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Research Article

Agroinfiltration-mediated transient assay for rapid evaluation of constructs in pigeonpea

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ABSTRACT

The process of generating stable transformants is time-consuming, labor-intensive, and genotype-dependent. In contrast, transient gene expression techniques, such as agroinfiltration, offer a rapid assessment of gene function and expression. Agroinfiltration, widely employed for studying gene function, has been extensively applied in leaf tissues of *Nicotiana benthamiana* and various other plant species. Despite its broad utility in various plants, to our knowledge, no prior investigation has been reported in pigeonpea. In this study, we developed an agroinfiltration method for transiently expressing a green fluorescent protein (*mGFP5*) reporter gene in four pigeonpea genotypes using syringe infiltration at the seedling stage under greenhouse conditions. The expression of the reporter gene *mGFP5* was assessed at 72-, 96-, and 120 h post-infiltration (hpi). Additionally, we assessed the effect of morphogenic genes, specifically *growth-regulating factor 4* (*GRF4*) and *GRF-interacting factor 1* (*GIF1*), from both rice and pigeonpea on the expression of *mGFP5* in four pigeonpea genotypes. Our findings demonstrate that *OsGRF4-GIF1* led to enhanced *mGFP5* expression compared to *CcGRF4-GIF1* in four diverse pigeonpea genotypes. Fluorescence could be detected till 120 hpi. Furthermore, PCR, RT-PCR, and fluorescence quantification confirmed the presence and expression of *mGFP5* at 72 hpi. Our results highlight the efficacy of agroinfiltration in quickly evaluating candidate genes in four genetically diverse pigeonpea genotypes, thereby reducing the time required for the initial assessment of constructs suitable for diverse molecular biology analyses.

1. Introduction

Pigeonpea (*Cajanus cajan* L.) is a C3 crop grown as a short-lived perennial shrub and an annual crop in developing nations. It serves as a crucial legume crop, addressing food security, reinforcing nutrition, promoting sustainable agriculture, and supporting livelihoods globally.[1] Despite its importance, pigeonpea productivity has remained stagnant for decades due to various biotic and abiotic stresses.[2,3] Conventional plant breeding has faced challenges in boosting pigeonpea yields due to genetic diversity and incompatibility between cultivated and wild varieties.[4] A notable distinction between conventional breeding and biotechnology lies in their respective speed, precision, reliability, and scope. The advent of plant transformation techniques represents a significant breakthrough in overcoming constraints and enabling precise genetic manipulation.[5]

Considerable genomic resources have been developed for pigeonpea, comprising numerous molecular markers of various traits,[6–8] genetic maps, and mapped QTLs for important traits.[9–12] Additionally, there are gene expression profiles[13] and a reference genome sequence assembly available for pigeonpea.[14–16] In addition, genetic transformation protocols and proof-of-concept gene editing have been established for pigeonpea.[17–19] Despite this significant progress, the rapid evaluation of the functional genes remains a challenge, particularly across diverse pigeonpea genotypes, some of them are highly recalcitrant. Recalcitrance to genetic transformation primarily arises from the incapacity to regenerate viable plants from an explant cultured *in vitro*, either through *de novo* organogenesis or somatic embryogenesis. Regeneration capability varies significantly among genotypes within a species, likely due to genetic variation[20,21] and response to environmental factors such as the chemical composition of the culture medium,[17–19] culture conditions such as temperature, photoperiod, and type of explant.[22,23] The genetic elements governing shoot regeneration mainly involve developmental regulators or morphogenic genes like *GRF (growth regulating factors)* and *GIF (GRF interacting factors)*, which are conserved across species.[24] In pigeonpea, the expression of *CcGRF4* and *CcGIF1* was significantly higher in immature embryos and mature seeds.[25] The use of a monocot

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fusion protein GRF4–GIF1 has notably enhanced regeneration efficiency, speed, and somatic embryogenesis in wheat and rice,[26] watermelon,[27] and lettuce,[28] suggesting the potential application of this approach in dicot crops as well.

Numerous researchers have made efforts to establish an effective stable *Agrobacterium*-mediated genetic transformation system for pigeonpea.[17–19] However, the process of obtaining stable transformants is both time-consuming and labor-intensive. In contrast to the creation of stable transformants, transient gene expression methods, including agroinfiltration, offer rapid assessment for gene expression and function.[29] Agroinfiltration has been effectively used for rapid analysis of gene function,[30] conducting protein-protein studies, and implementing gene-silencing approaches.[31] This technique has been successfully used in various plant species such as soybean,[32] cowpea,[33] tobacco,[34] tomato,[35] potato,[36] lettuce,[37] and Arabidopsis.[38] Despite its extensive use in other plants, agroinfiltration in pigeonpea has not been reported so far.

In this study, we used a simple, efficient, and reliable method for developing an agroinfiltration system to express the reporter gene - green fluorescent protein (*mGFP5*)[39,40] transiently within the primary leaves of pigeonpea seedlings using syringe-based infiltration. Furthermore, to assess the feasibility of this approach as a method to screen constructs, we targeted GRF4–GIF1 fusion protein from rice and pigeonpea to see whether the use of morphogenic genes, enhances *mGFP5* expression in four pigeonpea genotypes. This methodology can be effectively utilized for functional genomic studies, investigating protein-protein interactions, and characterizing promoters for targeted genes in various pigeonpea genotypes without generating transgenic plants.

