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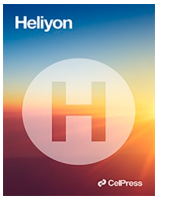

Research article

Molecular validation of genetically transformed *Catharanthus roseus* plants *via* different strains of *Agrobacterium tumefaciens*

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ABSTRACT

Medical plants play a crucial role in the pharmaceutical industry due to their natural synthesis of active compounds. Synthetic methods exist, which provide fewer effective molecules compared to those naturally occurring. *Catharanthus roseus*, a significant medicinal plant, synthesizes vital vinca alkaloids and various secondary metabolites widely used in cancer and hypertension treatment. However, genetic modification using *Agrobacterium tumefaciens*-mediated transformation, a common method for altering periwinkle plants, suffers from low efficiency and reproducibility. Factors like the Agrobacterium strain can influence transformation efficiency and post-transformation regeneration. This study compares the transformation and regeneration efficiency of three A. tumefaciens strains (LBA4404, EHA105, and GV3101) carrying the GUS gene and kanamycin selection via syringe infiltration in *Catharanthus roseus*. Molecular variations between mutants were examined using Inter-Simple Sequence Repeat (ISSR) and GUS expression via quantitative Real-Time PCR (qRT-PCR). Results revealed that GV3101 had the highest transformation efficiency (61.1 %) and LBA4404 the lowest (38 %). However, GV3101-infected explants had the lowest regeneration rate (10 %), the obtained mutants from it exhibited the highest GUS expression. ISSR analysis indicated 37 % polymorphism among mutants, highlighting the impact of Agrobacterium strains on plant genetics and potentially on phytochemical compositions. Overall, this study recommends using GV3101 for high transformation efficiency and LBA4404 for superior *in vitro* regeneration in *Catharanthus roseus*, suggesting a promising method for efficient gene transformation in this plant species.

Abbreviations

ANOVA	Analysis of variance
AS	Acetosyringone
BAP	6-Benzylaminopurine
bp	Base pair

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(*continued*)

GC-Mass	Chromatography-Mass Spectrometry
GUS	β-glucuronidase
IAA	Indole 3-acetic acid
ISSR	Inter-Simple Sequence Repeat
Kb	Kilo base
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NPTII	Neomycin phosphotransferase gene exhibiting resistance to kanamycin
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time PCR
SD	Standard deviation
SEM	Standard errors of mean
T-DNA	Transfer DNA
TBE	Tris Borate EDTA
Ti- plasmid	Tumor-Inducing Plasmid
TIA	terpenoid indole alkaloids
UPGMA	unweighted group average method

1. Introduction

Catharanthus roseus L. (*C. roseus*) also known as periwinkle, originated in India, and described native in Madagascar [1]. *C. roseus* belongs to the family Apocynaceae and has an estimated genome size of 69–124 mega-bases [2,3]. In addition to its decorative properties, folk medicine uses it for treating several diseases since 2600–1550 BC specially in ancient Egypt, Greece, India, and China [4,5]. It is considered as one of the most important medicinal plants due to its indispensable medicinal compounds, including phytols, phenolic acids, flavonoids and terpenoid indole alkaloids (TIA's) [6]. These naturally produced components are found in very small quantities. The pharmaceutical industry is making significant efforts to develop a synthetic equivalent of these natural components, but this technique is highly expensive. Hence, exertions are needed to develop *C. roseus* genetically modified plants with high content of medicinal active components [7]. Many of these alkaloids like (vindoline, ajamlicine, serpentine, catharanthine, vinblastine, and vincristine) are used in cancer drugs therapy and hypertension treatment [8]. Periwinkle leaves were found to be rich in alkaloids and carbohydrates, while the flowers were rich in tannins, triterpenoids, and alkaloids [9]. Despite the valuable and unique features of the periwinkle alkaloids and other components. They are produced naturally in very low level, approximately 0.0005 % of periwinkle active ingredients that make drugs extract from them have a high cost, making the extraction and purification process of its active ingredients is expensive [10]. Therefore, due to the significant market value of the medically significant anti-tumorigenic periwinkle alkaloids, there has been a strong interest in discovering alternative methods for the cost-effective and large-scale production of these pharmaceutical compounds. This includes utilizing callus cultures, cell cultures, and employing techniques such as elicitors, genetic modification, metabolic engineering, and biotechnology tools to produce these compounds via plant tissue culture techniques [11].

Plant tissue culture is a highly effective biotechnological method for quickly multiplying plants in a sterile environment, minimizing the chances of microbial contamination. Various *in vitro* investigations utilizing diverse tissue samples have effectively been carried out to investigate somatic embryogenesis and organogenesis in *C. roseus* [12]. Additionally, multiple efforts have been made to improve and enhance the production of periwinkle active chemicals by polyploidization, nano technology and genetic engineering [13–20]. Some studies have investigated the role of different *in vitro* culturing conditions to induce secondary metabolites production in periwinkle using tissue culture [21–24]. Other studies have used different physical stimulators such as radiation to enhance this plant active ingredients [25–29].

Another approach that has been adapted is the use of molecular tools to obtain genetically modified plants with high content of vinca alkaloids [30–32], in which Agrobacterium mediated transformation have been used as a genetic transformation tool. However, the absence of an efficient techniques for transforming and regenerating *C. roseus* via *Agrobacterium tumefaciens (A. tumefaciens)* has presented significant challenges in the genetic manipulation of this plant. Researchers commonly investigates the concentration of Acetosyringone (AS), explant type, the temperature and duration of co-cultivation, and the concentration of *A. tumefaciens* [33–35]. However, some other factors can also affect the transformation efficiency, such as Agrobacterium strain, transformation method and the used plant cultivar [36]. The selection of the Agrobacterium strain in use for plant transformation can significantly impact the effectiveness of transformation and the expression of foreign proteins. Therefore, it is crucial to carefully study this component during the process [37]. Studies in other plants have revealed that the choice of the used Agrobacterium strains can affect transformation efficacy [38–41]. Nevertheless, no study was conducted in *C. roseus* to investigate the differences in transformation efficiency and *in vitro* regeneration between various Agrobacterium strains. On the other hand, a study using callus as an explant have transformed *A. tumefaciens* strain LBA4404 and found that 98 % of the callus were GUS^{+}, however they have reported no *in vitro* regeneration of the transformed callus [33]. Another study has used hypocotyl as an explant using *A. Tumefaciens* strain EHA105 *via* sonication and showed around 80 % transformation efficiency however, the *in vitro* regeneration efficacy was around 11 % [34]. The use of *A. tumefaciens* strain GV3101 was implement via syringe and vacuum infiltration and however they have only investigated the transit GUS expression to be around 40 % [35].

Nevertheless, there has been no prior research utilizing serval strains of *A. tumefaciens* employing the same transformation

procedure and comparing their transformation effectiveness and *in vitro* regeneration rate on the resulting mutants. Here we have investigated the effect of three strains of *A. tumefaciens* (LBA4404, EHA105, and GV3101) on *in vitro* regeneration and transformation efficiency on *C. roseus*. Additionally, we have conducted molecular evaluation of the obtained mutant using ISSR to evaluate any variation between the obtained mutants. In addition, we have also used qRT-PCR to evaluate changes in *GUS* gene expression in the obtained mutants in strain specific manner.

