in cv. *MUM2*. These results underscore the genotype-dependent nature of hairy root transformation efficiency in mungbean under ex-vitro conditions (Fig. 5b).

Both in-vitro and ex-vitro methods employed in this investigation revealed significant interactions among the various parameters studied. Among these, the *Agrobacterium–host* interaction emerged as the most critical factor influencing the efficiency of *Agrobacterium rhizogenes*-mediated transformation [52]. Consequently, identifying the optimal combination of *Agrobacterium* strain and host genotype is essential for maximizing transformation efficiency. Notably, none of the previous optimization studies in mungbean have systematically examined this interaction, although such reports are available for soybean [38, 45, 53, 54]. In our current study, this interaction was found to play a crucial role in both in-vitro and ex-vitro transformation systems. Specifically, the combination of mungbean genotype cv. *K-851* and the *Agrobacterium rhizogenes* A4 strain yielded the highest transformation efficiency, highlighting this as the most effective *Agrobacterium–host* pairing (Figs. 5, 6, 7, 8).

The findings also demonstrated that the two-step ex-vitro inoculation method produced composite mungbean plants more rapidly than the in-vitro method, although with comparatively lower transformation efficiency. Nevertheless, the ex-vitro approach offers advantages in terms of simplicity and reduced technical requirements. This simplicity, however, may lead to fewer or less intimate interactions between bacterial cells and host tissues, potentially limiting transformation success [55].

To visually identify transgenic hairy roots in all seven mungbean genotypes, the *RUBY* reporter gene was employed in both transformation systems. *RUBY* expression was evident during the early stages of cell division and allowed for rapid, non-destructive screening. Unlike traditional reporters such as *GUS*, *GFP*, and *LUC* which require specialized substrates or detection equipment [29], the *RUBY* system provides a cost-effective and equipment-free alternative. Although anthocyanin-based reporters, like *VrMYB90*, have previously been used in mungbean [12], they may yield false-negative results due to variable pigment accumulation [27]. In contrast, *RUBY* consistently marked transformed tissues, as confirmed by PCR-based T-DNA detection in red hairy roots (Fig. 9). Moreover, expression of *RUBY* was uniformly distributed along the length of the transgenic roots (Fig. 3– 4), confirming its utility as a reliable and stable reporter.

Importantly, *RUBY* did not interfere with callus formation, regeneration, or overall plant development and fertility, as previously shown in *Arabidopsis thaliana*, *Nicotiana benthamiana*, and *Plukenetia volubilis* [56]. Beyond its role in gene functional studies, *RUBY* expression in ornamental plants or betalain-producing lines offers potential for developing novel horticultural traits [57]. To further validate this system and examine the relationship between *RUBY*-induced pigmentation and transgene copy number, Southern blot analysis would be a valuable next step, especially to confirm single-copy T-DNA insertions, a hallmark of high-quality transformation events.

Conclusion

The current study establishes a reliable and efficient hairy root transformation protocol for root specific gene functional studies in mungbean. This method offers distinct advantages over other approaches by eliminating the need for stringent aseptic conditions, thereby enabling the production of a large number of composite plants within three weeks. Under controlled conditions, the in-vitro method can effectively generate transgenic hairy roots, while the ex-vitro method serves as a rapid and straightforward approach for transgenic hairy root induction and in-root functional analysis. Using both transformation techniques, abundant red hairy roots were successfully generated after inoculation with *Agrobacterium rhizogenes* harboring the RUBY visible reporter gene. The *RUBY* gene facilitated the visual identification of transgenic hairy roots from the earliest stages of cell division to the development of fully formed hairy roots.

While the transformation efficiency was higher with the in-vitro method, the ex-vitro method provided a quicker route to producing composite plants. The optimized protocols are ideal for rapid and precise root specific functional studies of target genes, including overexpression, suppression, and knockout experiments. Additionally, the in-vitro protocol can be employed to assess the cleavage efficiency of designed gRNAs in a CRISPR/Cas9 system, offering a valuable tool for gene editing applications. This efficient, high-throughput transformation system is a versatile platform for gene function analysis and biotechnological advancements in various mungbean cultivars.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
SK: Conceptualization, Methodology, Investigation, original draft preparation, Supervision. SP: Assisted in performing experiments, PK: Assisted in performing plant tissue culture experiments, NSM: Providing resources, Review and editing, Project administration. SK & NSM: Funding acquisition. All authors have read and agreed for publication of the manuscript.

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Data availability
The original contributions presented in the current study are included in the manuscript/supplementary materials, further inquiries can be directed to the corresponding author/s.

Declarations

Ethics approval and consent to participate
This research did not contain any studies on human and/or animal participants performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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