2. Materials and methods

2.1. Construct development

The sequences for *GRF4* (Os02t0701300.01 and Cc_v2.0_08797) and *GIF1* (Os03t0733600.01 and Cc_v2.0_04050) were obtained from the rice (https://rapdb.dna.affrc.go.jp/) and pigeonpea (https://www.legumeinfo.org/taxa/cajanus/) databases, respectively using rice *GRF4-GIF1*.[25,41] Subsequently, the rice ubiquitin (*OsUbi*) (obtained from Addgene plasmid #126072, pRGEB32-BAR) promoter-driven *GRF4-GIF1* genes, along with NOS terminator genes, were synthesized and inserted into pUC57 plasmid individually (Genscript Biotech, Singapore) for both rice and pigeonpea genes. The pUC57 plasmid was then digested with *Sac*I and *Kpn*I to release the *OsUbi-OsGRF4-GIF1-NosT* gene, which was subsequently used for sequential cloning into the pCAMBIA2300-mGFP5 expression construct (Fig. 1A). Similarly, *OsUbi-CcGRF4-GIF1-NosT* was inserted into the pCAMBIA2300-mGFP5 construct using *Xba*I and *Sal*I restriction enzymes (Fig. 1B). The modified GFP (*mGFP5*) has a mutation, which is smaller in size with cryptic intron removed and codon optimized for expression in plants.[42] This study used four constructs: pCAMBIA2300 (empty vector), pCAMBIA2300-mGFP5, pCAMBIA2300-mGFP5::OsUbi-OsGRF4-GIF1-NosT, and pCAMBIA2300-mGFP5::OsUbi-CcGRF4-GIF1-NosT.

2.2. Agroinfiltration

2.2.1. Plant materials and growth conditions

Seeds of the four pigeonpea (*Cajanus cajan* L.) genotypes—ICPL87119, ICPL87, ICPV21333, and TS3R—were obtained from the Pigeonpea breeding unit at ICRISAT, India. Among these genotypes, ICPL87119, is a medium-duration, high-yielding genotype with large seeds and broad adaptability, resistant to fusarium wilt and sterility mosaic virus diseases. ICPL87 is characterized as a short-duration, high-yielding pigeonpea variety with wide adaptation, suitable for both single and multiple harvests, and exhibiting tolerance to fusarium wilt. ICPV21333 is an extra early genotype with synchronous maturity. TS3R stands out as a medium-to short-duration, high-yielding pigeonpea variety known for its resistance to fusarium wilt.

2.2.2. Preparation of Agrobacterium culture

The constructs were introduced into *Agrobacterium tumefaciens* strain C58 via electroporation. The pCAMBIA2300 vector carries genes encoding *aminoglycoside phosphotransferase (Apt)*, conferring kanamycin resistance in bacteria, and *neomycin phosphotransferase (NptII)* for kanamycin selection in plants. A single colony of *A. tumefaciens* grown on yeast extract peptone (YEP) media (Sigma-Aldrich Chemicals Pvt. Ltd, Bangalore, India), supplemented with 50 mg/L kanamycin (Sigma-Aldrich Chemicals Pvt. Ltd, Bangalore, India) and 25 mg/L rifampicin (Sigma-Aldrich Chemicals Pvt. Ltd, Bangalore, India), confirmed using polymerase chain reaction (PCR) with vector-specific primers was identified (Table S1). This single PCR-positive colony was cultured in 5 mL YEP starter broth containing 50 mg/L kanamycin and 25 mg/L rifampicin and incubated overnight at 28 °C with shaking at 200 rpm. The overnight starter culture was used to inoculate 25 mL fresh YEP broth with the same antibiotic concentrations and allowed to grow until the OD 600 reached 1.0. Before agroinfiltration, bacterial cells were collected by centrifugation at 5000×*g* for 10 min at room temperature, then resuspended in freshly prepared 5 mL infiltration buffer (5 M NaCl, 175 mM $CaCl_2$, 125 µL Tween 20, and 100 mM acetosyringone). The volume was adjusted to 50 mL with distilled water.