2. Methods

2.1. Plant in vitro establishment

Seeds of *Catharanthus roseus* (L) G. Don were provided from Horticulture Research Institute, Agriculture Research Center, Cairo, Egypt. Seeds were surface sterilized by submerging in 70 % (V/V) ethanol for 30 s followed by 10 % (V/V) bleach with 1–2 drop of Tween-20 for 15 min. Then seeds were rinsed using sterilized distilled water and incubated in sterilized distilled water for 48 h at 28 °C to enhance germination. After incubation seeds were plot dry and placed on Murashige and Skoog (MS) basal medium [42] supplemented 0.01 % (V/V) Proclin® 300 (Sigma, USA) to control contamination [43,44]. Seed cultures were germinated under 16 h light and 8 h dark photoperiod at 25 °C ± 2.

2.2. Agrobacterium strains and plasmid vector

For transformation, three *Agrobacterium tumefacien* strains LBA4404, EHA105, and GV3101 were transformed following manufacture protocol with pCAMBIA2301 plasmid (11,634 bp) (Fig. 1) (Intact Genomics, USA), differences between the strains in use are described in (Table 1). The vector contains β-glucuronidase (GUS) reporter gene and a selection marker gene NPTII (Neomycin phosphotransferase gene exhibiting resistance to kanamycin) which are driven by $CaMV_{35}S$ promoter. An intron set in the coding sequence of the GUS reporter gene to certify the expression of GUS activity is resulting in the eukaryotic cells.

2.3. Genetic transformation and co-cultivation

A 21-days-old seedling's cotyledons were agroinfiltrated using the three agrobacterium strains. For agroinfiltration preparation following [35], Agrobacteria single colonies were cultured in liquid Luria-Bertani (LB) both supplemented with 50 μg/mL of kanamycin for overnight at 26 °C at 250 rpm. Bacterial cells were pelleted and resuspended in Agrobacterium minimal media supplemented with 100 μM Acetosyringone (AS) for 3 h to induce the virulence genes. Then bacterial cells were centrifuges and resuspended in infiltration media containing (10 mM $MgSO_4$, 10 mM MES pH 5.6) freshly supplemented with 200 μM AS [45]. A needleless 5 mL syringe was used to infiltrate Agrobacterium into the abaxial side of individual seedling cotyledons. Later seedlings were kept on MS media and maintained in the dark for 48 h and then transferred back to a 16/8 h photoperiod for 24 h, steps described in (Figs. 2 and 3 a).

2.4. Plant regeneration

Agroinfiltrated cotyledons were separated from the hypocotyl and placed on callus induction medium containing half strength MS basal salt with vitamins supplemented with 0.5 mg/L BAP, 1 mg/L NAA,50 mg/L kanamycin and 150 mg/L timentin for 6 weeks. Induced Calli were transferred to shoot regeneration medium containing full strength MS basal salt with vitamins supplemented with 1 mg/L BAP, 1 mg NAA for selective media 6 mg/L kanamycin and 150 mg/L timentin for additional 6 weeks. Shoots were separated from callus and placed on the same media for elongation, then placed on half strength rooting media supplemented with 0.5 mg/L NAA, 2.5 mg/L IAA, and 150 mg/L timentin. We also had two controls for our *in vitro* experiment where un-infected explants were placed on anti-biotic free media as negative control and media with antibiotics as positive control. All cultures were maintained under same growth conditions as pre-described for seed germination.

The rooted shoots were acclimatized by gently rinsing the roots with warm water to remove any residues from the MS medium, reducing the risk of fungal contamination. Autoclaved soil, consisting of a mixture of equal parts peat moss and perlite, was prepared and placed into 5 cm plastic pots. The plants were transferred into the sterilized soil, and a plastic cover was placed over them to maintain high humidity. Gradual removal of the plastic cover followed. The plants were kept in a growth chamber under a 16-h light/

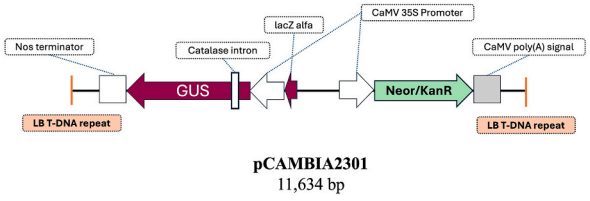

Fig. 1. Schematic drawing of pCAMBIA2301 plasmid transformed *into A. tumefaciens*. The plasmid carries β-glucuronidase (GUS) reporter gene and a selection marker gene NPTII under CaMV 35S promoter.

Table 1
Differences between the three *A. tumefaciens* strains in use (LBA4404, EHA105 and GV3101).

Feature	LBA4404	EHA105	GV3101	References
Chromosome background	Ach5	C58	C58	[89,90]
Marker genes on bacterial chromosome	Rifampicin	Rifampicin	Rifampicin and Gentamycin	[90]
Tumor-Inducing Plasmid (Ti Plasmid)	pAL4404	pEHA105	pMP90	[91]
Marker genes on Ti plasmid	Spectinomycin and streptomycin	Carbenicillin	Gentamycin	[92]
Virulence Genes	Octopine	Nopaline	Nopaline	[93]
Transformation Efficiency	Have high efficiency than GV3101 and EHA105, respectively in tobacco	It gives high transient efficiency than others in *Artemisia*. Henceforth, it gives high results *in Jatropha*	It gives high efficiency than EHA105 in tomato and potato	[94–98]
Transformation Efficiency in *C. roseus*	Using leaf, nodal segments and callus as explant by dipping method 98 % of total explants gave $^{+}$ve results.	Using seedlings as explant by sonication method gave transformation efficiency 11 %.	Using leaves as explant by vacuum infiltration method gave transformation efficiency 37 %.	[99–101]

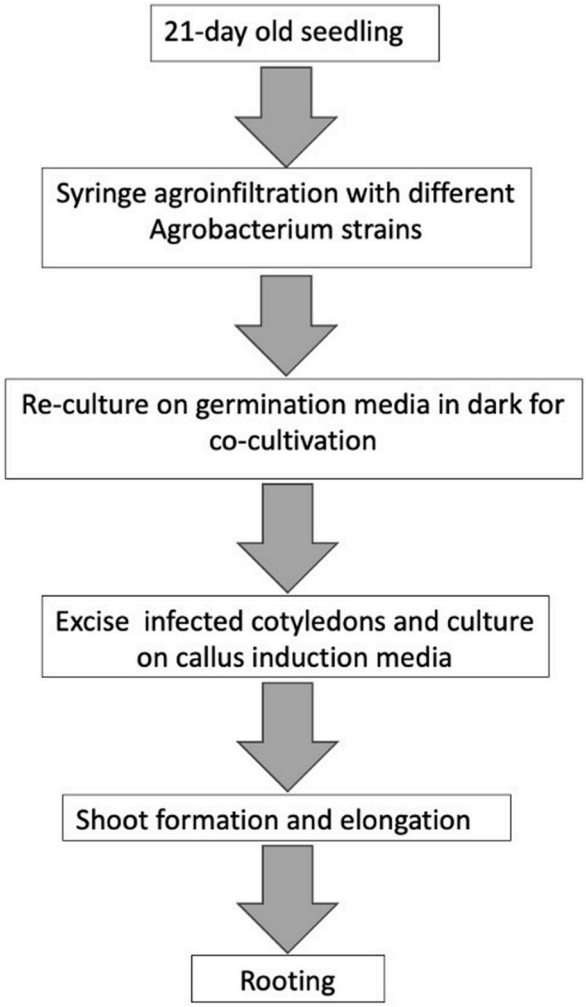

Fig. 2. *C. roseus* transformation and regeneration steps. Flow chart presenting the transformation procedures starting from the seedling inoculation with Agrobacterium strains (LBA4404, EHA105 and GV3101) till reaching a full regenerated plantlet.

