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Agrobacterium-Mediated Genetic Transformation and Cloning of Reference Genes in Suspension Cells of *Artemisia Pallens*

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Abstract

A reliable and stable *Agrobacterium*-mediated genetic transformation system has been developed using cell suspension cultures derived from *Artemisia pallens* cotyledon explants. Cotyledon, attached cotyledon, and compound leaf were found to be suitable for the induction of callus among five different types of explants tested. Yellow friable callus derived from attached cotyledon was used to initiate suspension cultures in Suspension Culture Medium (SCM) which was supplemented with 2.4-dichlorophenoxyacetic acid (2,4-D) and in combination with different concentrations of Zeatin (ZEA). Among the two different shock treatments, cold shock (at 4 ^{o}C) for 20 minutes and heat shock (at 45°C) treatment for 5 minutes, heat shock treatment increased the transformation efficiency. Supplementation of chemical additives such as Silwet L-77 (0.05%) and Pluronic F-68 (0.05%) significantly enhanced suspension cultures' transformation efficiency. The maximum GUS intensity was recorded with an optimal intensity of blue spots in the transformed cells. The highest GUS fluorometric activity was measured as 879.4±113.7 nmol 4MU/mg/min in transformed cell suspension cultures. The hygromycin-resistant callus derived from micro-calli showed intense blue colour in GUS histochemical assay. The transgene integration into the plant genome was confirmed by polymerase chain reaction (PCR) using *uidA* specific primers in six hygromycin-resistant cell lines. The cloned and mRNA expression levels of three candidate reference genes *ADP-ribosylation factor* (*Arf*), *β-actin* (*Act*), and *ubiquitin* (*Ubi*), and carotenoid biosynthesis pathway gene, i.e., *Phytoene desaturase* (*Pds*) along with transgene (*uidA*) were evaluated in transgenic callus lines. The present *Agrobacterium*-mediated genetic transformation protocol could help in better understand the metabolic pathways of this medicinally important plant and its genetic improvement.

Introduction

Artemisia pallens Walls ex. DC is an important medicinal and aromatic shrub which belongs to the family Asteraceae and found mostly in south India (Pala et al. 2016). Several medicinally important Artemisia species are distributed throughout various parts of Asia, Europe, the Middle East, and North Africa (Nigam et al. 2019; Jogam et al. 2020). It is commonly known as "Davana" and shows several important medicinal properties such as anti-inflammatory, anti-pyretic, anti-microbial, antioxidant, antidiabetic, antihelmintic, anti-malarial, antiseptic, antihypertensive, antidepressant, balsamic, choleretic, digestive, depurative, diuretic, emmenagogue, insecticidal, and also used in the treatment of leukemia, wound healing, and some skin infections especially sclerosis (Suresh et al. 2011; Haider et al. 2014; Shreyas et al. 2018; Hiremath et al. 2020). It has also been used to treat measles, cough, and cold in the Indian traditional systems of medicines (Pavithra et al. 2018). The leaves and flowers of this aromatic shrub are used in decorations, making garlands, bouquets, floral decorations, and chaplets due to its rich magnificent fragrance and also in religious ceremonies (Narayana et al. 1998). The leaves and flowers are also used in the extraction of essential oil called "Davana oil." Davana oil is mainly used to prepare various food items and beverages as a flavoring agent, cosmetics, and high-grade perfumes (Mallavarapu et al. 1999; Rekha and Langer 2007; Ruikar et al. 2011).

This plant has gained considerable importance in food and pharmaceutical industries (Rekha and Langer 2007) due to the presence of several secondary metabolites, including a vital sesquiterpene lactone (artemisinin) (Mallavarapu et al. 1999; Shukla et al. 2015; Pala et al. 2016; Shreyas et al. 2018; Hiremath et al. 2020). Artemisinin and its derivatives are used to treat various diseases such as malaria, cancer, hepatitis, and schistosomiasis (Salehi et al. 2018; 2019). There is a high demand for large quantity of this plant due to its industrial importance. A substantial amount of biomass is required for the extraction and large-scale production of the compounds of interest. Initially, the artemisinin and its derivatives were extracted from aerial (mainly leaves) parts of *Artemisia* plants, but this process was limited due to the availability species, biomass requirement, and low product yield (< 1%) (Mannan et al. 2010). Therefore, the development and optimization of plant regeneration and genetic transformation protocols for *A. pallens* will be useful for engineering the metabolic pathway for increased production of the valuable compounds (Alok et al. 2016). Attempts have been made to develop plant regeneration system (Nathar and Yatoo 2014), *Agrobacterium*-mediated genetic transformation (Alok et al. 2016), induction of hairy roots using *Agrobacterium rhizogenes* (Pala et al. 2016) to improve this medicinally important herb.

Plant cells suspension cultures are the valuable and renewable resource of biological material that can be used for several applications, including production of potential secondary metabolites (Yue et al. 2016; Salehi et al. 2018; 2019; Santos et al. 2019). The plant cells suspension cultures are gaining popularity as a host system for the production of recombinant proteins (Tekoah et al. 2015; Yue et al. 2016) and have several advantages such as post-translational modifications, a slight risk of viral contamination, low cost of plant culture media, and cost-efficiency of bacterial expression systems (Santos et al. 2016; Zagorskaya and Deineko 2017; Permyakova et al. 2019). Metabolic engineering requires a deep understanding of its molecular and genetic architecture to produce targeted secondary metabolites successfully. Genetic and metabolic engineering in plant cell suspensions is in high demand to increase the biosynthesis of a compound of interest. The suspension cells can be developed from any explant type; however, most preferably, an embryogenic callus is used. Several successful cell suspension cultures were reported from various plants such as *Artemisia annua* (Salehi et al. 2019), *Catharanthus* (Saiman et al. 2018),

Taxus (Wilson et al. 2018), *Capsicum* (Brito-Sanchez et al. 2019), *Medicago* (Santos et al. 2019), *Arabidopsis* (Permyakova et al. 2019), *Gentiana* (Rybczynski and Wojcik 2019), and *Panicum* (Ondzighi-Assoume et al. 2019).