2.2.3. Agroinfiltration

Pigeonpea agroinfiltration was conducted following a protocol reported for *Medicago*[43] with slight modification. The 10-day-old seedlings were grown in a temperature-controlled greenhouse at 25-28 °C with a 14-10 h light-dark photoperiod. The two-leaf stage seedlings were chosen for agroinfiltration (Fig. 2A). Leaves with well-expanded and soft abaxial surfaces were selected to facilitate efficient infiltration. One day before the infiltration treatment, irrigation was stopped. The leaf was supported from the adaxial side with a finger, and using a 1 cc needleless syringe containing *Agrobacterium* solution, gentle pressure was applied to the underside of the leaf (abaxial side), slowly pressing the syringe to infiltrate the plant tissue to ensure uptake of the solution through stomata (Fig. 2B and C). It is known that local wounding can cause patterns of bright autofluorescence.[44] Therefore, the data generated needs to be interpreted with care. Care was taken not to apply excessive pressure during infiltration, as it could result in bruising and tearing of the leaf tissue, leading to potential loss and also minimizing autofluorescence of the infiltrated region. To assess the pattern of fluorescence two regions were targeted: one at the base and the other at the mid-leaf, both adjacent to the midrib. Successful infiltration was indicated by a water-soaked appearance of the leaf (Fig. 2D and E). Infiltration of buffer (1X) served as a negative control. Proper labeling of all infiltrated plants ensured easy identification during sample collection. Following treatment, all treated plants, including the control, were promptly covered with dark plastic covers and placed beneath a table to prevent direct light exposure to the treated regions (Fig. 2F and G). The experimental setup was left undisturbed until sample collection. The infiltrated leaves were wiped with ethanol before sample collection. Expression levels were assessed using a fluorescent stereo microscope (Serial No- Leica M165 FC, Leica Microsystems, India), with fluorescence checked at various time points starting from 72 h (h), 96 h, and 120 h post-infiltration (hpi). Samples were collected in sampling covers and placed in a dark box to prevent light exposure. Subsequently, samples were examined under the fluorescence microscope using a GFP filter with a wavelength of 520 nm and a diameter of 2 mm. The minimum level of exciting light was used to avoid bleaching and damage to infiltrated leaves. Images are acquired with image-processing software - LAS V4.13. In addition, the intensity of the fluorescence was quantified using ImageJ software.[45] For each construct, two leaves were inoculated, and the entire experiment was repeated three times. The data was statistically analyzed using a one-way ANOVA in PRISM 10 (GraphPad