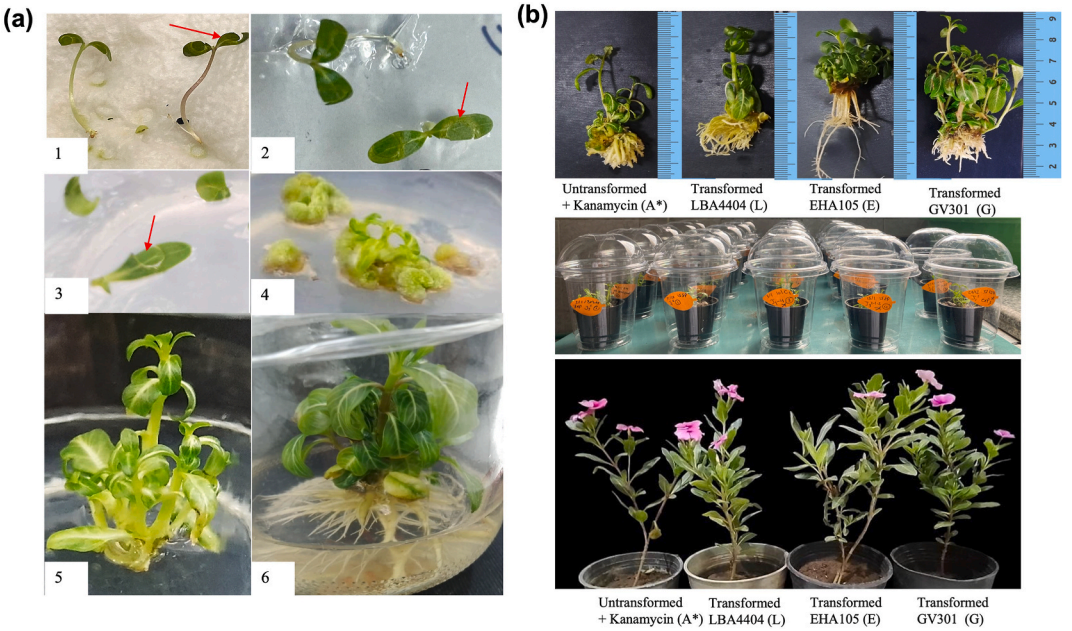

Fig. 3. ***C. roseus in vitro*** **regeneration and acclimatization initiated from cotyledons agroinfiltrated by three different Agrobacterium tumefaciens strains harboring pCAMBIA2301 vector. a)** *In vitro* transformation-regeneration procedures including: **1)** 21-day old seedling agroinfiltrated with syringe. **2)** incubation of seedlings on MS medium. **3)** excised cotyledon on callus induction media. **4)** shoot formed from callus on shood regeneration medium after 30 days. **5)** shoot excised from callus and elongating on shoot regeneration medium, and **6)** rooting and plant development in rooting medium. Red arrows showing wounds on cotyledons from syringe infiltration. **b)** Mutant lines acclimatization procedures including plants removed and washed from MS media **(Top),** followed by placing them in 5 cm pots containing 1 peatmoss: 1 perlite soil **(middle),** and finally plants at full maturity stage **(lower)**. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

8-h dark photoperiod at 25 °C ± 2 °C until the cover was completely removed. They were then transplanted into larger 20 cm pots and maintained under greenhouse conditions. Humidity was monitored with a humidity meter, and plants were watered as needed. A fungicide solution (1 mL/L) was applied weekly to protect against fungal contamination. Acclimatization steps are shown in (Fig. 3 b).

2.5. *Examination of the putative transformants using GUS*

Histochemical GUS activities in the agroinfiltrated cotyledons and leaves of the control and putatively transformed plantlets, using the three Agrobacterium strains, were investigating according to Jefferson et al. [46] with some modifications in two courses of time (a) initial GUS: 4 days after the cotyledon infection. (b) stable GUS: approximately 20–30 days after shoots formation. Three random selected leaves were taken and analysed using same approaches reported by Ref. [46] with some modifications, adjusting pH at 7.0, one of the most crucial factors that should be consider is thermal stability of incubation; here we use 35 °C for 16 h. As previously reported that overexpression or reduction of beta-glucuronidase enzyme adjusted by heat, they suggested that the ideal temperature should be 35 °C [47]. Tissues were placed in freshly prepared GUS stain solution containing (10 mM Na_2EDTA, 0.07 M NaH_2PO_4, 0.1 M Na_2HPO_4, 15 mM sodium azide, 0.45 % V/V B-mercaptoethanol, 0.1 % V/V Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$. $3H_2O$ with (pH 7.0) and 100 mg/L X-Gluc) with incubation at 37 °C for 16 h. After that, the plant tissue was thoroughly washed by 70 % EtOH to eliminate any remaining chlorophyll. The leaves were then examined under OPTIKA® 2X magnification stereo microscope and imaged using camera. A black background was then added by photoRoom program. The transformation efficiency was calculated based on GUS staining activity.

The Prescence of the *GUS* gene in the putative transgenic plants were assessed using Polymerase chain reaction (PCR) after 15–30 days of shoot formation, once shoot become 1–2 cm long, random leaves were taken for PCR analysis. Around 100 mg of plant leaves were grinded using liquid nitrogen, DNA was isolated using DNeasy plant DNA isolation kit (QIAGEN) according to manufacture protocol. DNA was measured using Nanodrop and ran on 1 % (W/V) Agarose gel along with GeneRuler1Kb plus DNA ladder (Thermo Scientific) at 150 V, the gel was stained with 10 μg/mL Ethidium bromide for 30 min. The primer set in use were GUSF- 5'-CGACTGGGCAGATGAACATG-3' and GUSR 5'-TACTCCACATCACCACGCTT-3' [48]. The PCR reaction was conducted in 25 μl final volume containing 1.5 ng of genomic DNA and 12.5 μl Easytaq master mix (Transgene biotech, China) and 0.2 μM of each primer. The cycling conditions was carried out in applied biosystems (Life technology, U.S.A.) thermal cycler programmed at initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturing at 95 °C for 30 Sec, annealing at 62 °C for 30 Sec, extension at 72 °C for 30 Sec, and then a final extension at 72 °C for 5 min.

The PCR product was then evaluated using gel electrophoresis on 1.5 % (W/V) Agarose gel using 150 V in 0.5X TBE, the gel was

stained with 10 μg/mL Ethidium bromide for 30 min, then visualized using UV transilluminator. The product size was then compared with GeneRuler1Kb plus DNA ladder (Thermo Scientific).