Agrobacterium-mediated genetic transformation depends on factors like types of explants, bacterial density, co-cultivation duration, the temperature of co-cultivation, concentration of acetosyringone, etc. (Tiwari and Tuli 2012). Plant cells suspension culture is a sustainable system, but various factors may reduce the yield of product. Hence, it is necessary to develop metabolic engineering methods to optimize the production for different plant systems (Wilson et al., 2014). A reliable and stable gene transfer method must be designed for metabolic engineering of cell suspension cultures (Wilson et al., 2018), which could enable the successful utilization of suspension cultures for a wide range of studies such as molecular biology, biochemistry, and genome editing (Permyakova et al., 2019). Various factors like optical density (OD) of bacterial culture, co-cultivation time, co-cultivation temperature, silwet, pluronic concentration, acetosyringone, etc., were evaluated to develop a reliable and high-throughput stable transformation method. Apart from this, the quantification of transgene within transformed cells and other genes involved in the specific pathway would contribute to the genetic improvement of *A. pallens*. Gene expression analysis is a valuable and extensively used approach to reveal transcriptional regulatory networks, expression profiles and to identify a novel function of gene (Huggett et al. 2005; Thompson et al. 2015; Itoh et al. 2016). The candidate reference genes are ubiquitously expressed and generally involved in relatively stable expression of housekeeping processes (Thellin et al. 1999; Pfaffi et al. 2004). These genes are thought to be expressed stably in various tissues, different growth stages, and other environmental conditions. Hence, reference genes can be used to quantify the expressions of the target genes (Wang et al. 2017; 2019; Chen et al. 2019). Therefore, the quantification of gene expression using RT-qPCR requires stable reference or housekeeping genes. There is a lack of genomic or transcriptome data to the best of our knowledge in *A. pallens*, so, cloning and identification of nucleotide sequence and verification of reference genes in this plant are obligatory which can help in screening the suitable reference genes in *A. pallens*. We have cloned three candidate reference genes, i.e., *ADP-ribosylation factor* (*Arf*), *β-actin* (*Act*) and *ubiquitin* (*Ubi*), and the functional gene which involves carotenoid biosynthesis pathway, i.e., *Phytoene desaturase* (*Pds*), for comparing the expression of the transgene as well as *A. pallens* genes.

In the present study, we report a reliable, stable, and high-throughput *Agrobacterium*-mediated genetic transformation protocol using cell suspension cultures obtained from cotyledon explants of *A. pallens.* Moreover, an attempt has been made to clone reference genes and quantify transgene in transgenic cell lines of *A. pallens*. This protocol would be starting material for future metabolic engineering studies this aromatic and highly medicinal plant.

Materials And Methods

Seed sterilization, germination, and culture conditions

Seeds of *A. pallens* were washed with tap water and kept for soaking for 2-3 hr. Surface sterilization of imbibed seeds was done using 0.1% mercuric chloride-containing water for 2 min. The sterilized seeds were washed 3-4 times with autoclaved water under aseptic conditions to remove all traces of mercuric chloride. Seeds were spread onto Murashige and Skoog (Murashige and Skoog, 1962) agar (MSA) semisolid medium (Table 1) and kept for 2 days at 4ºC in dark conditions for stratification. Then the plates were moved under the light for seed germination at 26 ± 2ºC temperature.

Explant preparation and callus initiation

Newly emerged cotyledons from *in-vitro* grown seeds were dissected and kept for callus induction. The MS basal medium supplemented with different concentrations (1, 2, 3 mg L^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D) was tried for this experiment. In another combination, 2 mg L^{-1} 2,4-D was kept constant with different concentrations of zeatin (0.25, 0.5, and 0.75 mg L^{-1}) supplemented in media tested for callus induction. The attached cotyledons, individual cotyledon, compound leaf, stems, and roots were used as explants and compared to find out the best explant source (Fig. 1). The explants were kept onto the respective medium in the dark at 25 ± 2°C. At every 18-20 days, the fresh medium was used for subculturing of the callus.

Initiation of cell suspension culture

The friable calli were used for the initiation of suspension cells (SC) in a liquid medium. The compositions of four different SC liquid media, namely SC1, 2, 3, and 4, were tabulated in Table1. Different sets of friable calli were inoculated in 10-15 ml SC1, 2, 3, and 4 liquid media in 100 ml flask and kept at 25 ± 2ºC temperature in the dark with constant shaking at 85 rpm. The cells were subcultured by replacing old liquid media with fresh medium in a sterile condition after every 8-10 days. Few suspension cell lines were discarded due to slow growth after the 4th and 5th subcultures. The cell lines of higher rate of multiplication were maintained in a 30-35 ml liquid medium in a 250 ml flask.

***Agrobacterium*-mediated cell suspension transformation**

The *Agrobacterium tumefaciens* strain AGL1 has a pCambia1301 plant expression vector grown in Luria broth (LB) liquid media with appropriate antibiotics as described by Alok et al. (2016). The vector carries the *β-glucuronidase* gene (*uidA*) as a reporter marker, whereas the *hygromycin phosphotransferase* gene (*hptII*) is a plant selectable marker. The bacterial pellet was suspended in MSL medium corresponding to OD_{600} 1.0. Finely filtered cells, 5-7 ml, were mixed with 5-7 ml *Agrobacterium* suspension, keeping final $O.D_{600}$ 0.5. The cells were allowed to co-culture for 4 hr at 25ºC temperature in a 50 ml Tarson tube under dark conditions with 80 rpm constant shaking. Cells were allowed to settle down, and the bacterial solution was removed. Cells were taken out with the help of a cell scraper and spread over Whatman filter paper. Further, this filter paper was kept onto MSA supplemented with 2 mg L^{-1} 2,4-D medium and kept in the dark for 3 days at 24 ºC temperature. Transformed cells were washed thrice with SC3 medium containing 250 mg L^{-1} cefotaxime. The cells were again spread on Whatman filter paper overlaid onto SM (Cef) medium (Table1). After two months, the grown micro calli were transferred onto SM (10H) medium (Table 1) with 10 mg/L hygromycin for induction of transgenic calli.