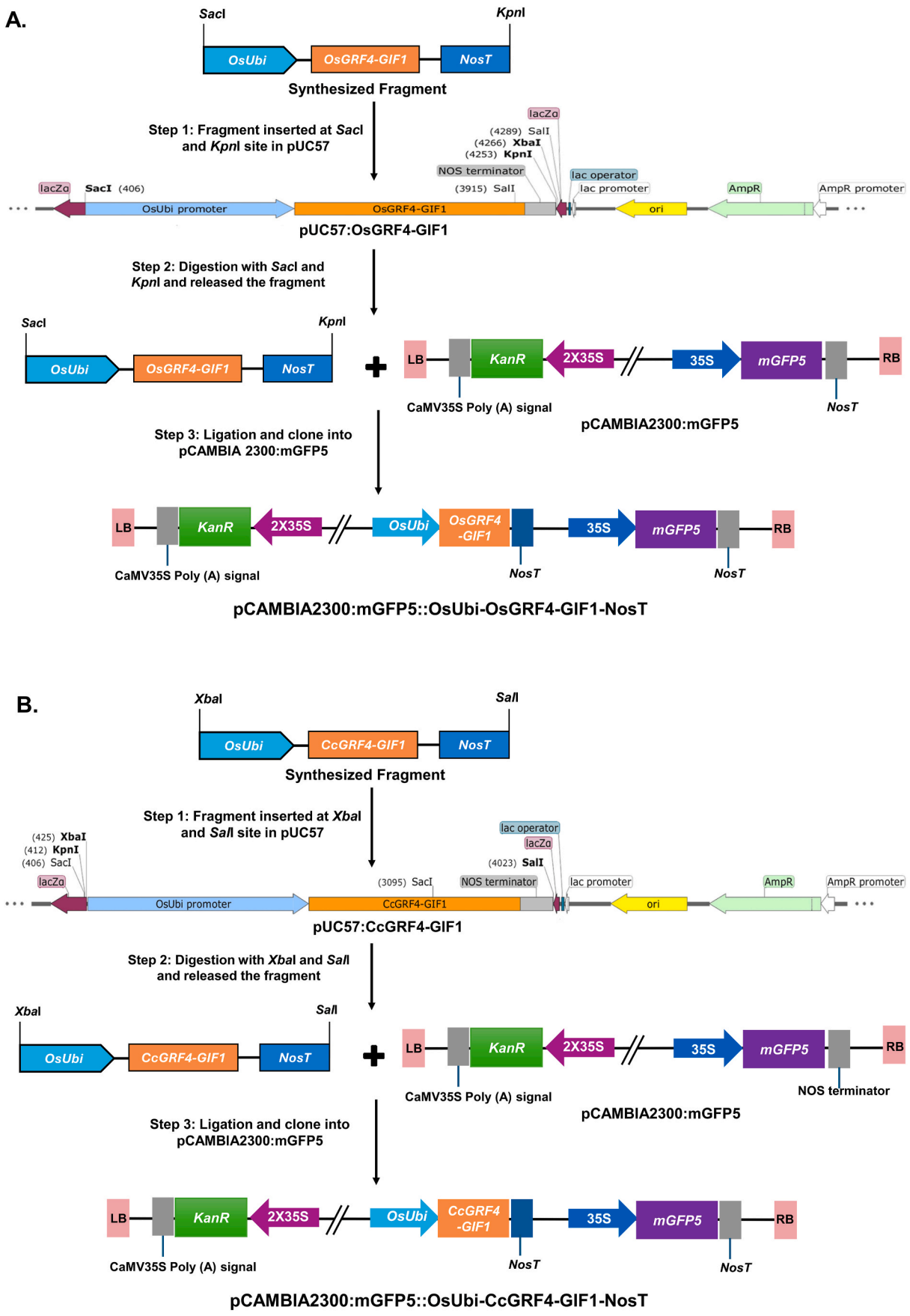

Fig. 1. Schematic diagram showing the cloning of (A) *OsUbi-OsGRF4-GIF1-NosT* and (B) *OsUbi-CcGRF4-GIF1-NosT* into pCAMBIA2300-mGFP5 binary vector.

Fig. 2. Overview of the agroinfiltration protocol in pigeonpea. (A). Selection of two leaf stages (B and C). Agroinfiltration syringe on the abaxial surface of the leaf (D and E). Water-soaked lesion on abaxial and adaxial leaf surface after agroinfiltration (F and G). Agroinfiltrated plants were covered and placed under the table.

Software Inc.), and the significance of fluorescence values was assessed using Tukey's test, with a p-value threshold of $p < 0.05$.

2.3. Confirmation of the mGFP5 gene in the infiltrated plants

Genomic DNA was isolated from infiltrated leaf tissue from 72 hpi, following the DNA extraction buffer made of 2 % hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 1.4 M sodium chloride (NaCl), 2 % polyvinylpyrrolidone (PVP)-40 (Sigma-Aldrich Chemicals Pvt. Ltd, Bangalore, India). The quality of the DNA was assessed through 1 % agarose gel electrophoresis. The presence or absence of *mGFP5* was determined via PCR amplification using *GFP*-specific primers (GFP-FP: 5′-GGAGTTGTCCCAATTCTTGT-3′ and GFP-RP: 5′-ATGCCGTTCTTT TGCTTGTC-3′) with genomic DNA as the template and pigeonpea *GAPDH* gene (Table S1) as an internal reference. The PCR reaction was carried out using Emerald Amp® GT PCR 2 X Master Mix (Takara Bio Inc, USA) according to the manufacturer's instructions. The PCR conditions - 2 min at 98 °C, 35 cycles of 10 s at 98 °C, 30 s at 55 °C and 1 min at 72 °C, followed by 5 min at 72 °C, were used. Gel electrophoresis was performed on a 1.5 % agarose gel containing 5 μL/100 mL SYBR safe stain (Invitrogen), and gel images were captured using a Gel Doc XR$^+$ Gel Documentation system (Bio-Rad, Bangalore, India) for visualization and recording of PCR products.

For Reverse Transcriptase (RT)-PCR analyses, total RNA was extracted from infiltrated sites of pigeonpea leaves using the RNeasy Plant Mini kit (Qiagen, Tokyo, Japan) with three replications. A 2.0 μg portion of purified RNA was used for cDNA synthesis following the recommended protocol (Thermoscript RT-PCR system, Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using *GFP*-specific primers (Table S1), and gel images were captured, as mentioned in the earlier section.

2.4. Quantification of mGFP5 fluorescence

To measure GFP fluorescence in infiltrated leaves, infiltered leaf tissue (100 mg) was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. The protein extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA) was added at a 1:4 (g/ml) ratio to the powdered tissue and mixed thoroughly. Centrifuged the homogenate at 12,000 g for 15 min at 4 °C, and the supernatant containing the total soluble proteins was collected.[46] Further, the GFP fluorescence in the total protein was measured using a Tecan Spark® Microplate Reader (Tecan Trading AG, Männedorf, Switzerland) at an excitation/emission wavelength of 488/528 nm. Relative fold changes were determined by comparing the fluorescence values obtained for the constructs with the values obtained for the empty vector.

3. Results

3.1. Design of constructs

To test whether the presence of morphogenic genes *GRF4* and *GIF1* enhances the expression of the reporter gene *mGFP5* in pigeonpea, four

different constructs were tested. The pCAMBIA2300-mGFP5 vector was modified as mentioned in the materials and method (Fig. 1). Briefly, the *OsUbi* promoter driving the expression of an *OsGRF4-GIF1* and *CcGRF4-GIF1* (Fig. 1) was incorporated into the binary vector pCAMBIA2300-mGFP5, resulting in the creation of the pCAMBIA2300-mGFP5::OsUbi-OsGRF4-GIF1-NosT (Fig. 1A) and pCAMBIA2300-mGFP5::OsUbi-CcGRF4-GIF1-NosT constructs (Fig. 1B).