2.6. Transgenic plant molecular variation evaluation using inner simple sequence repeats (ISSR)

Around 100 mg of plant leaves were grinded using liquid nitrogen, DNA was isolated using DNeasy plant DNA isolation kit (QIAGEN) according to manufacture protocol. DNA was measured using Nanodrop and ran on 1 % (W/V) Agarose gel along with GeneRuler1Kb plus DNA ladder (Thermo Scientific) at 150 V. The PCR reaction was conducted in 25 μl final volume containing 1.5 ng of genomic DNA and 12.5 μl of TOPsimple master mix (Enzynomics, Korea) and 0.2 μM of each primer, the ISSR primers used are mentioned in (Table 2).

The cycling program was set at initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturing at 95 °C for 30 Sec, annealing for 30 Sec, extension at 72 °C for 30 Sec, and then a final extension at 72 °C for 5 min.

The PCR product was then evaluated using gel electrophoresis on 2 % (W/V) Agarose gel using 150 V in 0.5X TBE, the gel was stained with 10 μg/mL Ethidium bromide for 30 min, then visualized using UV transilluminator. The product size was then compared with GeneRuler1Kb plus DNA ladder (Thermo Scientific). The clear and reproducible bands were considered for counting alleles in the ISSR analysis.

The ISSR amplificon profile for each of the obtained mutants and control was assessed in the same loci by the precent or absence of the band. A binary data matrix was generated as the presence of the band loci was recorded as “1” and the absence was recorded as “0”.

The genetic similarity coefficient of SM was calculated using the biological software NTSYS-pc2.10, and the unweighted group average method (UPGMA) was used to cluster the ISSR-PCR amplified bands for genetic similarity analysis and used construct a dendrogram.

2.7. GUS expression analysis using qRT-PCR

Leaf tissue was collected from all mutants and pooled as one biological replicate for each treatment. Since both controls (uninfected with kanamycin and without kanamycin) used in this study showed negative GUS histochemical, one negative control (uninfected without kanamycin) was utilized (Control, LBA4404, EHA105, and GV3101). A total of three replicates were conducted for each treatment. Tissue was collected by cutting leaves directly into liquid nitrogen to preserve mRNA. Total RNA isolation was preformed using GeneJet plant genomic RNA purification kit (Thermo, U.S.A) according to manufacture protocol. RNA was then measured using nanodrop for quantification and 5 μl of the isolated RNA samples were mixed with RNA loading dye (Thermo, U.S.A.) and heated to 56 °C, then placed on ice for gel electrophoresis. Then samples were run on 0.7 % (W/V) agarose gel at 100 V for 30 min following that the gel was stained using 10 μg/mL ethidium bromide for 30 min. After verification of the isolated RNA by nano-drop and gel electrophoresis, 10 ng of each RNA sample was used for quantitative real time PCR (qRT-PCR) analysis via GoTaq 1-step RT-qPCR system (Promega, U.S.A.), the reaction was made to a final volume of 20 μl following manufacture protocol. The qRT-PCR primers for the detection of GUS expression were F: 5’-GAATACGGCGTGGATACGTTAG-3’, R: 5’-GATCAAAGACGCGGTGATACA -3’ [49], while using Actin as a reference gene F: 5’-GTGCAACGCCTTCTCCGTTC-3’, R:5’-TGGCTGATGGAGCACAGAGG-3’ [50].Each reaction was made in triplicates, in addition to a no-template control for each primer, and placed in STRATAGENE (mx3000P) real-time PCR system using the following cycling parameters one cycle at 37 °C for 15 min (reverse transcription), one cycle at 95 °C for 10 min (Reverse transcriptase inactivation and 1 GoTaq® DNA Polymerase activation) and 40 cycles of 95 °C for 10 Sec (denaturation), 60 °C for 30 Sec (annealing) and 72 °C for 30 Sec (extension). The relative gene expression in *C. roseus* mutants was calculated using the $2^{-\Delta\Delta CT}$ method [51].

2.8. Data analysis

The callus induction data was collected by dividing the number of explants produced calluses by the total number of explants multiplied by 100.The browning percentage was calculated by dividing the number of brown Calli by the total number of Calli then multiplied by 100.The shoot regeneration data was recorded 4–6 weeks after transfer to shoot regeneration media. The regeneration efficiency percentage was calculated as described by Ref. [52]. The GUS activity percentage was calculated as the number of plants expressed GUS/the total number of plants evaluated x 100 [53]. Statistical analysis for all data was performed by GraphPad Prism 6 using ANOVA (Tukey post hoc test) at 0.05 significance level.

Table 2
ISSR primers sequence, length, and annealing temperature.

Primer name	Sequence	Length	Annealing temperature	Reference
ISSR-2	$(AG)^8T$	150-1000 bp	52 °C	[102]
HB-13	5’-GAGGAGGAGGC -3’	600-2000 bp	36 °C	[84]
ISSR-5	CCC $(GT)^7$	150-1000 bp	52 °C	[102]

3. Results

3.1. Effect of different agrobacterium strains on C. roseus in vitro callus induction

The effect of the three Agrobacterium strains (LBA4404, EHA105, and GV3101) on different *in vitro* stages including callus induction stage was evaluated here (Table 3). presents the results of callus induction and browning percentages under various Agrobacterium strains along with two sets of control treatments. In the control group without kanamycin, callus induction reached 100 %, indicating a robust response, while browning was relatively lower at 23 %. Interestingly, the addition of kanamycin to the control still resulted in a high callus induction of 97 %, although there was a noticeable increase in browning to 41 %. The strains LBA4404, EHA105, and GV3101 exhibited varying effects on callus induction and browning. LBA4404 showed a reduction in both callus induction (79 %) and browning (48 %), suggesting a potential influence on the Agrobacterium strain in use. Similarly, EHA105 demonstrated a moderate impact, with callus induction at 86 % and browning at 50 %. In contrast, strain GV3101 exhibited the lowest callus induction at 67 % and a relatively higher browning rate at 54 %. The browning percentage data showed no significant differences between the with and without kanamycin control treatments. Also, there was a significant difference between the three-strain compared to the controls. While all strains had no significant differences when compared to each other. These findings highlight the callus induction differential responses to the various Agrobacterium strains, emphasizing the need for further investigation Agrobacterium strain-specific effect on *C. roseus in vitro* regeneration.

3.2. Effect of different agrobacterium strains on C. roseus in vitro shoot regeneration

Shoot regeneration is a critical parameter that measures the success of many plant tissue culture protocols. During our experiment shoot number was recorded and regeneration efficiency was calculated for all treatment. Data showed that regeneration efficiency was the highest value for the negative control (27 %) while the lowest value was for the strain (GV3101) treatment (10 %) (Fig. 4). Among the strain treatments, LBA4404 strain had the highest regeneration (19 %) and there was not significant different when compared to the negative and the positive controls. While GV3101 was significantly lower in shoot regeneration when compared to both controls.