Optimization of various factors for efficient transformation

In our previous report, we optimized the effect of strains and acetosyringone on transformation efficiency, and therefore we used strain AGL1 and 200 µM acetosyringone in MSL media (Alok et al., 2016). In the present study, we checked the effect of heat and cold shock to plant cells before co-culture with *Agrobacterium*. For heat shock, the filtered suspension cells in 50 ml falcon were kept in a water bath for 5 min at 45°C temperature, and the cells were kept for 20 min at 4 °C temperature for a cold shock. The effect of two chemical additives, namely, Silwett L-77 (0.05%) and Pluronic F-68 (0.05%), on transient transformation was assessed. These chemical additives are well known to reduce surface tension and enhance bacterial entry into plant cells (Deguchi et al., 2020). Transformation efficiency was calculated based on GUS spots visible under a microscope in 200 µl transformed SC. After optimization, all these factors were kept constant for the final transformation protocol and repeated thrice.

Genomic DNA isolation and PCR of transgenic callus lines

Transformed and untransformed calli of weight 200-300 mg were taken out from the plate for DNA isolation using the CTAB method (Doyle and Doyle, 1987). The 0.8 % agarose gel was used to check the DNA quality, and quantification was done by BioSpectrometer (Eppendorf, Germany). The Polymerase Chain Reaction (PCR) was performed using 2x G9 Taq Readyload PCR master Mix (GCC Biotech, India), 10 µM primers set, and 80-100 ng of DNA. The *hptII* and *VirG* specific primers were used to confirm the transgenic callus and to confirm that none of the bacteria has adhered to transgenic calli. The condition of PCR cycle was initial denaturation 94 °C for 5 min (one cycle), 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s (for 35 cycles) with a final extension at 72 °C for 5 min. The amplified PCR products were electrophoresed in 0.8 % agarose gel.

GUS histochemical assay

GUS activity of transiently transformed suspension cells and stable expression calli was performed as reported previously (Jefferson et al., 1987; Balhotia et a., 2016; Alok et al., 2020). The transformed suspension cells were kept in a 2 ml tube, and the liquid media were discarded. The 500 µl X-Gluc solution [0.1% X-Gluc (Sigma, USA), 100 mM Na_2HPO_4 p^H 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X and 20% Methanol] was added to the tube. The hygromycin-resistant callus was also incubated into 1 ml of X-Gluc solution in a 50 ml Tarson tube. These tubes were incubated at 37°C for overnight in the dark. Further, cells and calli were washed with 70% ethanol. The gus activity in suspension cells was visualized under a microscope, whereas stable calli visualized by naked eyes.

RNA isolation, cDNA preparation, and quantitative real-time PCR

Total RNA was extracted from callus and suspension cells using a CTAB extraction buffer containing activated charcoal, PVPP, and β-mercaptoethanol, as described earlier by Rajakani et al. (2013). The contamination of genomic DNA was removed using NEB DNAseI as per manufacturer protocol. First-strand cDNA synthesis was carried out using an oligo-dT primer of SuperScript III reverse transcriptase (Invitrogen, USA). Quantitative real-time PCR of the *uidA* gene was carried out using RT-specific primers (Table 2b) of *A. pallens Arf* (*ApArf*), *ApAct*, and *ApUbi* as reference genes. The expression was also assessed for one of the *A. pallens* gene, namely the *Phytoene desaturase* (*pds*) gene, Apart from transgene (*uidA*). The iTaq Universal SYBR Green Supermix (Bio-Rad, USA), with a final primer concentration of 0.3 µM forward and 0.3 µM reverse using Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, USA). Three biological replicates were amplified separately in real-time PCR assays. The PCR cycle was as follows: 95°C for 30 sec for 40 cycles, followed by 95°C for 5 sec and 56°C for annealing and extension. The melt curve plot was done from 65°C to 95°C with a 0.5 increase in temperature at each step.

Cloning of reference gene of *A. pallens*

Three housekeeping genes *Arf*, *Act*, and *Ubi*, and one *Pds* gene were cloned using degenerate primers. The degenerate primers were designed based on conserved regions of the candidate genes from monocot and dicot plants such as *Artemisia annua*, *Arabidopsis thaliana*, *Nicotiana tabacum,Solanum lycopersicum, Brachypodium distachyon*, *Triticum aestivum*, and *Oryza sativa.* The accession numbers of all nucleotide sequences from Gene Bank are presented in Table 2. The PCR reaction was set up using genomic DNA as a template, degenerate primers, and Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, USA). The cycle was as followed at 98°C temperature for 2 min (one cycle), 98°C for 30 sec, 55°C for 20 sec, 72°C for 30 sec (for 35 cycles), and a final extension at 72 °C temperature for 2 min. The amplified PCR products were purified and ligated into pJET1.2/blunt cloning vector using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA) as per manufacture protocol. The ligated products were then transformed into competent cells of *Escherichia coli* (*E. coli*) strain Top10. The plasmid was isolated, digested by the *Bgl*II restriction enzyme, and sent for sequencing.

Results

Effect of explant type on callus induction

Five different types of explants such as attached cotyledon, individual cotyledon, compound leaf, stem, and root explants were subjected to induce callus on various concentrations and combinations of media. Out of five types of explants, attached cotyledon, individual cotyledon, and compound leaf showed efficient callogenesis (Fig. 1a, b & c). Callus initiation and formation were observed after 7–10 days of culture initiation. Whereas, stem and root did not respond, or in few cases, callus formation was noticed onto root tip. Three different types of calli were formed independently of explant types. The MS medium supplemented with 2,4-D at 2.0 mg/L was found to be the optimal concentration for callus induction from three different explants (Fig. 1e). These are morphologically distinguished as (1) a friable cream yellow (Fig. 1d), (2) soft watery and non-embryogenic type (Fig. 1f), and (3) light blackish and hard nodular type (Fig. 1g). Cent percent callus induction was found with attached or individual cotyledons, whereas leaf showed 60% of callus induction.