3.2. Agroinfiltration of pigeonpea to test the efficacy of constructs by measuring GFP fluorescence

Agroinfiltration was conducted using four pigeonpea genotypes: ICPL87119, ICPL87, ICPV21333, and TS3R. The infiltration buffer was used as a negative control. Additionally, the following constructs were used for testing agroinfiltration efficacy as measured by GFP fluorescence: pCAMBIA2300 (empty vector), pCAMBIA2300-mGFP5, pCAMBIA2300-mGFP5::OsUbi-OsGRF4-GIF1-NosT, and pCAMBIA2300-mGFP5::OsUbi-CcGRF4-GIF1-NosT. The infiltration process involved using a 1 cc syringe to infiltrate the abaxial surface of leaves at the 2-leaf stage of 10-day-old plants. Specifically, leaves at this stage were broad and fully opened. In pigeonpea, leaf tissue wetting was minimal, similar to soybean, where it did not cover the entire leaf but extended to some degree beyond the diameter of the syringe head.[32] After infiltration, the infiltrated leaves were covered with plastic covers to prevent direct exposure to light and were left undisturbed for 72 h or until *GFP* measurement was conducted (Fig. 2).

GFP fluorescence was assessed using a Leica fluorescent stereo microscope, with the *GFP* filter set to a wavelength of 520 nm and a diameter of 2 mm. Measurements were taken at three different time intervals: 72-, 96-, and 120 hpi, with three replicates for each genotype (Fig. 3). Initially, fluorescence was observed primarily in the border regions of the infiltrated area where wetting occurred during agroinfiltration. However, over time, fluorescence extended to adjacent cells where wetting did not initially occur. The level of fluorescence was the same at 72-, 96-, and 120 hpi (Figs. S1 and S2). Furthermore, the experiment investigated the impact of introducing morphogenic *GRF4-GIF1* genes from rice and pigeonpea on *GFP* expression levels. The objective was to recognize any variations in fluorescence intensity and whether the addition of morphogenic genes affected expression levels. Our findings indicate that *GFP* expression was higher in the presence of *OsGRF4-GIF1* than *CcGRF4-GIF1* across genotypes (Fig. 3A).

Additionally, GFP quantification in the total protein extracted from infiltrated leaves was measured using the Tecan Spark® Microplate Reader with excitation and emission wavelengths of 488/528 nm. Quantification of fluorescence (measured relative fluorescence unit (RFU)) revealed variation across genotypes. The fold change difference ranged from 4.80 to 152.46. Among the genotypes, ICPL87119 exhibited the highest GFP levels. Moreover, the rice morphogenic genes *OsGRF4-GIF1* displayed greater GFP levels compared to other constructs including construct carrying pigeonpea morphogenic genes (Fig. 3B). On the other hand, the presence of pigeonpea *GRF-GIF* led to reduced *mGFP5* expression in three genotypes - ICPL87, ICPV 21333, and TS3R. The image quantification using Image-J analysis also supports the observation of higher GFP levels in the *OsGRF4-GIF1* construct (Fig. S3).

3.3. Molecular analysis of agroinfiltrated pigeonpea expressing mGFP5

In this study, a particular variant of *GFP*, known as *mGFP5*, served as the reporter gene. PCR using gene-specific primers confirmed the presence of *mGFP5* gene in the infiltrated leaves of pigeonpea genotypes (Fig. 4A). Our results demonstrate the presence of *GFP* in all four genotypes, compared to the empty vector and infiltration buffer controls (Fig. 4B). Furthermore, RT-PCR analysis of the infiltrated leaves confirmed the expression of *mGFP5* in all the four pigeonpea genotypes (Fig. 4C).

4. Discussion

Plant transformation serves as a crucial method for unraveling the complexities of plant biology. However, obtaining stable transformants proves to be time-consuming and labor-intensive. Agroinfiltration emerges as a potent technique that has transformed the landscape of plant science. Recognized as a quick method for assessment of gene function,[30] generating recombinant proteins,[47] and investigating plant-pathogen interactions.[48] Agroinfiltration presents a relatively straightforward, rapid, and cost-efficient approach to manipulating plant cells for diverse research and development endeavors.[29] Its successful implementation spans various plant species, including soybean,[32] cowpea,[34] tobacco,[34] tomato,[35] potato,[36] lettuce,[37] and Arabidopsis.[38] Despite its widespread application in other plant species, the utilization of agroinfiltration in pigeonpea has not been reported so far. Hence, our efforts are directed towards developing and optimizing agroinfiltration in pigeonpea utilizing the modified reporter gene, *mGFP5*.[42] This modified plant codon-optimized *mGFP5* lacks 84 bp cryptic introns and has mutations V163A, S175G, and S167T, which allows better folding and, hence, detection in plants.[39]

Transformation studies in highly recalcitrant crop species such as pigeonpea encounter notable obstacles, including issues related to chimera formation, genotype specificity, extended crop duration, and low regeneration efficiency.[49] Despite previous reports indicating effective stable regeneration and transformation in pigeonpea,[17] creating stable transgenic lines takes time and effort. Despite numerous efforts from various research groups to address this challenge, the successful establishment of an efficient rapid screening transformation system for pigeonpea still needs to be established.[18] In contrast to the production of transgenic plants, transient gene expression offers a rapid and straightforward alternative for analyzing gene function. Additionally, it also allows for an opportunity to check the response to diverse genotypes to transformation protocol. Among transient expression methods, agroinfiltration is widely used, typically conducted by introducing an *Agrobacterium* suspension through the stomata on the underside of leaves using a syringe. Transgenes are subsequently transferred from *Agrobacterium* into the leaf parenchyma cells. Although only a fraction of these T-DNA copies integrate into the plant chromosomes, the non-integrated T-DNAs transiently express for several days.[29] The primary advantage of transient assays is the generation of initial experimental results which would reduce the number of constructs to be tested by stable transformation within a few days rather than months. Despite its utilization in various plants, to our knowledge, no prior investigation has reported agroinfiltration in pigeonpea.