3.3. Analysis of the presence of the GUS gene using histochemical analysis and direct PCR

GUS activity analysis was performed on the collected shoots from the mutants created by the three Agrobacterium stains (LBA4404, EHA105, and GV3101). A histochemical assay was performed at two stages, the initial transient expression, on the infected cotyledon, and the stable transformation, on a detach leave originated from each transgenic mutant line. The initial expression represented the success of the transformation at different efficiency among different treatments (Fig. 5 a), while the stable transgenic lines demonstrated strong GUS activity in all generated mutant line (Fig. 5 b). The presented (Table 4) outlines the *GUS* expression percentages, with standard deviations, for the three different Agrobacterium strains at initial and stable stages. Strain LBA4404 exhibited an initial *GUS* expression of 59.3 %, which decreased to 38 % in the stable stage, indicating a decline in GUS activity over time. In contrast, Strain EHA105 demonstrated a higher initial *GUS* expression of 77.8 %, showing a robust initial response, and maintained a relatively stable *GUS* expression at 55.6 % in the stable stage. Strain GV3101, on the other hand, had an initial *GUS* expression of 63 %, which slightly increased to 61.1 % in the stable stage. Interestingly, there was no significant differences in the initial *GUS* expression between strains, while the strain LBA4404 showed significantly lower *GUS* expression than the GV3101 strain. These results suggest differential temporal dynamics in *GUS* expression among various *A. tumefaciens* strains, emphasizing the need for careful consideration of strain-specific characteristics in designing and interpreting *C. roseus* transformation studies. The observed variations underscore the complexity of gene expression regulation and highlight the importance of evaluating GUS activity over time for a comprehensive understanding of the intended genetic modifications due to the plant transformation.

The success of the transformation was further evaluated by the insertion of the *GUS* gene into the plant genome. This was conducted by the presence or absence of the GUS amplification PCR product (approximately 216 bp) in the mutants. Gel Electrophoresis illustrating some of the obtained GUS $^{+}$ mutant plants are presented in (Fig. 5 c). The presented data shows a PCR amplification band with variable intensity in obtained mutant treatments; G1 (GV3101mutant), E1:E4 (EHA105 mutants), and L1-L5 (LBA4404 mutants) samples with no amplification in the negative control plants A (mock transformation) and A* (mock transformation grown in kanamycin media). We have also observed no bands detected in the no template control reaction (NTC). However, a primer dimer was noticeable >100 bp in all the samples which was consistent with the research paper in which we have obtained the primer sequence

Table 3
Calli inducted from *C. roseus* cotyledon explants agroinfiltrated with three different Agrobacterium strains.

Treatments	Total number of explants produced callus/total number of explants	Callus Induction (%± SD)	Browning (%± SD)
Control (-Kanamycin)	73/73	100 (±0.00)^{a}	23 (±0.44) ^{ab}
Control (+Kanamycin)	47/48	97 (±0.14)^{a}	41 (±0.49)^{a}
Strain (LBA4404)	89/112	79 (±0.40) ^{bc}	48 (±0.49)^{b}
Strain (EHA105)	97/112	86 (±0.34) ^{ab}	50 (±0.50)^{b}
Strain (GV3101)	76/112	67 (±0.46)^{c}	54 (±0.49)^{b}

Treatments with similar letters have no significant differences.

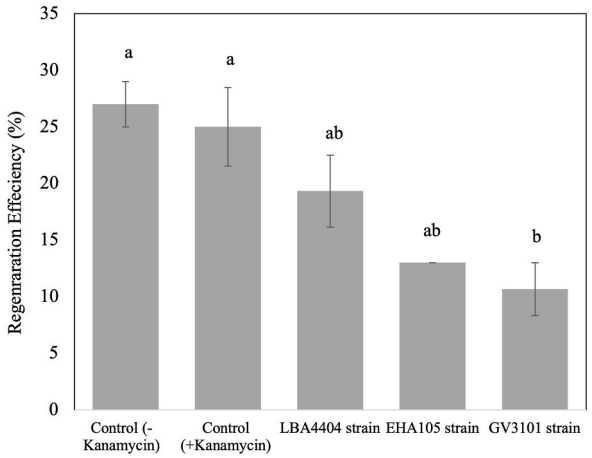

Fig. 4. Regeneration efficiency (%) of *C. roseus* shoots from different Agrobacterium strains. Treatments with similar letters have no significant differences, error bars showing SD.

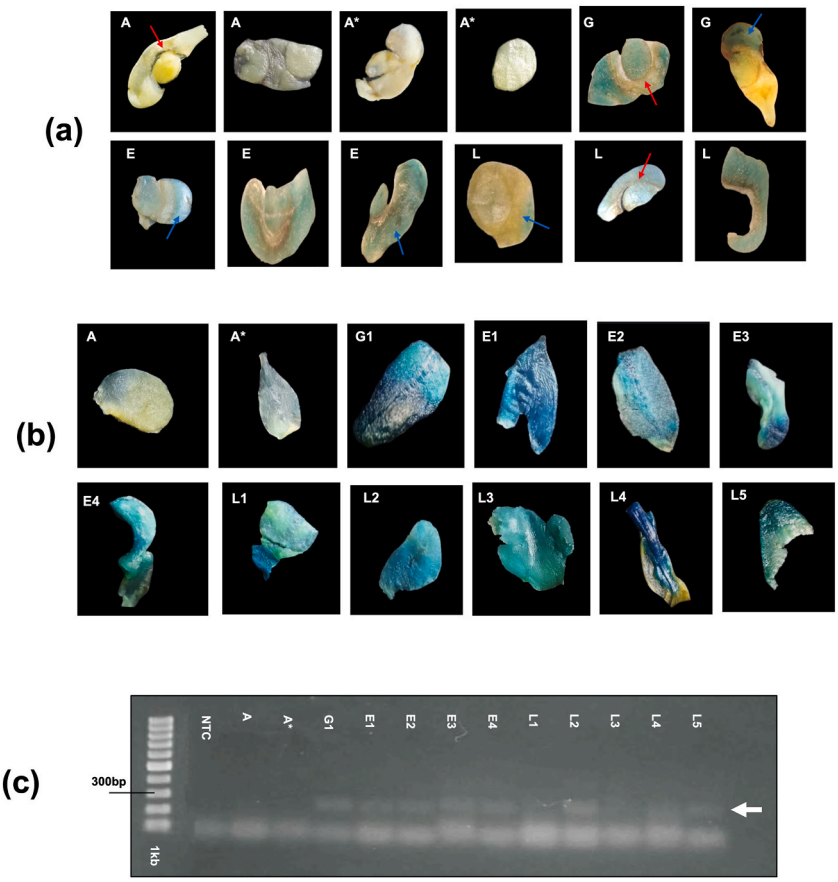

Fig. 5. GUS screening for some of the putative transformants using histochemical analysis and PCR in the controls and Agrobacterium transformed *C. roseus* plants. where -A- mock transformation plants cultivated in antibiotic-free media, **A*** and kanamycin-containing media. **G1** plant generated by GV3101 strain infection, **E1:E4** generated by EHA105 strain infection, **L1:L5** plants generated by LBA4404 strain infection. **(a)** initial GUS histochemical assay on agroinfiltrated cotyledons, **(b)** Stable GUS histochemical assay for regenerated shoots, **(c)** 1%Agrose gel stained by 10ug/ml Ethidium bromide along with 1 kb DNA ladder showing a 216-amplification product of the GUS gene indicated by white arrow, with no detected bands in both controls and no temple control (NTC).