Establishment Of Suspension Cells

Yellow friable calli (Fig. 1d) developed from attached cotyledon were transferred to different SCM to initiate suspension cells. Most of the friable calli dispersed and proliferated into cell suspension (Fig. 2a), but few lines were not appropriately proliferated and, therefore, discarded. It was observed that 80% of the callus developed from cotyledons was developed into fast proliferating suspension cells. Whereas, friable calli produced from leaf showed lower multiplication rate and few lines were released more phenolic and due to this, the medium got blackish color. The fast and uniformly dividing cells were subcultured after every 10 days, and efficient cell suspensions were established within 3 months. When the size of cells increased more than the size of mustard seeds, it was filtered to separate fine or small cells. The fine cells were maintained for a continuous supply of suspension cells (Fig. 2b). Whereas, the filtrated, which consisted of big size cells, were discarded (Fig. 2c). Out of all four SC media, the best growth of suspension cells was observed in the SC3 medium (Table 1). Fully developed and fast multiplying cell lines were diluted by transferring of 5 ml packed cell volume (Fig. 2d) from the old culture into 35 ml of fresh SC medium into a 250 ml conical flask.

Factors affecting Agrobacterium**-mediated transformation**

The protocol for suspension cell transformation has been optimized in present study, and the transformation was confirmed with the help of the intensity of blue coloration due to its GUS reporter gene. The GUS histochemical assay of non-transformed suspension cells did not show any color (Fig. 2e). Dark blue colored spots were noticed in suspension cells under the stereomicroscope when suspension cells were subjected to heat at 45°C as compared to normal transformation (Fig. 2f). Whereas, in the case of a cold shock to plant cells before co-culture, the significant changes were not noticed as compared to cells transformed in normal conditions (Fig. 2h). The addition of Pluronic F-68 (0.05%) in MSL medium during transformation also increased the transient transformation efficiency and showed more prominent blue spots within suspension cells (Fig. 2i). The addition of Silwett L-77 (0.05%) in MSL medium during transformation leads to more browning of cells, which might not be suitable. However, the blue coloration was a little higher as compared to expected. The GUS intensity was lower in cells transformed without including above factors. The maximum GUS intensity was observed as more blue spots in the transformed cells, including all these factors. The GUS fluorometric activity of suspension cells transformed with bacterial solution normally (without any factor) showed 267.8 ± 61.9 nmol 4MU/mg/min activity (Fig. 3). Pluronic acid and heat significantly improved transformation efficiency, and showed 513.1 ± 55.0 and 626.0 ± 32.5 nmol 4MU/mg/min activity, respectively (Fig. 3). The highest GUS activity (879.4 ± 113.7 nmol 4MU/mg/min) was recorded when all optimized factors were included (Fig. 3).

Development of micro calli and selection of stable calli lines

The final transformation was done with the optimized conditions such as heat, pluronic, and 2 days co-cultivation. The transformed fine suspension cells were spread onto the Whatman filter. The cells were taken out with the help of a cells scraper and kept in SC media supplemented with 250 mg L^{-1} cefotaxime in a 50 ml falcon tube. Washing with 250 mg L^{-1} cefotaxime for 30 min resulted in bacterial growth after 5–7 days onto plates. Whereas, washing with 250 mg L^{-1} cefotaxime and 90 rpm constant shaking for 6 hours did not show any bacterial contamination. The increase in the concentrations of cefotaxime up to 350 mg L^{-1} and 500 mg L^{-1} with 30 min also unable to inhibit bacterial growth. Transformed cells were allowed to grow for two months onto SM (Cef) media having filter paper (Fig. 4a). All fine cells were grown into small yellowish creamish micro-calli after 2–3 months (Fig. 4b). This micro-calli (2–3 mm) was sifted onto SM (10H) medium with 10 mg L^{-1} hygromycin directly onto the medium without filter paper. Untransformed micro-calli were turned brown after two to three subcultures and became dead (Fig. 4c). During every subculture, the dead calli were removed from the plates. Simultaneously, the stably transformed calli were whitish and pooled (Fig. 4d and e). The GUS histochemical staining of this stable calli showed intense blue color (Fig. 4f).

Confirmation Of T-dna Integration

The random screening of 6 hygromycin-resistant calli was done using *uidA* gene-specific primers. Agarose gel electrophoresis showed no bands in negative template PCR (L2), whereas as control, positive control plasmid PCR (L3) gave an amplification of 1500 bp amplicon. Hygromycin resistance calli also showed amplification of 1500 bp (Lane, L5 to L10) corresponding to the *uidA* gene, whereas no amplification was observed in non-transformed calli (L4) (Fig. 5). The *VirG* gene-specific primers were used to detect the presence of bacteria in hygromycin-resistant calli. All positive calli lines and control calli did not amplify *VirG* gene bands in PCR.

S**equencing and bioinformatics analysis of reference genes**

The draft genome, transcriptome, and EST sequences were not available for A. *pallens*, therefore we could perform a blast search using the reference gene sequences from *Arabidopsis*. The PCR product sizes of the *ApAct*, *ApArf*, *ApUbi* reference genes, and *PDS* gene amplified by degenerate primers were 175, 235, 300, and 400 bp in length, respectively (Supplementary material 1a). The sequencing result of the nucleotide sequence of positive clones showed the exact sequence (Supplementary material 1b). The BLASTn of sequenced *ApAct* showed the highest 99% similarity with *Chrysanthemum lavandulifolium actin* (JN638568.1), whereas 89.71 % similarity with *Arabidopsis*. *ApArf* showed 90.99% and 81.97% similarity with *Helianthus annuus (*XM_035986453.1) and *Arabidopsis ADP-ribosylation factor 2*, respectively. The *ApUbi* showed the highest similarity with *Prunus mume polyubiquitin*-A (XM_016792839.1), while with *Arabidopsis*, it showed 88.28% similarity. The *ApPDS* showed 96.45% and 79.39% identity with *phytoene desaturase* of *Chrysanthemum boreale (*KC202430.1) and *Arabidopsis* (NM_001340908.1), respectively. All sequences were submitted to NCBI Gene Bank, and the assigned accession numbers are MW579540, MW579541, MW579542, and MW579543.