Our research findings reveal that *GFP* fluorescence was detected in four pigeonpea genotypes at varying levels across three different time points, a result further confirmed by PCR, RT-PCR, and quantification of GFP. Furthermore, we used two morphogenic genes in a single reading frame without stop codon, namely *GRF4-GIF1* (leading to the production of fused protein), derived from rice and pigeonpea, respectively, to assess whether the presence of morphogenic genes leads to enhanced expression of the reporter gene *mGFP5*. Our investigation revealed that the *OsGRF4-GIF1* complex substantially enhanced *GFP* expression compared to *CcGRF4-GIF1* across the four pigeonpea genotypes tested. Two pigeonpea genotypes (ICPL87119 and ICPL87) have been used for various transformation experiments so far.[19,23] Testing of genotypes with different genetic backgrounds and duration with the current protocol suggests that *OsGRF4-GIF1* is working consistently (though at different levels), at least in the transient assay. This preliminary assessment has served as the baseline information for using a short duration (ICPV21333) in our ongoing endeavor to generate alleles using gene editing in pigeonpea (unpublished data). However, the presence of pigeonpea *GRF4-GIF1* reduced the expression of *mGFP5* in three (ICPL87, ICPV21333, and TS3R) out of four genotypes tested in the current study. Our previous study reported that the expression of *CcGRF4* and *CcGIF1* in two pigeonpea genotypes was significantly