Table 4
Transformation efficiency based on GUS histochemical analysis for both initial GUS expression (in agroinfiltrated cotyledons explants) and stable GUS (in regenerated shoots) using three *A. tumefaciens* strains in *C. roseus*.

Treatment	GUS Activity (% ± SD)	
	Initial	Stable
Strain (LBA4404)	59.3 ± 0.064[a]	38 ± 0.019[a]
Strain (EHA105)	77.8 ± 0.0[a]	55.6 ± 0.096 [ab]
Strain (GV3101)	63 ± 0.128[a]	61.1 ± 0.096 [b]

Treatments with similar letters have no significant differences.

from Ref. [48].

3.4. GUS expression analysis in C. roseus obtained mutants

qRT-PCR analysis was used to access the expression level of the *GUS* gene in the obtained mutants via *Agrobacterium tumefaciens* mediated transformation in *C. roseus* plants. Transgenic plants generated from *A. tumefaciens* were pooled for each strain separately (LBA4404, EHA105 and GV3101) for *GUS* gene expression analysis in comparison with control untransformed plants in antibiotic-free media (A). The relative expression of GUS (β-glucuronidase) is shown in (Fig. 6**).** The analysis of the data reveals clear differences in *GUS* gene expression among the samples. The LBA4404 strain exhibited the lowest increase in expression, followed by the EHA105 strain, which showed a substantial upregulation. Notably, the GV3101 strain demonstrated the highest level of *GUS* expression, significantly surpassing all other samples. This pronounced increase in the GV3101 strain indicates a strong enhancement of gene expression, making it the most efficient strain for driving *GUS* expression compared to the others. Specifically, the LBA4404 strain produced a modest 5.55-fold increase in *GUS* expression, which was not significantly different from the control group. In contrast, the EHA105 strain displayed a marked increase, reaching 19.29-fold, significantly higher than both the control and LBA4404 strains, though not statistically different from the GV3101 strain. The GV3101 strain exhibited the highest expression level, with a 54.27-fold increase compared to the control. These results highlight the varying efficiency of each strain in inducing *GUS* gene expression, with GV3101 proving to be the most effective.

3.5. Transgenic plants molecular variation evaluation using inner simple sequence repeats (ISSR)

In Agrobacterium-mediated transformation the Agrobacterium strain's genetic background can impacts the behavior of transformed plants. This study used ISSR to examine molecular variations in mutants transformed by three Agrobacterium strains, a common method for assessing genetic diversity in *in vitro* propagated plants [54,55]. We conducted ISSR-PCR analysis on mock-transformed plants grown in antibiotic-free media A and kanamycin-containing media A*, along with 12 GUS^{+} mutant plants. The G1 plant was generated by GV3101 Agrobacterium strain infection, four plants E1:E4 by EHA105 Agrobacterium strain, and five

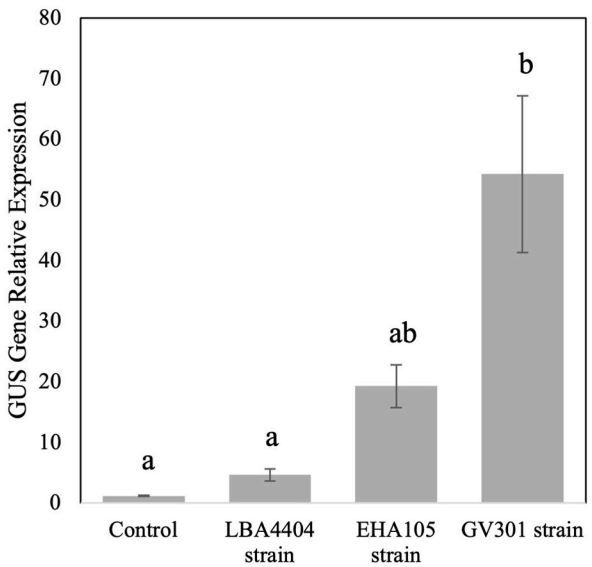

Fig. 6. Showing *GUS* relative expression of *C. roseus* obtained mutants via three *Agrobacterium tumefaciens* strains (LBA4404, EHA105 and GV3101). Error bars showing SD.

plants L1: L by LBA4404 Agrobacterium strain. Here in the ISSR analysis we have used three primers ISSR-2, ISSR-5, and HB-13, with amplification data and primer size ranges detailed in (Table S1).

The amplification profiles of three ISSR primers (ISSR-2, ISSR-5, and HB-13) across 12 samples of transgenic and non-transgenic *Catharanthus roseus* plants, as depicted in (Fig. 7), exhibited band sizes ranging from 300 to 2000 bp. There were no significant differences in the ISSR patterns between the untransformed control samples A: without kanamycin and A*: with kanamycin), as both displayed similar patterns with all tested primers. However, noticeable differences emerged in the transformed plants compared to the controls, particularly with primer HB-13 in samples E1, E3, L2, and L4. Additional bands were observed around 1000 bp in samples E1, E3, and L2, while a distinct band was identified around 900 bp in samples E3 and L4. Furthermore, another band was detected around 450 bp in samples E3 and L4. These additional bands, highlighted in Fig. 7, were marked with yellow squares and may indicate T-DNA insertion into the plant genome. Therefore, we conclude that the transformed plants generated by the Agrobacterium strains EHA105 (E1, E3) and LBA4404 (L2, L4) exhibited the greatest variations among all transgenic plants compared to the controls. Collectively, the primers amplified 116 bands, of which 43 were polymorphic, yielding an overall polymorphism ratio of 37 %, indicating a reasonable level of genetic diversity in the obtained transgenic *C. roseus* samples (Table 5). These findings emphasize the varying effectiveness of different primers in revealing genetic diversity among the analysed samples.

A similarity matrix using a 0/1 binary comparison to the control samples analysed relationships among transformed plants. The UPGMA dendrogram split the 12 plants into two main clusters (Fig. 8). The dendrogram showed that the control plants A and A* were genetically distinct from the transformed GUS^{+} plants, with the G mutant from the GV3101 strain being more closely related to the controls. In contrast, the L and E samples from the LBA4404 and EHA105 strains were more closely related to each other and displayed