Cq values of candidate reference genes and expression of UidA **transgene**

The value of the quantification cycle (Cq) represents the accumulated level of mRNA transcript in tissue. The mean Cq values of *ApUbi, ApAct*, and *ApArf* were 16.8, 19.0, and 21.1, respectively. The presence of a single peak also confirmed the primer specificities in melt curve analysis by RT-PCR (Fig. 6a, b, c). Further, a single DNA band confirmed these reference genes' primer specificities onto 1.5 % agarose gel electrophoresis (Fig. 6d). The relative expression level for the *uidA* transgene and *ApPDS* gene were quantified according to the 2-ΔΔCT method (Schmittgen and Livak, 2008; Alok et al., 2015). The *uidA* transcript mRNA of the selected callus (Line 1) was set to 1.

Discussions

Metabolic and genetic engineering in medicinal plants are in huge demand to enrich pharmaceutically important metabolites (Santos et al., 2016; Pourianezhad et al., 2019; Rodríguez-Sánchez et al., 2020). Suspension cell culture of *Sophora flavescens*, *Taxus baccata*, and *Morinda citrifolia* were used to produce sophoraflavanone G, taxol, and anthraquinone, respectively (Bassetti and Tramper, 1995; Kajani et al., 2010; Zhao et al., 2003). *Agrobacterium*-mediated genetic transformation is a more efficient and easy way of genetic transformation using different explants. Suspension cultures were successfully employed for *Agrobacterium*-mediated genetic transformation in several medicinally important plant species such as *A. anuua* (Sallets et al., 2015), *Taxus* sp. (Wilson et al., 2018), and *Gentiana* sp. (Rybczynski and Wojcik, 2019). Suspension cells can generate a large number of plants, and it has been used for various medicinal plants. *A. pallens* is an important medicinal plant in which protocols for cell suspension development and genetic transformation is still unreported. Therefore, in the current study, we optimized the best medium for suspension cell development and its *Agrobacterium*-mediated genetic transformation. Embryogenic calli were transparent or white friable, whereas non-embryogenic calli were blackish and globular-like structures in this case. In the present study, 2,4-D was used in SC media, and the same has been used for various other medicinal plants such as *Orthosiphon*

stamineus (Wai-Leng and Lai-Keng, 2004), *Peganum harmala* (Khafagi, 2007), *Jatropha curcas* (Soomro and Memon, 2007), and *A. annua* (Salehi et al., 2019). Embryogenic calli grow fast as compared to the non-embryogenic calli in suspension cells and similar result was observed in case of sweet potatoes (Yang et al., 2011).

The *Agrobacterium*-mediated genetic transformation was dependent upon various factors affecting transformation efficiency. In our earlier report in *A. pallens*, the factors such as bacterial density, acetosyringone, and strains were optimized (Alok et al., 2016). The AGL1 strain for the transformation of suspension cells was used, which is a supervirulent strain and used for various crop plants such as *Artemisia annua* (Sallets et al., 2015), *Musa* spp. (Shivani et al., 2018), and *Triticum aestivum* (Alok et al., 2015; 2020) for transformation studies. Heat treatment before the bacterial infection could enhance the transformation capacity (Tripathi et al., 2008). Similarly, in this study, the GUS staining and specific activity were higher in heat-treated cells than the normally transformed cells (Fig. 2&3). There were no significant changes observed in transient transformation efficiency on the addition of Silwet L-77; however, it increased GUS expression in *Cannabis* sp. (Deguchi et al., 2020). However, the addition of Pluronic F-68 in bacterial solution increased the GUS intensity and activity (Fig. 2 &3). Similarly, the addition of Pluronic F-68 surfactants effectively increased the transformation efficiency in *Theobroma cacao* (Fister et al., 2016) and *Cannabis sativus* (Deguchi et al., 2020). After transformation, cefotaxime was used in the selection medium to kill adhered *Agrobacterium*. The overgrowth of bacteria might influence cell growth negatively. Similarly, cefotaxime is better than other antibiotics, which do not block plant regeneration in the case of sweet potatoes (Yang et al., 2011).

Transgene expression analyses are generally done by the RT-qPCR method due to their unique advantages. However, for this, a stable reference gene should be known. Due to unknown genome information of *A. pallens*, another strategy is to clone using degenerate primers. Various medicinal plants, whose reference genes were not reported, used degenerate primers (Wang et al., 2017). *ApArf*, *ApAct*, and *ApUbi* are the most commonly used reference genes in various plant species (Alok et al., 2015; Flowerika et al., 2016, Wang et al., 2017; 2019; Alok et al., 2020). Here, for the first time we, reported the nucleotide sequences of three reference genes from *A. pallens*. The melt curve and amplification on agarose gel showed that a single band was amplified (Fig. 6a, b, c & d). It is crucial to select reference genes in a specific experiment and avoid using multiple genes that participate in related biological processes (Wang et al., 2017). These findings will provide more insight into suspension cell transformation and the expression profile for the transgene as well as indigenous target genes in *A. pallens*.

Conclusions

This study successfully demonstrated a reliable, stable, and transient genetic transformation of *A. pallens* using cell suspension cultures derived from cotyledonary explants with *A. tumefaciens* strain AGL1 harboring pCAMBIA1301. Transformation efficiency was enhanced by optimizing various factors like temperature, silwet, and pluronic in suspension cultures confirmed by the stable expression of the β-glucuronidase (*uidA*) reporter gene. Further, three candidate reference genes *Arf*, *Act*, and *Ubi* were cloned using degenerate primers and quantified the uidA gene expression in transgenic lines. The optimized *Agrobacterium*-mediated genetic transformation protocol could help better understand the metabolic pathways and improve this valuable medicinal herb.