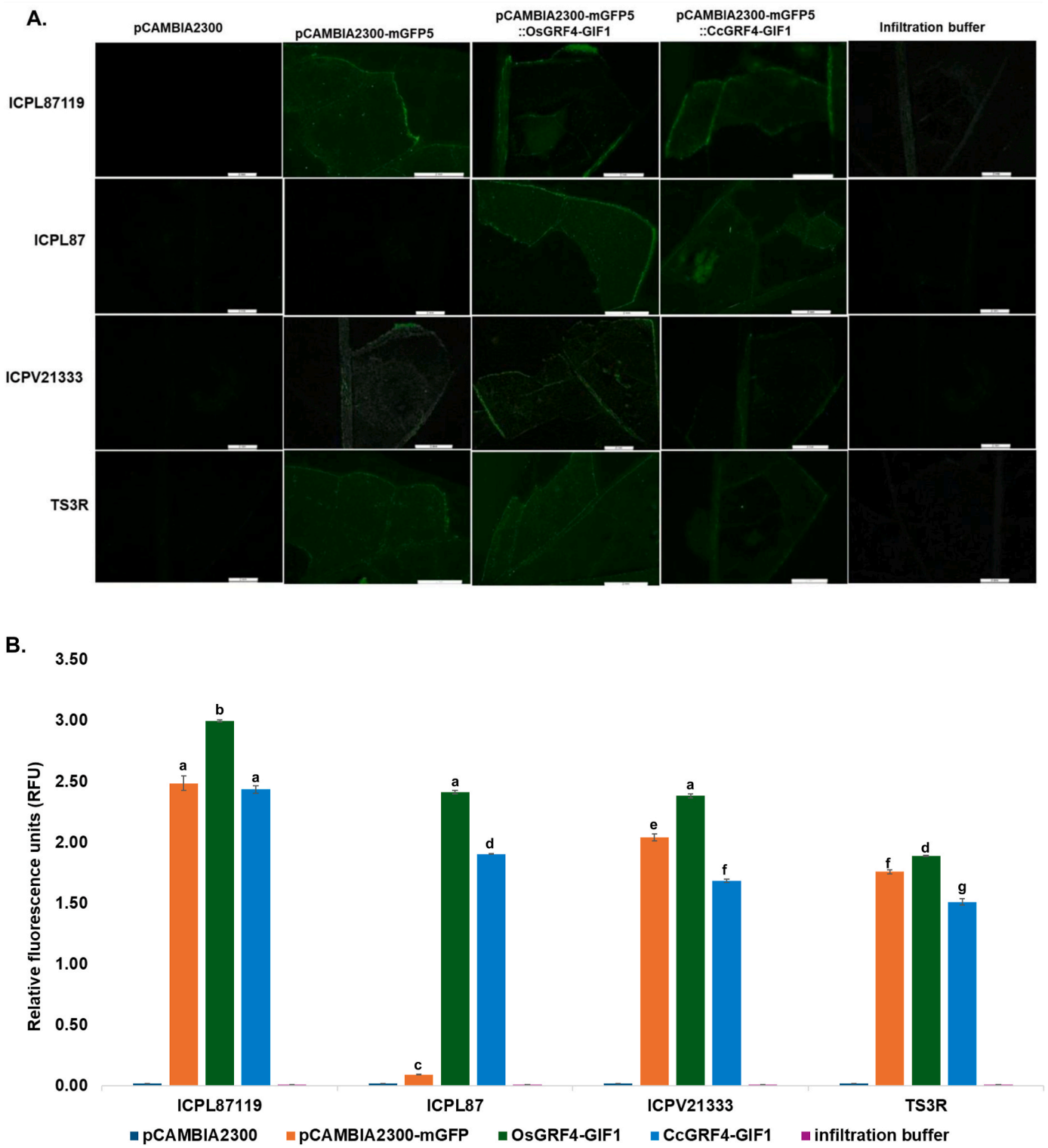

Fig. 3. Quantification of m*GFP5* fluorescence in four agroinfiltrated pigeonpea genotypes at 72 h after infiltration using a microplate reader. (A). An overview of m*GFP5* fluorescence observed in four pigeonpea genotypes- ICPL87119, ICPL87, ICPV21333, and TS3R. (B) GFP fluorescence quantification was measured at an excitation/emission wavelength of 488/528 nm, and an empty vector and infiltration buffer were used as a reference and negative control, respectively. The fluorescence levels detected in a sample are relative to the levels in reference and infiltration buffer and represented as RFU. The Y-axis represents mean values of normalized fluorescence intensity (RFU), while the X-axis indicates the genotypes. The results are representative of three independent samples, and values indicate the normalized mean ± standard error of replicates. The mean values followed by the same alphabet on top indicate no difference (Tukey's Test, $p < 0.05$). All the means are statistically significant as determined by one-way ANOVA ($p < 0.05$). Total four constructs - pCAMBIA2300 (empty vector), pCAMBIA2300-mGFP5, pCAMBIA2300-mGFP5::OsUbi-OsGRF4-GIF1-NosT, pCAMBIA2300-mGFP5::OsUbi-CcGRF4-GIF1-NosT, along with infiltration buffer (negative control) were used for measuring *mGFP5* levels.