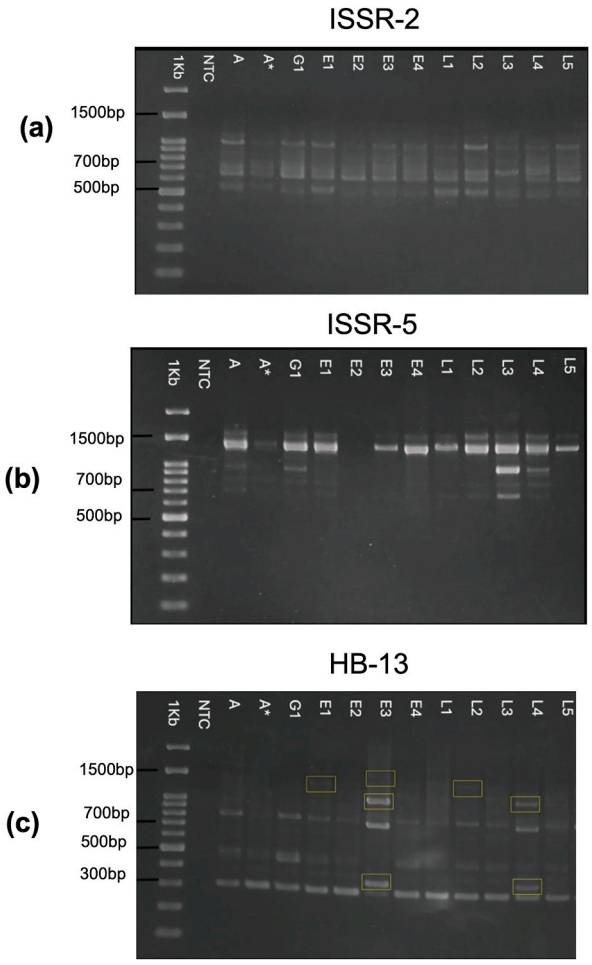

Fig. 7. Amplification profile of ISSR primers across the *in vitro* propagation *C. roseus* obtained transformed mutants. (a) ISSR-2 primer **(b)** ISSR-5 primer**,** and **(c)** HB-13 primer. All amplification products on 2%Agrose gel stained with 10 μg/mL ethidium bromide along with 1 kb DNA ladder, **-A-**mock transformation plants cultivated in antibiotic-free media, **A*** and kanamycin-containing media. **G1** plant generated by GV3101 strain infection, **E1:E4** plants generated by EHA105 strain infection, **L1:L5** plants generated by LBA4404 strain infection. no bands were obtained in the no template control sample (NTC). Additional bands detected compared to the control samples are marked with yellow squares which may indicate Ti-DNA insertion into the plant genome. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Showing polymorphism ration between ISSR-2, ISSR-5 and HB-13 in the transgenic *C. roseus* samples.

Primers	No. of amplified bands	No. of polymorphic bands	Polymorphism ratio (%)
ISSR-2	42	12	28.5
ISSR-5	26	15	57.6
HB-13	48	16	33.3
Total	**116**	**43**	**37.0**

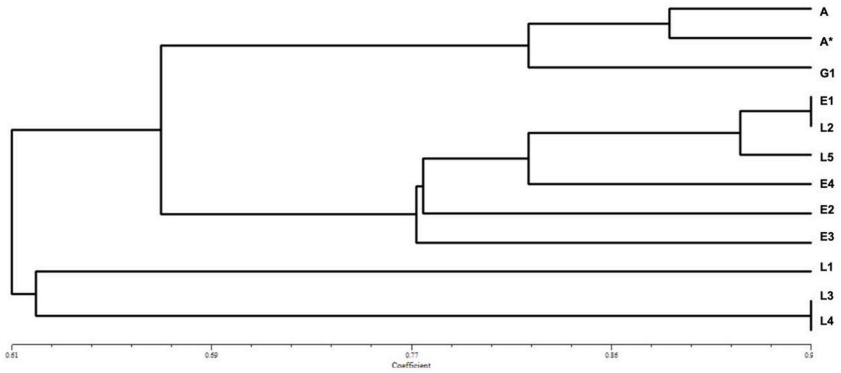

Fig. 8. UPGMA dendrogram showing the clustering of the 12 *in vitro* propagartion obtened *C. roseus* plants based on SM similarity coefficient calculated from ISSR markers. (A) mock transformation plants cultivated in antibiotic-free media, **(A*)** and kanamycin-containing media. **(G1)** plant generated by GV3101 strain infection, **(E1:E4)** generated by EHA105 strain infection, **(L1:L5)** plants generated by LBA4404 strain infection.

the greatest variations compared to the controls. In conclusion, the ISSR data confirm genetic variation among the mutants and controls, suggesting that the Agrobacterium strains EHA105 and LBA4404 were more effective in inducing genetic changes in the transformed plant genome.

4. Discussion

A. tumefaciens mediated gene editing is one of the commonly used methods to develop genetically modified plant through plant tissue culture [56]. Several studies have been conducted to develop an optimized Agrobacterium transformation protocol for *C. roseus* [57–59]. Most of these protocols were focused on different optimization parameters including bacterial intensity, incubation time, using different plant parts and using different infection methods. However, these studies have neglected the choice of the Agrobacterium strain in the transformation process, which could have a direct impact on *in vitro* plant regeneration and transformation efficiency. No research has been undertaken to demonstrate the impact of various strains of *A. tumefaciens* on *C. roseus* plants transformation. In this study, we showcased the impact of the three commonly employed Agrobacterium strains on the *in vitro* regeneration and transformation efficiency of the periwinkle. Additionally, plant regeneration following Agrobacterium infection is crucial step in the development of transgenic mutant, this is due to high plant cell death post-transformation.

In this study, we employed three Agrobacterium strains, namely LBA4404, EHA105, and GV3101, for the transformation of *C. roseus*. We evaluated various *in vitro* parameters, including callus induction, callus browning, and shoot regeneration efficiency. Initially, callus induction stage exhibited variations among the three agrobacterium strains showing highest for EHA105 (86 %) followed by LBA4404 (79 %) then lastly GV3101 (67 %) which was significantly different when compared to the non-infected control (100 %). Callus browning following Agrobacterium infection can be attributed to a multifaceted interplay of factors. The release of phenolic compounds from wounded plant tissues induced by *A. tumefaciens* is a primary contributor. The subsequent oxidation of these phenolics can result in the formation of brown pigments, causing discoloration in the callus. Cellular damage incurred during the infection process, including wounding and the introduction of foreign genes, triggers a stress response in plant cells, leading to the production of phenolic compounds as part of their defense mechanisms [60]. Here we found that strain GV3101 gave the highest browning ratio (54 %), followed by EHA105 strain (50 %) and lastly the LBA4404 strain gave around 48 % browning which is relatively high when compared to the uninfected control (23 %). Another factor can be attributed to the callus browning is the addition of the kanamycin as a selective marker, which will subsequently slow the growth of any untransformed cells. Resulting to its death, hence the brown discoloration [61,62]. In conclusion, there were significant differences between the transformed treatments and both control groups and as we have used the same bacterial density among the three Agrobacterium strains for the infection. Henceforth, the differences in the callus browning can also be attributed to the strain virulence and the specific interaction with the *C. roseus* cells [63].

Shoot regeneration following Agrobacterium-mediated transformation is a pivotal stage in plant biotechnology, marked by the recovery and development of shoots from transformed plant tissues. The success of this process is influenced by various factors.

Selection pressure is exerted through the incorporation of selectable marker genes, enabling the identification and proliferation of transformed cells during subsequent regeneration. Additionally, some studies in other plants have indicated that the Agrobacterium strain in use can affect shoots and callus regeneration [64]. Here we found that different patterns displayed in shoot regeneration in *C. roseus* among different Agrobacterium strains, LBA4044 strain showed higher regeneration efficiency (19 %) compared to EHA105 and GV3101 strains at 13 and 10 % respectively. LBA4044 showed no significant differences when compared to the uninfected control. Beside no significant differences was noticed between negative and positive control assuring that the shoot regeneration differences are due to different Agrobacterium strain treatment. These data are constant with another study by Bakhsh et al., in which they have demonstrated variation in callus and shoot regeneration in tobacco plant when using five different agrobacterium strains. The highest calli, shoot regeneration and transformation efficiency was 92, 25, and 20 % respectively observed with LBA4404 strain compared to other strains including EHA1015 which had calli, shoot regeneration and transformation efficiency 73, 16 and 13.3 % respectively [64].