Declarations

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Author Contribution

AA and VRA conceived the idea, designed the experiments, analyzed the results, and finalized the manuscript. PJ and DS performed experiments and wrote the manuscript. KS and VRA provided the facilities and improved the manuscript. VP and MSS conceptualization, data curation, and revision of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Research involving human participant and/or animals

No human participants and/or animals have been involved in this research.

Informed consent

No human participants have been involved in this research.

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Tables

Table 1. Different media and composition

Name of Media	Composition of media
MSA	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8 (KOH)
SC1	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, and 0.8 % (w/v) agar, pH 5.6 (KOH)
SC2	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, 5 mg/L Ascorbic acid, and 0.8 % (w/v) agar, pH 5.6 (KOH)
SC3	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, 0.25 mg/L Zeatin, 5mg/L Ascorbic acid, and 0.8 % (w/v) agar, pH 5.6 (KOH)
SC4	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, mg/L 0.5 Zeatin, 5mg/L Ascorbic acid, and 0.8 % (w/v) agar, pH 5.6 (KOH)
MSL	4.33 g/L Murashige and Skoog basal salts, 20 mg/L glucose, 195.2 mg/L MES, pH 5.6 (KOH)
SM(Cef)	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, 0.25 mg/L Zeatin, 250 mg/L cefotaxime, 0.8 % (w/v) agar, pH 5.85 (KOH)
SM(10H)	4.33 g/L Murashige and Skoog basal salts, 3 % (w/v) sucrose, 2 mg/L 2, 4 D, 0.2 mg/L Zeatin, 10 mg/L hygromycin , 250 mg/L cefotaxime, 0.8 % (w/v) agar, pH 5.8 (KOH)

Table 2a: Degenerate primers of reference genes for cloning in *A. pallens*.

Genes	Forward primer sequence	Reverse primer sequence	Amplicon size
ApAct	5’AYGAYATGGARAARATHTGGCAYCAY3’	5’GCYTGRATRGCVACATACATRGCHGG3’	175 bp
ApArf	5’AATGAYMGRGABCGYGTTGTKGARGC3’	5’RCCARTCMAGVCCYTCRTAVARH3’	235 bp
ApUbi	5’AAGCARCTKGARGAYGGMMGNACYY3’	5’RVACMARRTGVARSGTBGAYTCYTT3’	300 bp
ApPDS	5’ARGATGAWGATGGNGAYTGGTAYGA3’	5’GCCTTKGACATDGCAATRAAMACCTCR3’	400 bp

Table 2b: RT-PCR primers of used for real time expression of transgene in *A. pallens*.

Genes name	Forward primer sequence	Reverse primer sequence
ApAct	5’GGCATCACACTTTCTACAACGAGC3’	5’CCTGGATGGCAACATACATAGCG3’
ApArf	5’GGGAACGTGTTGTTGAGGCAAG3’	5’CCCTCGTACAGTCCTTCGCCAG3’
ApUbi	5’GGAATCGACCCTCCATTTGGTTC3’	5’GTCAGCAAGGGTACTTCCATCCT3’
ApPDS	5’CTGCCTGCACCACTCAATGGAA3’	5’CACCTCAGTCGTAACTCGATCC3’
UidA_RT	5'AGGGCCTCGGAAAAGTCATTCGT	5’CCCGGTTGGGCCATTGAAGTC3’
UidA	5’TACCCGATCAACACCGAGACCCG3’	5’CCACGCGTGAAATTCCTGGCGG3’

Table 2C: Accession numbers of reference and *PDS* genes in different plants in Gene Bank.

Genes	*Arabidopsis thaliana*	*Nicotiana Tobaccum*	*Solanum lycopersicum*	*Artemisia annua*	*Brachypodium distachyon*	*Triticum aestivum*	*Oryza sativa*
ApAct	NM_121018	XM_016658880	NM_001330119.1	EU531837.1	XM_003574140.4	AK457872	XM_015759418
ApArf	NM_001331919	XM_016584354	XM_026030464	Not found	XM_003557321	AK446928	XM_015791548
ApUbi	NM_001084884	XM_016597143	NM_001345879	EU258763	XM_003574817	AK446626	XM_015780523
ApPDS	NM_001340907	XM_016642616	XM_019212594	KM892862	XM_010230778	FJ517553	XM_015777615

Figures

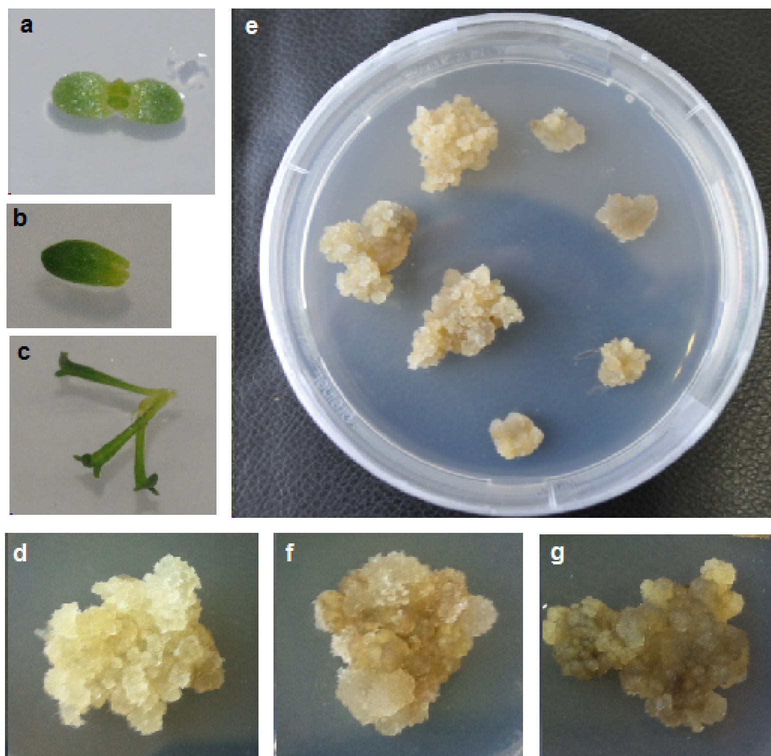

Figure 1

Callus induction from different explants of Artemisia pallens on MS medium supplemented with 2,4-D (2. 0 mg L-1). (a) attached cotyledon explant, (b) individual cotyledon explant, (c) compound leaf explant, (d) friable cream yellow callus, (e) 2 months old calli onto medium obtained from cotyledon explant, (f) non-embryonic callus, and (g) hard nodular callus.