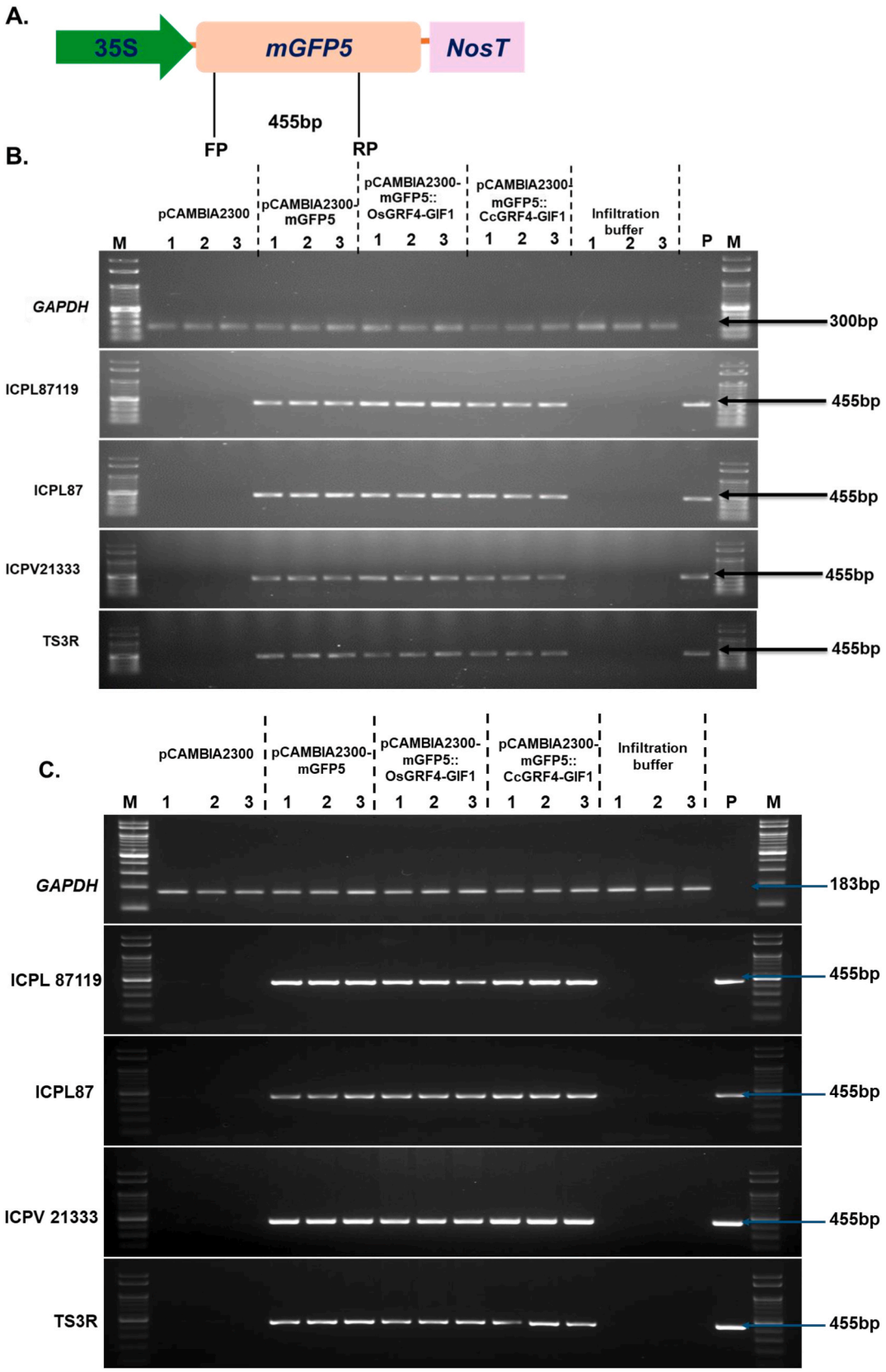

Fig. 4. Confirmation of the presence and expression of *GFP* in infiltrated pigeonpea plants. (A). Schematic representation of the pigeonpea *GFP* gene sequence targeted for primer design (FP - forward primer lies in UTR and RP - reverse primer lies in the exon). (B). Confirmation of the presence of *GFP* during the transient assay with PCR amplification using *GFP* gene-specific primers with pigeonpea *GADPH* gene as the internal reference. (C). RT-PCR confirmation of the expression of *GFP* using *GFP* gene-specific primers with pigeonpea *GADPH* gene as the internal reference. Four pigeonpea genotypes - ICPL87119, ICPL87, ICPV21333, and TS3R were infiltrated using a total of four constructs -pCAMBIA2300 (empty vector), pCAMBIA2300-mGFP5, pCAMBIA2300-mGFP5::OsUbi-OsGRF4-GIF1-NosT, pCAMBIA2300-mGFP5::OsUbi-CcGRF4-GIF1-NosT along with infiltration buffer (negative control) and P - Plasmid as a positive control. M – 100 bp DNA ladder.

higher in immature embryos and mature seeds.[25] Based on this, we hypothesize that the *CcGRF4-GIF1* plays a diverse role in plant growth and development and may not be an ideal morphogenic gene to be used for transformation studies. Additionally, species-specific variation, if present, especially in the regulatory regions, might affect the binding of *trans*-acting factors that negatively interact with *GRF-GIF*, leading to reduced *mGFP5* expression.

The effectiveness of agroinfiltration may be linked to variations in leaf architecture among genotypes. Moreover, since the pigeonpea *GRF4-GIF1* genes were driven by the rice Ubi promoter, this suggests that the promoter-gene combination might need further optimization. Achieving successful agroinfiltration depends on a multitude of factors that require optimization to enhance transformation efficiency.[50,51] These factors influence the transfer of T-DNA from *Agrobacterium* to plant cells[52] and encompass variables such as plant genotype, explant type, *Agrobacterium* strain, cell density in the inoculation medium, inoculation conditions, and co-culture techniques.

The *GRF-GIF* complex constitutes a plant-specific transcriptional complex crucial for regulating various aspects of plant growth and development, including leaf, stem, root, seed, and flower development.[53] Notably, introducing a GRF4-GIF1 fusion protein has demonstrated significant improvements in regeneration efficiency, speeding of regeneration, and somatic embryogenesis in both wheat and rice.[26] Furthermore, studies have demonstrated that a chimera of a monocot *GRF-GIF* in dicots has similarly improved regeneration efficiency in citrus and watermelon and increased transformation efficiency in lettuce,[26–28] suggesting the potential applicability of this approach to dicotyledonous crops as well.

4.1. Conclusion

Our findings offer a model approach that could facilitate transformation in other challenging plant species, particularly those where achieving stable expression of *Agrobacterium* proves to be inefficient, inconsistent, time-consuming, and labor-intensive. Our investigation revealed that the *OsGRF4-GIF1* complex substantially enhanced *mGFP5* expression compared to *CcGRF4-GIF1* across the four pigeonpea genotypes tested. Examining genotypes with different genetic backgrounds and duration with the current protocol suggests that *OsGRF4-GIF1* is working across genetic backgrounds (though at different levels), at least in the transient assay. The simplicity and effectiveness of this technique also render it highly suitable for validating plasmid constructions for genetic transformation and assessing sgRNA targeting efficiency for CRISPR/Cas9 gene editing across genetic backgrounds. Moreover, its potential in virus-induced gene silencing within economically significant pigeonpea varieties can be further explored. The inherent advantages of agroinfiltration underscore its importance as a valuable and sustainable source of plant material for various molecular biology analyses.

CRediT authorship contribution statement

Kalenahalli Yogendra: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Harika Gadeela:** Validation, Methodology, Formal analysis. **Koppula Nithya Sree:** Validation, Methodology, Formal analysis. **Wricha Tyagi:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

The dataset supporting the findings of this article is included within the article.

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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

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