For detecting transgenic plants, numerous reporter genes or screening systems are utilized, choosing among them depend on which is more effective, accurate, and safety. One of which is beta-glucuronidase enzyme that expressed in the presence of substrate called (X-GLUC) that turned blue color in existence of gene that encode for β-glucuronidase known as GUS histochemical assay. As shown by Ref. [65], *GUS* gene isolated from *Escherichia coli* is ecologically safe. Hence [66], reported that GUS assay considered as a conscious and functional approach for screening transgenic plants. Various studies used GUS assay widespread including *C. roses* as screening tool for transgenic lines transformed by Agrobacterium; as mentioned by previous studies [67,68], Thus, we adopted this method for transient and stable transgenic plant detection, followed by PCR for detecting and confirming GUS gene insertion, via GUS- specific primer reported by Ref. [69]. The histochemical analysis showed that the highest GUS positive percentage was achieved by GV3101 strain with 61.1 % while the lowest was observed by LBA4404 strain with 38 %. Similar transformation pattern was previously reported in *Bacopa monnieri* plant transformed with the same three strains harboring pCAMBIA2301 vector [70].Also [71], reported that GV3101 strain harboring pBI121 plasmid had the highest transformation rate (60 %), followed by EHA101 (40 %). Although it was reported in other studies that GV3101 have highest transformation efficiency with the infiltration method among plants when compared to LBA4404 due to it virulence proteins [45,72], however due to plant species specificity other results was observed in other plant species. For instance, GV3101 strain showed the lowest transformation efficiency when compared to EHA105 and LBA4404 in a biofuel plant (Jatropha *curcas*) [73]. Another study on potatoes showed LBA4404 strain had better GUS positive percentage (60 %) followed by EHA105 (47 %), then GV3101 (7 %) [72]. Thus, more plant species studies need to be addressed in the aim of enhancing plant transformation, in particular important and low transformation efficiency plant species.

Furthermore, the three agrobacterium strains, incorporated in our study, were represented previously in research by utilizing only the use of one strain at a time. These studies have showed variation in transformation frequency of *C. roseus*. For instance, Wang et al. used EHA105 harboring pCAMBIA2301 vector for transforming *C. roseus* cultivar Pacific cherry red hypocotyl and reported 11 % transformation efficiency [59]. On the other hand, Srivastava et al., 2009 used LBA4404 harboring pBI-S1 to transform callus, nodal and leaf segments of *C. roseus* var alba and reported unsuccessful regeneration and 90 % GUS transient expression on survived callus [58]. In addition to a recent study, researcher used infiltration method with GV3101 strain harboring pSB97 plasmid to enhance transgene expression in *C. roseus* which demonstrated increase in GUS expression in transformed seedlings [57]. These three separate studies for three different strains demonstrated efficient transformation of *C. roseus* and neglecting the impact of Agrobacterium strain effect. Addressing that, these three agrobacterium strains are genetically different and have been demonstrated to have significant effect on other plant species transformation including tomato, tobacco, and some medicinal plants such as bacopa and caragana plants.

In our study, The GV3101 strain treated mutants showed significantly higher GUS gene relative expression compared to the other two strains. This data along with the initial and stable GUS histochemical assay suggest that GV3101 strain is highly compatibility with *C. roseus* transformation. Similar results were observed in other plant species including mulberry tree, hemp and Caragana [74,75]. Additionally a research on *Cannabis sativa* found that strain GV3101 showed higher expression of GUS than LBA4404 and EHA105 [76]. However, that is not the general case as different plant species show different compatibility to other Agrobacterium strains [75], which present the importance of similar studies toward optimizing plant transformation.

The integration of the Ti-plasmid into the transfected plant genome causes molecular changes in the plant genome this change depends on several key factors. One critical factor is the specific strain of *Agrobacterium tumefaciens* carrying the Ti plasmid, as different strains may exhibit variations in their virulence genes and transfer DNA (T-DNA) regions. The T-DNA region plays a central role in the integration process, and its structure and composition impact how foreign genes are incorporated into the plant genome. Additionally, the integration site within the plant genome is crucial, as different locations can result in varying stability and expression of the introduced genes. The plant species and cultivar being targeted also play a significant role, as their genetic and physiological characteristics affect the success of transformation [77]. Moreover, the presence of regulatory elements on the Ti plasmid, such as promoters and enhancers, influences the expression of the inserted genes [78–81]. Therefore, here we investigated the variation between the obtained mutant via implementing the ISSR as a molecular marker. As previous study has shown that transformation using *A. tumefaciens* can result in genetic variations among the obtained in transformed plants [82]. We have used ISSR based PCR profiling as a tool to evaluate the diversity among the *GUS* $^{+}$ obtained transformed plants, here we have used three ISSR primers (ISSR-2, ISSR-5 and HB-13) previously used in *C. roseus* genetic studies in Egypt [83,84]. ISSR is frequently used in many fields as in detection of genetic diversity among cultivars of *C. roseus* as reported by Refs. [85–88]. Our study found 37 % polymorphism in ISSR amplification, which is considered acceptable for micro-propagation-generated plants. Similarly, a study by Ref. [82] reported 27.9 % polymorphism *in C. roseus* GUS $^{+}$ mutants transformed with the LBA4404 strain, aligning with our results. In contrast, ISSR analysis of *Stevia rebaudiana* GUS $^{+}$ mutants using the EHA105 strain showed a higher polymorphism of 56 % (Reference missing), likely due to differences in plant species and ISSR primers used. In conclusion, The ISSR data confirm genetic variation between the mutants and

controls, showing that the Agrobacterium strains EHA105 and LBA4404 were more effective in inducing genetic changes in the transformed plant genome. These results demonstrate the success of Agrobacterium-mediated transformation in generating genetic diversity in *C. roseus*. Understanding these relationships is essential for selecting plants with desirable traits for research and agriculture. Future studies are needed to explore the phenotypic effects of the observed genetic variations to better understand their implications in transgenic plants.

5. Conclusion

Based on the *in vitro* regeneration data and the *GUS* expression analysis of *C. roseus* transformation using three different *A. tumefacien* strains. Our study indicated that the *A. tumefacien* GV3101 strain performed better toward the highest transformation efficiency, however it showed the lowest callus induction and shoots regeneration efficiency. On the other hand, *C. roseus* mutants transformed using LBA4404 *A. tumefacien* strain showed both moderate transformation, callus, and shoots regeneration efficiency. Further analysis using ISSR method provided evidence for obtaining a stable transformed C. *roseus* mutants from the regenerated plants using agroinfiltrated cotyledons. Thus, choosing the *A. tumefacien* strain for transforming different plant species is an important parameter that is required for optimizing the transformation protocol. Furthermore, the genetic transformation method described in this study may be useful to other plant species with several selective agents and explant type.

CRediT authorship contribution statement

Rania El-Tanbouly: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Hend Hassan:** Writing – review & editing, Validation, Methodology, Investigation. **Lojina M. Awd:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Azza Ali Makhlouf:** Writing – review & editing, Validation, Supervision. **Hani G. Shalabi:** Writing – review & editing, Validation, Supervision. **Sarah El-Messeiry:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40589.

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