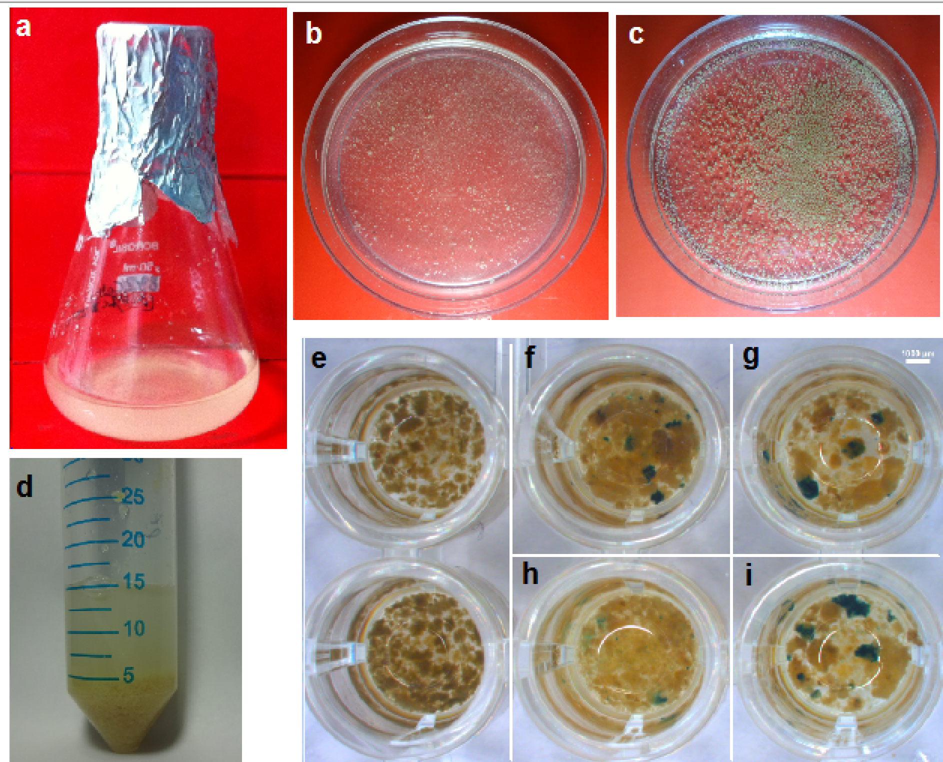

Figure 2

Initiation and proliferation of suspension cultures from callus derived from cotyledon explants of Artemisia pallens on MS medium supplemented with 2,4-D (2.0 mg L-1) and ZEA (0.25 mg L-1). (a) Initiation of suspension cultures, (b) proliferated fine cells after two to three months of cultures initiation, (c) filtered suspension cells or clumps, (d) transformation of suspension cultures with Agrobacterium tumefaciens, (e) non-transformed suspension cells and micro-calli (control) after gus histochemical assay, (f) transformed suspension cells and micro-calli showing blue color after gus histochemical assay, (g) effect of temperature on gus histochemical assay, (h) effect of cold shock on gus histochemical assay, and (h) effect of pluronic acid F-68 on gus histochemical assay,

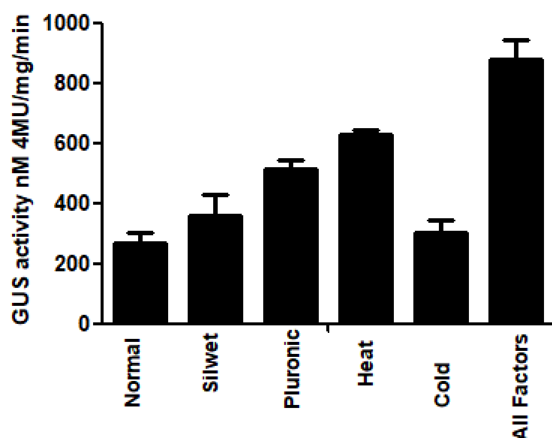

Figure 3

Effect various factors on GUS fluorometric activity nmol (4MU/mg/min) of suspension cells transformed with Agrobacterium tumefaciens.

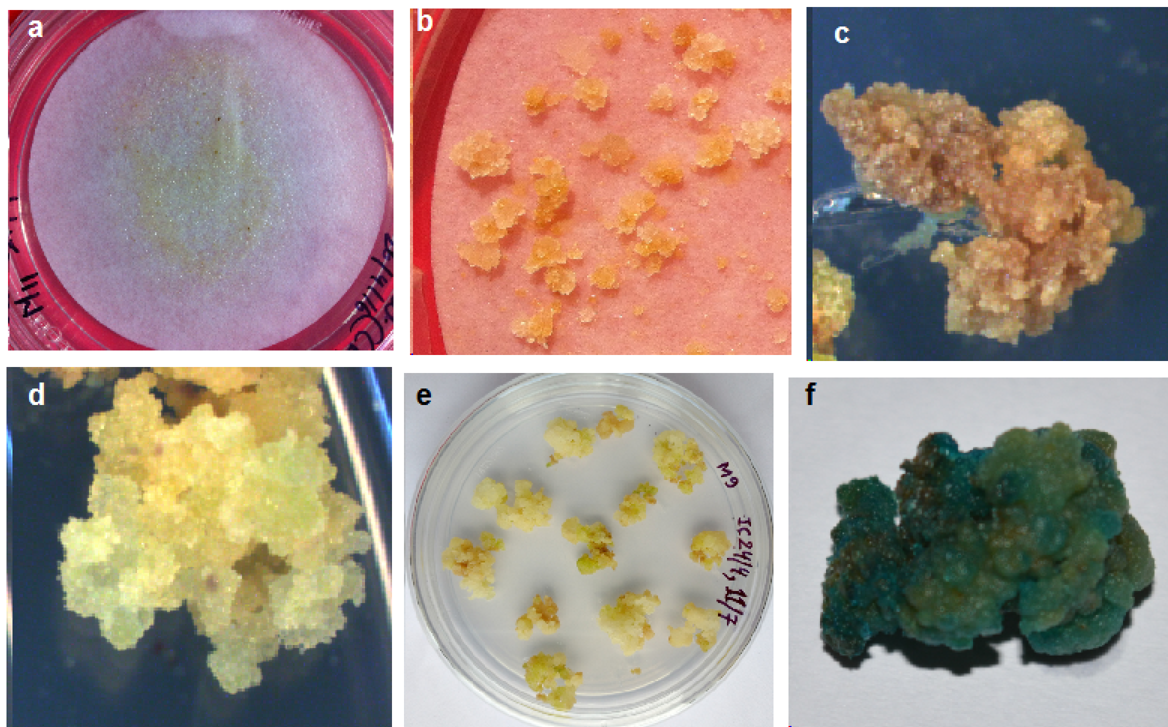

Figure 4

Proliferation of transformed suspension cultures and development of micro-calli of Artemisia pallens. (a) transformed cells onto filter paper, (b) yellowish and creamish micro calli after 3 months of cultures, (c) untransformed calli dead after two to three subcultures on selection medium, (d and e) transformed micro-calli on selection medium after two to three subcultures, and (f) transformed micro-calli showing blue color in gus histochemical assay.

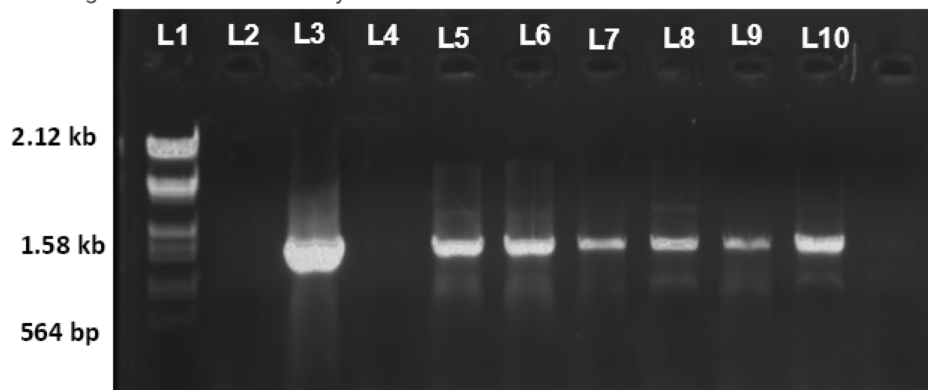

Figure 5

Molecular confirmation of transgenic callus lines Artemisia pallens analysis for gusA gene presence in hygromycin-resistant callus lines using gusA gene-specific primers. L1: Marker DNA ladder (2.12 kb ladder, Thermo Scientific); L2: No template (Negative control); L3: Positive control (plasmid DNA); L4: Wild type (control plant DNA); L5-L10: Hygromycin resistant callus lines

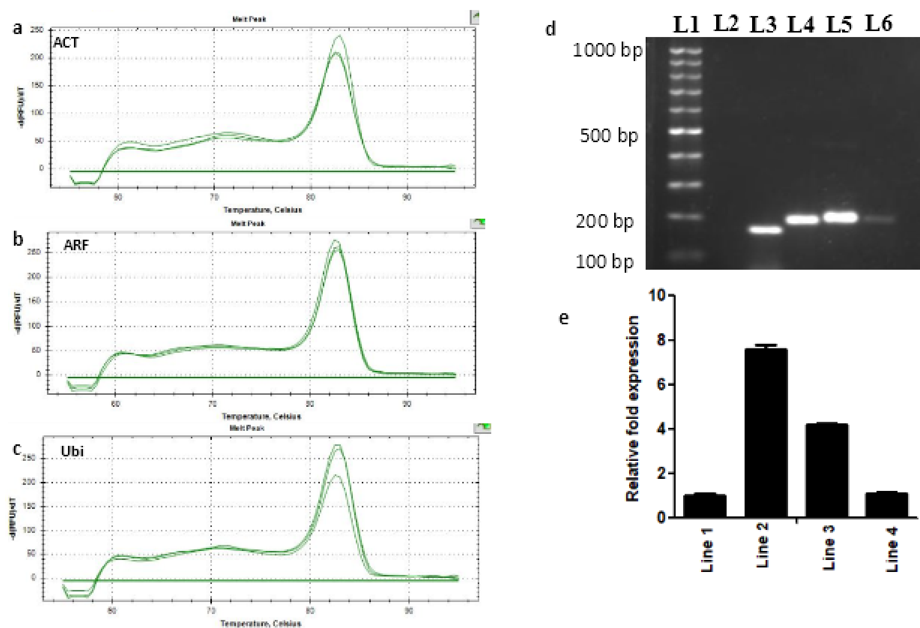

Figure 6

Confirmation for the presence of a single peak in melt curve analysis by RT-PCR. Melt curves of 3 candidate reference genes showing single peaks (a) ApAct (β-actin), (b) ApArf (ADP-ribosylation factor), (c) ApUbi (ubiquitin) (d) L1: 100 bp DNA ladder, L2: No template (Negative control), L3: ApAct, L4: ApArf, L5: ApUbi, and L6: Appds (e) the relative expression of uidA gene in transgenic callus lines of Artemisia pallens.

Supplementary Files

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- groupimage3.png